Identification of a Transforming Growth Factor-β1/Bone Morphogenetic Protein 4 (TGF-β1/BMP4) Response Element within the Mouse Tissue Transglutaminase Gene Promoter*

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Tissue transglutaminase is a calcium-dependent, protein cross-linking enzyme that is highly expressed in cells undergoing apoptosis. The expression of tissue transglutaminase is regulated by a variety of molecules including retinoids, interleukin-6, and transforming growth factor-β1 (TGF-β1). Retinoid and interleukin-6 inductions of tissue transglutaminase expression are mediated by specific cis-regulatory elements located within the first 4.0 kilobase pairs of the promoter of the gene. The present studies were designed to identify the molecular mechanisms mediating the regulation of tissue transglutaminase gene expression by TGF-β family members. Transient transfection of Mv1Lu cells with transglutaminase promoter constructs demonstrated that 0.2 nM TGF-β1 maximally induced the activation of the promoter through a 10-base pair TGF-β1 response element (TRE; GAGTGTGTC) located 868 base pairs upstream of the transcription start site. This same element mediated an inhibitory activity of TGF-β1 on the transglutaminase promoter in MC3T3 E1 cells. The TRE through which TGF-β1-regulated the activity of the transglutaminase promoter was necessary and sufficient for bone morphogenetic protein 2- (BMP2) and BMP4-dependent inhibition of the tissue transglutaminase promoter. The TGF-β1, BMP2, and BMP4 regulation of the transglutaminase promoter activity was similar to the responses we observed for the endogenous transglutaminase activity of Mv1Lu and MC3T3 E1 cells. For BMP2 and BMP4, this regulation was paralleled by a decrease in tissue transglutaminase mRNA in MC3T3 E1 cells. The results of these experiments suggest that TGF-β1, BMP2, and BMP4 regulation of mouse tissue transglutaminase gene expression requires a composite TRE located in the 5′-flanking DNA.

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‡ The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; CAT, chloramphenicol acetyltransferase; TRE, transforming growth factor-beta response element; bp, base pair(s); PAI-1, plasminogen activator inhibitor-1; PCR, polymerase chain reaction; Luc, luciferase; MEM, minimum Eagle’s medium; NaB, sodium butyrate.

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Transglutaminase TGF-β1 Response Element

EXPERIMENTAL PROCEDURES

Materials

Mv1Lu cells, an epithelial-like cell line derived from normal mink lungs, were purchased from the American Type Culture Collection (ATCC, Rockville, MD). MC3T3 E1 cells, a mouse preosteoblastic cell line, were a gift from Dr. Renee Franchesci (University of Cincinnati). Recombinant human TGF-β1 (rhTGF-β1) was purchased from R & D Systems (Minneapolis, MN), and recombinant human BMP2 and BMP4 were generous gifts from Genetics Institute, Inc. (Cambridge, MA). The pCAT-Basic, SV-β-gal, and pRL-SV40 vectors were purchased from Promega Corp. (Madison, WI). The Quick Change mutagenesis kit was purchased from Stratagene Cloning Systems (La Jolla, CA). Oligonucleotide primers were purchased from Genosys (Houston, TX).

Cell Cultures

Mv1Lu cells were maintained in Eagle's MEM supplemented with 10% heat-inactivated fetal bovine serum. MC3T3 E1 cells were maintained in α-MEM containing 10% heat-inactivated calf serum. Both cell lines were cultured in the presence of 100 units/ml penicillin and 50 mg/ml streptomycin.

Plasmid Constructs and Site-directed Mutagenesis

The regulation of transglutaminase gene expression by TGF-β family members was investigated in transient transfection experiments with mouse tissue transglutaminase promoter constructs (pMT4G.0-Luc, pMTG1.8-CAT, pMTG6.2-CAT, and pMTG1.8-ΔSmal-CAT; Fig. 1A) (8). pMTG1.65-CAT, pMTG1.3-CAT, and pMTG1.0-CAT were constructed using a Quick Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) to introduce a HindIII restriction site at positions −1647, −1287, and −1006, respectively, followed by excision of the DNA fragment with HindIII (−1647, −1287, or −1006 to −1842; Boehringer Mannheim) and religation with T4-DNA ligase (Boehringer Mannheim). pMTG1.8-ΔMut-CAT was generated by site-directed mutagenesis of the TGF-β1 regulatory element (TRE) within pMTG1.8-CAT using the Quick Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) and complementary primers that converted the TTGG (5′ → 3′) tetranucleotide sequence at position −871 to CTAG.

