Transforming growth factor-β1 Regulation of Collagenase-3 Expression in Osteoblastic Cells by Cross-talk between the Smad and MAPK Signaling Pathways and Their Components, Smad2 and Runx2*

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Transforming growth factor-β (TGF-β) plays a key role in osteoblast differentiation and bone development and remodeling. Collagenase-3 (matrix metalloproteinase-13) is expressed by osteoblasts and seems to be involved in osteoclastic bone resorption. Here, we show that TGF-β1 stimulates collagenase-3 expression in the rat osteoblastic cell line UMR 106-01 and requires de novo protein synthesis. Dominant-negative Smad2/3 constructs indicated that Smad signaling is essential for TGF-β1-stimulated collagenase-3 promoter activity. Inhibitors of the ERK1/2 and p38 MAPK pathways, but not the JNK pathway, reduced TGF-β1-stimulated collagenase-3 expression, indicating that the p38 MAPK and ERK1/2 pathways are also required for TGF-β1-stimulated collagenase-3 expression in UMR 106-01 cells. These inhibitors did not prevent nuclear localization of Smad proteins, but they inhibited Smad-mediated transcriptional activation. We have shown for the first time that Runx2 (a bone transcription factor and a potential substrate for the MAPK pathway) is phosphorylated in response to TGF-β1 treatment in osteoblastic cells. Cotransfection of Smad2 and Runx2 constructs had a cooperative effect on TGF-β1-stimulated collagenase-3 promoter activity in these cells. We further identified ligand-independent physical interaction between Smad2 and Runx2. Taken together, our results provide an important role for cross-talk between the Smad and MAPK pathways and their components in expression of collagenase-3 following TGF-β1 treatment in UMR 106-01 cells.
form functional interactions with critical transcriptional adapter proteins (25). The inhibitory Smad proteins (Smad6 and Smad7) directly inhibit the TGF-β type I receptor serine/threonine kinase and the transcriptional machinery (19–21). MAPKs also represent another major type of signaling intermediate for TGF-β.

Because TGF-β1 is a local regulator of bone cell function and collagenases appear to be involved in osteoclast-mediated bone resorption, we postulated that TGF-β1-stimulated collagenase-3 expression in osteoblastic cells could play a pivotal role in bone remodeling. Hence, it was of interest to dissect and identify the molecular mechanisms responsible for TGF-β1-stimulated collagenase-3 expression in UMR 106-01 cells. The Runx family of transcription factors is encoded by three distinct genes, Runx1 (polyoma enhancer-binding protein-2B/core-binding factor-α2/AML1), runx2 (polyoma enhancer-binding protein-2A/core-binding factor-α1/AML3), and runx3 (polyoma enhancer-binding protein-2C/core-binding factor-α3/AML2). Runx2 plays an essential role in osteogenesis (26–28). We show here the functional interaction of Smad2 and Runx2 as signaling components for the Smad and MAPK pathways and that they are necessary for TGF-β1-stimulated collagenase-3 promoter activity in osteoblastic UMR 106-01 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—TGF-β1 was purchased from Promega (Madison, WI). Radionuclides were obtained from PerkinElmer Life Sciences. Synthetic oligonucleotides were synthesized by Invitrogen. 3H[Chloramphenicol was obtained from Amersham Biosciences. Tissue culture media and reagents were obtained from Invitrogen. Fetal bovine serum was a product of JRH Biosciences (Lenexa, KS). The MEK1/2 (0.38 MAPK, and JNK inhibitors were purchased from Calbiochem. All other chemicals were obtained from Sigma or Fisher. Anti-phosphothreonine antibodies were obtained from Sigma. Anti-phospho-Smad2 antibody was purchased from Zymed Laboratories Inc. Anti-phospho-Smad3 antibody was obtained from Upstate Biotechnology, Inc. Phosphorylated Smad3 was detected using a purified rabbit polyclonal antibody raised against a 13-amino acid C-terminal phosphorylated Smad3 peptide as described previously (29). Anti-phosphoarginine and anti-phosphothreonine antibodies were obtained from Sigma. Anti-phosphotyrosine antibody was purchased from Calbiochem. Anti-α-tubulin and anti-Cdk2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Culture**—UMR 106-01 cells were maintained in monolayer in Eagle’s minimal essential medium (with Earle’s salts) supplemented with nonessential amino acids, 25 mM HEPES (pH 7.3), 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO2 and 95% air.