The nucleotide sequence for each of the mouse tissue transglutaminase gene promoter constructs was determined. Samples were processed according to the standard protocol for the T7 Sequenase Quick-Denatur Plasmid Sequencing Kit (Amersham Pharmacia Biotech). Following the sequencing reaction, samples were resolved using a 6% denaturing polyacrylamide gel and a GenomyxLR programmable DNA sequencer (Genomyx Corp., Foster City, CA).

Transient Transfections

Co-transfection assays with test and control plasmids were conducted using approximately 70% confluent Mv1Lu or MC3T3 E1 cells and a 12-well dish format. Mv1Lu cells were washed with serum-free Eagle's MEM prior to Lipofectin-mediated transfection (Life Technologies, Inc.) of 1 μg of test plasmid along with 0.1 μg of pRL-SV40. Cells were transfected for 3 h prior to addition of Eagle's MEM plus 10% heat-inactivated fetal bovine serum containing treatments described within figure legends. MC3T3 E1 cells were co-transfected using 3 μg of DOSTER (Boehringer Mannheim), 1 μg of test plasmid, and 0.05 μg of pR-L-SV40. MC3T3 E1 cells were transfected for 6 h prior to addition of α-MEM plus 10% heat-inactivated calf serum containing treatments. Mv1Lu and MC3T3 E1 cells were cultured for 48 h in the presence of the treatment(s), washed once with phosphate-buffered saline and cell extracts were prepared as described for enzyme assays.

Enzyme Assays

Chloramphenicol Acetyltransferase Assay—Chloramphenicol acetyltransferase (CAT) activity was determined using the method of Gorman et al. (19). Mv1Lu or MC3T3 E1 cells were scraped into 250 μl of 0.25 M Tris-HCl (pH 7.5) and lysed by three rounds of freeze-thawing. The extracts were briefly centrifuged, and the supernatant was used for CAT and renilla-luciferase (LucRen) assays. Duplicate 100-μl cell extracts were incubated with 200 μM of [3H]chloramphenicol, 32 μl of 1 M Tris-HCl (pH 7.5), 10 μl of 8 mM acetyl-CoA for 20 h at 37 °C. The reaction was terminated with 300 μl of xylenes and extracted with 500 μl of ethyl acetate. 700 μl of the organic phase was removed, dried, and separated by thin layer chromatography (Whatman silica gel) in chloroform:methanol (97:3) for 1 h. Acetylated products were detected by exposure to a phosphorimaging screen and developed using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Di-acetylated chloramphenicol was quantified using ImageQuant version 1.1 software (Molecular Dynamics). CAT activity was normalized to LucRen activity to correct for any difference in transfection efficiency or generalized alterations in transfection.

Luciferase and Renilla Assay—Cells were lysed with 250 μl of cell lysis buffer (Promega Corp., Madison, WI), and 20 μl of the extract was assayed for firefly-luciferase (Lucfire) activity using a Luciferase Assay kit (Promega Corp., Madison, WI) according to the manufacturer's suggested protocol. Lucfire activity was determined in 20 μl of the Mv1Lu cell extract using a Dual Luciferase Assay kit (Promega Corp., Madison, WI) according to the manufacturer's suggested protocol. The total protein concentration of each extract was determined using a Bio-Rad Protein Assay kit (Bio-Rad), and luciferase activity was normalized to the total protein concentration of the cell extract.

Tissue Transglutaminase Assay—Tissue transglutaminase activity in Mv1Lu and MC3T3 E1 cell extracts was determined as described previously (26). Cells were washed once with phosphate-buffered saline, scraped into 250 μl of Tris-HCl (pH 7.5), and lysed with three cycles of freeze-thawing. Cell lysates were incubated in the presence of [3H]Hypersine and N,N'-dimethylcysine. Ca2+-dependent conjugation of [3H]Hypersine to N,N'-dimethylcysine was determined by trichloroacetic acid precipitation followed by scintillation counting.