**Northern Blot Analysis**—Ten μg of total RNA was separated by formaldehyde-agarose gel electrophoresis and transferred to Zeta probe (Bio-Rad). Hybridization was carried out as described previously (30). The cDNAs for rat collagenase-3 and 18 S ribosomal RNA were labeled using either random primer or nick translation kits (Promega). The filters were scanned and quantitated using a PhosphorImager system.

**Real-time Quantitative Reverse Transcription (RT)-PCR**—Total RNA was prepared using the QIAGEN RNeasy kit. Reverse transcription was carried out using TaqMan reverse transcription reagents (Roche Applied Science). PCRs were performed using a real-time PCR DNA Engine Opticon (MJ Research, Inc., Watertown, MA) according to the manufacturer’s instructions, which allow real-time quantitative detection of the PCR product by measuring the increase in SYBR green fluorescence caused by binding of SYBR green to double-stranded DNA. The SYBR green kit for PCRs was purchased from PerkinElmer Life Sciences. Primers for human collagenase-3 and β-actin were designed using PrimerExpress software (PerkinElmer Life Sciences).

**Transient Transfections**—The plasmid DNAs were transiently transfected into UMR 106-01 cells using LipofectAMINE 2000 (Invitrogen). Briefly, cells were plated at 3 × 105 cells/well in 6-well plates in Eagle’s minimal essential medium containing 10% fetal bovine serum. The following day, the cells were transfected with 1 μg/particle DNA and 5 μg/particle LipofectAMINE in 1 ml of serum-free Eagle’s minimal essential medium. After 16 h, 1 ml of Eagle’s minimal essential medium containing 10% fetal bovine serum was added. After 24 h, the cells were treated with either control or TGF-β1-containing medium for 24 h. Chloramphenicol acetyltransferase (CAT) activity was measured by reacting 50 μl of cell lysate in duplicate in a 100-μl reaction volume consisting of final concentrations of 250 μM α-butyryl-CoA and 23 μM 3H[Chloramphenicol (0.125 μCi/assay). Butylated chloramphenicol was removed by pre-extraction with 200 μl of mixed xylene. The xylene phase was back-extracted with 100 μl of 0.25 M Tris-HCl (pH 8.0). Butylated chloramphenicol retained in the final organic layer was determined by scintillation counting. A standard curve using purified CAT was performed every experiment to determine the linear range of the enzyme assay. Renilla luciferase activity was monitored as an internal control of transfection efficiency using a dual luciferase assay system (Promega).

**RESULTS**

To study the effect of TGF-β1 on expression of collagenase-3 in the rat osteoblastic osteosarcoma cell line UMR 106-01, cells were treated with TGF-β1 either at different concentrations for 24 h (Fig. 1A) or for different time periods with 10 ng/ml TGF-β1 (Fig. 1B). Total cellular RNAs were purified and analyzed by Northern blotting using a rat collagenase-3 cDNA. The filters were scanned and quantitated using a PhosphorImager. As shown in Fig. 1A, collagenase-3 expression was maximally stimulated (3.8-fold) by 10 ng/ml TGF-β1 at 24 h in UMR 106-01 cells (Fig. 1B). An equal amount of RNA loading and transfer was verified by detecting 18 S ribosomal RNA on the same filter. To determine whether the TGF-β1-mediated increase in collagenase-3 mRNA is a primary response, UMR 106-01 cells were treated with control medium or medium containing TGF-β1 for 24 h in the presence or absence of 30 μg/ml cycloheximide added 1 h before treatment. Total RNA was subjected to Northern blot analysis using labeled rat collagenase-3 or 18 S ribosomal RNA as a same filter. To determine whether the TGF-β1-stimulation of collagenase-3 expression is secondary and that de novo protein synthesis is required for this purpose. An enzyme-linked immunosorbent assay was then performed using an antibody against rat collagenase-3, which confirmed stimulation of collagenase-3 from UMR 106-01 cells into the medium (data not shown).

Smad proteins have been identified as intracellular mediators for members of the TGF-β superfamily. To determine the involvement of the Smad pathway in TGF-β1-stimulated collagenase-3 expression, we first examined phosphorylation patterns of Smad2 activated by TGF-β1. UMR 106-01 cells were treated with TGF-β1 for different times as indicated. Whole cell extracts were prepared and subjected to Western blot analysis. As shown in Fig. 2A, Smad2 was rapidly phosphorylated within 15 min of TGF-β1 treatment, which persisted for 4 h in these cells. Similarly, Smad3 was also phosphorylated in response to TGF-β1 treatment for 1 h (Fig. 2B). To examine the functional significance of the Smad pathway activated by TGF-β1 in regulating collagenase-3 expression in these osteoblastic cells, we utilized dominant-negative Smad2 and Smad3 expression plasmids. The rat -500 collagenase-3 promoter construct was transiently transfected with either pCMV-Smad2Mutant or pCMV-
Smad3Mutant into UMR 106-01 cells and then treated with control or TGF-β1-containing medium for 24 h and assayed for CAT activity. The transfection efficiency was normalized by cotransfection with Renilla luciferase reporter gene construct. Both Smad2/3 mutants reduced, but did not completely abolish, the basal and TGF-β1-stimulated collagenase-3 promoter activities in UMR 106-01 cells (Fig. 2C), indicating that the TGF-β1 response for collagenase-3 promoter activity requires an additional signaling pathway.

To identify whether the MAPK signaling pathway is involved in TGF-β1-stimulated collagenase-3 expression, we used MAPK pathway inhibitors. UMR 106-01 cells were pretreated with Me2SO, PD98059 (MEK/ERK1/2 inhibitor), SB203580 (p38 inhibitor), or SP600125 (JNK2 inhibitor) at different concentrations for 20 min and then treated with or without TGF-β1 for 24 h. Total RNA was isolated and subjected to real-time quantitative RT-PCR using the sense or antisense oligomers for rat collagenase-3 and β-actin (Fig. 3, A–C). The ERK1/2 and p38 MAPK inhibitors decreased TGF-β1-stimulated collagenase-3 expression from 10.0±1.8-fold to 3.0±0.6-fold at 25 μM and to 1.7±0.4-fold at 10 μM, respectively. These results suggest that, in addition to the Smad pathway (Fig. 2), the MAPK pathway is also required for TGF-β1-stimulated collagenase-3 expression in UMR 106-01 cells.
was no effect with the JNK2 inhibitor (Fig. 3C). To show the action and specificity of the MAPK inhibitors, we pretreated the UMR 106-01 cells with those inhibitors for 20 min and then treated them with control or PTH (10⁻⁸ M)-containing medium for 24 h. Total RNA was subjected to real-time RT-PCR, and the results show that ERK1/2 (Fig. 3D) and p38 (data not shown) inhibitors had no effect on PTH-induced collagenase-3 expression in UMR 106-01 cells, whereas the JNK2 inhibitor blocked PTH-induced collagenase-3 expression in UMR 106-01 cells (Fig. 3E).

We next examined whether the Smad and MAPK pathways act independently or involve some level of intracellular cross-talk. Smad2 translocates to the nuclear compartment after TGF-β1 stimulation. Activation of MAPK pathways has been shown to both activate and induce nuclear translocation of Smad2 (34, 35). We therefore tested whether specific inhibitors of MAPK pathways inhibit TGF-β1-dependent nuclear translocation of the Smad proteins in UMR 106-01 cells. Cells were pretreated with the ERK1/2 and p38 MAPK inhibitors for 30 min, followed by TGF-β1 treatment for 1 h, and nuclear extracts were prepared and subjected to Western blot analysis using anti-phospho-Smad2 and anti-phospho-Smad3 antibodies. When UMR 106-01 cells were stimulated with TGF-β1, there was increased nuclear accumulation of phospho-Smad2 and phospho-Smad3, which were detected by their respective antibodies (Fig. 4A). Pretreatment with p38 MAPK and ERK1/2 inhibitors did not inhibit nuclear translocation of phospho-Smad2 and phospho-Smad3 proteins. These observations demonstrate that nuclear translocation of Smad2 and Smad3 proteins is independent of MAPK signaling pathways.