Tissue Transglutaminase mRNA Measurement

Total RNA Preparation—Total RNA was prepared from MC3T3 E1 cells treated with either TGF-β1, BMP2, or BMP4 according to the QIAshredder and RNeasy protocols (Qiagen, Inc., Santa Carita, CA) and quantified by measuring the absorbance at 260 nm.

Quantification of Selected mRNA—50 ng of total RNA from MC3T3 E1 cells was reverse-transcribed according to a standard protocol (Current Protocols in Molecular Biology) using Superscript Reverse Transcriptase (Life Technologies, Inc.) with an oligonucleotide primer (300 nt) for either mouse tissue transglutaminase (CCAGATCTCCCTCTCCACAT; Genesys, Houston, TX) or mouse 36B4 (ATATGAGGCCAGCTTTTCCACAG; Genesys, Houston, TX) in the presence of 4 mM MgCl2 in 50 μg/ml DNTPs. Following reverse transcription, the cDNA product was PCR-amplified using 300 nt forward primers for either mouse tissue transglutaminase (AGACTTACCTTTCGCTGTCTG; Genesys, Houston, TX) or human 36B4 (AGATGCAAGCATCCGGCAT; Genesys, Houston, TX) and 100 nt of an oligonucleotide probe for either tissue transglutaminase (ACCGGCCAGATCTCCGAGGAA; Synthetic Genetics, San Diego, CA) or mouse 36B4 (AGGCTGTTGGCTGTATGGCGGAGAC; Applied Biosystem, Foster City, CA). Probes were labeled with 6-carboxyfluorescein and 6-carboxytetramethylrhodamine (5′ and 3′, respectively). The PCR amplification was accomplished with Taq DNA-polymerase (Boehringer Mannheim) in an ABI Prisim 7700 “real-time” PCR analysis instrument (Applied Biosystems, Foster City, CA), monitoring product generation throughout each of 40 cycles. Synthetic RNA was used to generate standard concentration curves for each mouse tissue transglutaminase (20 pg to 0.2 fg) or human 36B4 (20 pg to 2 fg). The point at which the fluorescent signal rose above the background (Ct; 10 S.D. above the base-line values) was used to quantitate the amount of the specified mRNA in the starting sample. Quantification of the sample template from the determined Ct was accomplished by comparison with cRNA standards run in parallel.

Statistics

Differences within and between data sets were determined using SigmaStat for Windows (SPSS Inc., Chicago, IL). One-way analysis of variance and subsequent Tukey's or Dunnett's mean separation procedure were performed at a significance level of p ≤ 0.05.

RESULTS

TGF-β1—TGF-β1 has been reported to increase the transglutaminase activity of several cell types (14, 15). To assess the ability of TGF-β1 to regulate the activity of the tissue transglutaminase gene promoter in the mouse transgenic mink lung epithelial cell line, Mv1Lu, and mouse preosteoblastic cells, MC3T3 E1, with reporter constructs that included segments of the mouse tissue transglutaminase promoter linked to a reporter gene (either CAT or LucRen) and a normalization plasmid (LucRen), following transfection, cells were treated with recombinant human TGF-β1 or control media, and 48 h later cells

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were lysed and assayed for reporter gene (CAT or Luc<sub>r</sub>) and normalization gene (Luc<sub>r</sub>) activities. In each assay, differences in transfection efficiency were corrected by normalizing reporter gene activity to Luc<sub>r</sub> activity. Previous studies from our laboratory have demonstrated that pmTG3.8-Luc, a reporter gene construct containing 3.8 kilobase pairs of the mouse tissue transglutaminase promoter, possesses all the necessary information to direct physiological expression of tissue transglutaminase in vivo (16). We therefore transfected Mv1Lu cells with pmTG3.8-Luc (Fig. 1A) and measured the effect of TGF-β1 on the activity of the promoter. The results with Mv1Lu cells (Fig. 1B) indicate that TGF-β1 induces a dose-dependent increase in pmTG3.8-Luc activity.