To determine whether these MAPK pathways affect the transcriptional activity of the Smad complex, the −500 collagenase-3 promoter construct was transiently transfected into UMR 106-01 cells along with FLAG-tagged Smad2/4 expression constructs. The cells were then pretreated with Me2SO or with p38 MAPK and ERK1/2 inhibitors, followed by TGF-β1 treatment. The lysates were prepared, and CAT activity was measured. The transfection efficiency was normalized by co-transfection with Renilla luciferase reporter plasmid. As shown in Fig. 4B, Smad2/4 increased both the basal and TGF-β1 responses for collagenase-3 promoter activity in UMR 106-01 cells. Pretreatment with SB203580 and PD98059 inhibited both TGF-β1-stimulated and Smad-mediated collagenase-3 promoter activity in these cells. The level of expression of FLAG-Smad2 and FLAG-Smad4 proteins in the transfected cells was analyzed by Western blot analysis. These results indicate that both the Smad and MAPK pathways merge their signals within the nucleus or at the transcriptional level for TGF-β1-stimulated collagenase-3 expression in UMR 106-01 cells.

Runx2 is a bone-related transcription factor and is essential for the differentiation of osteoblasts from mesenchymal precursors and for bone formation (26–28). Runx transcription factors have been shown to interact with Smad proteins to confer TGF-β and bone morphogenetic protein (BMP) signaling pathways (36, 37). Because the −500 collagenase-3 promoter construct contains a Runx-binding site (31) and Runx proteins interact with Smad proteins (38, 39), we wanted to first delineate the role of Runx2 in TGF-β-stimulated collagenase-3 promoter activity in UMR 106-01 cells. The rat −500 collagenase-3 promoter construct was transiently transfected into UMR 106-01 cells with increasing amounts of an AML-1/ETO expression plasmid. AML/ETO is a repressor protein that does not contain the transactivation domain proline, serine, and threo-
compared with the control. The expression of FLAG-Smad2/4 proteins was also verified by Western blot analysis using anti-FLAG antibody.

Both increases and decreases in osteoclast formation, bone resorption, osteoblast proliferation, and osteoblast differentiation by TGF-β1 have been reported (4–8). Collagenase-3 produced by osteoblasts seems to be involved in osteoclast-medi-
regulated bone resorption (17, 18). Both TGF-β1 and collagenase-3 could function as putative coupling factors between bone formation and bone resorption. In this study, we have shown that TGF-β1 stimulates collagenase-3 expression in rat osteoblastic UMR 106-01 cells. The decreased collagenase-3 mRNA upon 30 ng/ml TGF-β1 treatment for 24 h could be due to the instability of collagenase-3 mRNA (Fig. 1A). There was both an inhibition and a stimulation of collagenase-3 mRNA expression by TGF-β1 at early and later time periods, respectively, suggesting that there might be an intermediate protein that would be responsible for the secondary effect on collagenase-3 expression (Fig. 1B). The decreased collagenase-3 mRNA at 10 ng/ml TGF-β1 treatment for 48 h could also be due to the prolonged exposure of TGF-β1 in the medium, resulting in degradation of TGF-β1 (Fig. 1B). There are intermediate proteins that would be responsible for stabilization of collagenase-3 mRNA, as recently suggested by Rydziel et al. (41). Either a high dose or prolonged treatment with TGF-β1 could have altered these intermediate proteins, resulting in destabilization of collagenase-3 mRNA. Inhibition of collagenase-3 mRNA expression by cycloheximide indicated that de novo protein synthesis is required for TGF-β1-stimulated collagenase-3 expression (Fig. 1C). The activator protein-1-binding sites are responsible for early gene activation, and many TGF-β1-inducible genes contain these sites, which have been functionally linked to transcriptional activation by TGF-β (42, 43).

The TGF-β1 signaling pathway for collagenase-3 stimulation in bone appears to be significantly different from fibroblast and chondrocyte collagenase-3 expression (44, 45) and is regulated by several intracellular pathways and components. In human gingival fibroblasts, MAPK inhibitors block nuclear translocation of Smad3 (44). Our data indicate that MAPK inhibitors do not inhibit nuclear translocation of Smad2/3 proteins in osteoblastic UMR 106-01 cells (Fig. 4A). It appears that nuclear translocation of Smad proteins by TGF-β1 is cell type-specific. Smad proteins are the main cytoplasmic signaling pathways in TGF-β1-stimulated collagenase-3 expression in osteoarthritic chondrocytes (45). The enhancement of collagenase-3 expression by TGF-β is dependent on p38 MAPK activity in human gingival and skin fibroblasts, transformed human epidermal keratinocytes, and the human cutaneous squamous cell carcinoma cell line (44, 46). We have shown here that TGF-β1-induced activation of p38 MAPK and ERK1/2 is essential for Smad-mediated collagenase-3 promoter activity and that the interaction between the Smad and MAPK pathways is necessary for maximal promoter activation in UMR 106-01 cells. The explanation for cross-talk between the Smad and MAPK pathways for TGF-β-stimulated collagenase-3 expression is that components of these pathways interact directly in the transcriptional complex. Runx2 is a substrate for the MAPK pathway, and this pathway can be stimulated by a variety of signals (47). The PST and C-terminal regions of Runx2 are important for its activity (48–53). It appears that Runx2 expression and activity are regulated by post-translational modifications and protein-protein interactions. Here, we demonstrated that TGF-β1 stimulated Runx2 phosphorylation at threonine and tyrosine residues in rat osteoblastic cells (Fig. 5D). Because Runx2 phosphorylation also occurs at tyrosine
residues, the epidermal growth factor receptor could also be responding to TGF-β1, as evidenced by epidermal growth factor receptor transactivation in TGF-β1-mediated fibronectin expression in mesangial cells (54). Runx2 phosphorylation at threonine residues after TGF-β1 treatment strongly suggests that Runx2 is a substrate for the ERK1/2 signaling pathway that is required for collagenase-3 promoter activity. ERK1/2 is a common target of TGF-β and BMP-2 and activates Runx2-dependent transcription without affecting the expression of Runx2 (53). The activation of p38 MAPK is also involved in the induction of Runx2 by TGF-β1 and BMP-2 stimulation (55). Because both the activator protein-1 and Runx2 transcription factors are important targets of interleukin-induced p38 MAPK, leading to collagenase-3 expression in a human chondrosarcoma cell line (56), it is possible that Runx2 could also act as a substrate for the p38 MAPK pathway. It is likely that Runx2 phosphorylation leads to conformational changes in its structure that could protect Runx2 from degradation and/or allow interaction with other proteins for transactivation of its target genes, including collagenase-3.

So far, the significance of the Smad and Runx2 proteins and the mechanisms of their interactions with other proteins for collagenase-3 expression in either fibroblasts or osteoblasts have not been studied. We have shown the requirement of Runx2 for TGF-β-stimulated collagenase-3 promoter activity in rat osteoblastic cells (Fig. 5A). In fibroblasts, Smad3 mediates the induction of collagenase-3 expression by TGF-β, whereas Smad2 is not involved in this context (44). In osteoblasts, only Smad2 (not Smad3) mediates its effect for collagenase-3 promoter activation (Fig. 6). We have provided evidence of functional cross-talk between the Smad and MAPK pathways by cotransfection of their components (Smad2 and Runx2) along with the collagenase-3 promoter construct in UMR 106-01 cells (Fig. 6A). The increased basal response by Runx2 could be due to interaction with activator protein-1 factors. Even though the interaction of Smad2 and Runx2 is ligand-independent (Fig. 6C), TGF-β1 treatment is required to confer maximal collagenase-3 promoter activity (Fig. 6A), indicating that TGF-β1-induced, TGF-β1-repressed, or TGF-β1-modified factors may be necessary for interaction between Smad2 and Runx2 proteins. This functional interaction may be stabilized or mediated by CAMP-responsive element-binding protein-binding protein and p300, which could act as transcriptional adapter proteins (57, 58).

Overall, we have shown that TGF-β1 stimulates collagenase-3 expression in UMR 106-01 cells and have provided evidence for cross-talk between the Smad and MAPK pathways and their components in expression of collagenase-3 following TGF-β1 treatment in these cells. In view of the importance of Runx2 as a pivotal transcription factor of bone and bone-related genes (collagenase-3), this study further advances our understanding of how Runx2 physiologically functions in bone metabolism.

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