To locate the regions of the transglutaminase promoter responsible for the induction by TGF-β1, we transfected Mv1Lu cells with a series of reporter constructs in which segments of the transglutaminase promoter were deleted, pmTG1.8-CAT, a construct containing 1834 bp of transglutaminase promoter linked to a CAT reporter gene, demonstrated the same dose-dependent increase in transcriptional activity as we obtained with pmTG3.8-Luc (Fig. 1B). However, the ability of TGF-β1 to induce the activity of this promoter above the activity of the untreated controls was greater than what we observed with the pmTG3.8-Luc construct. In a series of experiments (n = 8), 0.2 mM TGF-β1 induced a 2.6-fold increase in reporter gene expression of the pmTG1.8-CAT construct (p < 0.001). Deletion of an additional 187 or 547 bp from the 5′-end of the promoter (pmTG1.65 and pmTG1.3-CAT, respectively) had no deleterious effect on the TGF-β1 inducibility of the tissue transglutaminase gene promoter (Fig. 1B). However, deletion of the promoter region 3′ to the Smal site at −1094 (pmTG0.2-CAT and pmTG1.8-Smal-CAT) eliminated the TGF-β1 inducibility (Fig. 1B). These studies indicated that the TGF-β1 induction of the transglutaminase promoter depended on the sequence located between −1094 and −256.

TGF-β1 has been found to increase the activity of a number of promoters, but a precise TRE for TGF-β1-inducible genes has not been identified (21, 22). In contrast to its stimulatory activity, TGF-β1 has also been demonstrated to inhibit the expression of several genes (23–28). Comparison of the promoter regions that were necessary for TGF-β1-mediated inhibition of gene expression led to the identification of a conserved sequence (GnnTGGHGnG; n is A, G, C, or T; H is C, A or T; D is G, A, or T). This canonical TRE was suggested to be a necessary component for the inhibitory effects of TGF-β1 on gene expression (29). Examining the sequence of the mouse tissue transglutaminase promoter necessary for TGF-β1 activity (−1094 to −256) led to the identification of a motif (GAGTTGGTCC; −877 to −868) similar to the canonical TRE (Fig. 1C). Although this TRE was identified as an inhibitory motif for TGF-β1-regulated gene expression, we investigated whether this element mediated TGF-β1-induced activation of the transglutaminase promoter in transfected Mv1Lu cells. Alteration of the transglutaminase TRE was accomplished by mutating two of the positions common between the transglutaminase TRE and the canonical TRE (Fig. 1D), mutant transglutaminase TRE). Comparison of the effect of TGF-β1 on the wild-type sequence (pmTG1.8-CAT) and the mutated sequence (pmTG1.8-Mut-Cat) (Fig. 1C) indicated that mutation of these two nucleotides resulted in a complete loss of the TGF-β1-inducible expression. In a series of experiments (n = 8) 0.2 mM TGF-β1 did not induce an increase in the activity of the mutated construct (p > 0.1), whereas it induced a 2.6-fold increase in the activity of its non-mutated parent construct (p ≤ 0.001). These results indicate that TGF-β1-induced activation of the tissue transglutaminase promoter in Mv1Lu cells requires a canonical TRE located at position −868 of the mouse transglutaminase promoter.

BMP2 and BMP4—To determine if the effects of TGF-β1 on the transglutaminase promoter activity could be extended to other members of the TGF-β superfamily, we examined the effects of BMP2 and BMP4 on the activity of the tissue transglutaminase promoter. Expression of BMP2 and BMP4 in the developing limb coincides with tissue transglutaminase, occurring in regions such as the interdigital web and the apical ectodermal ridge (16, 30, 31). Mv1Lu cells, co-transfected with pmTG3.8-Luc and SV<sub>g</sub>-β-galactosidase, were treated with recombinant human BMP2, BMP4, or control media for 48 h. After 48 h, the luciferase and β-galactosidase activities were determined in the cell extracts. Both BMP2 and BMP4 inhibited the activity of the transglutaminase promoter to a level that was 9 and 13%, respectively, of control (Fig. 2A; p < 0.001, n = 2).

The basal level of expression of the pmTG3.8-Luc construct in the Mv1Lu cells was low; thus, accurately assessing the level of BMP-mediated inhibition was difficult. Recently, we observed that inhibitors of histone deacetylation, such as sodium butyrate (NaB), greatly increased the basal level of activity of transiently transfected transglutaminase promoter constructs. In the absence of NaB, the basal level of luciferase activity in Mv1Lu cells transfected with pmTG3.8-Luc was 291 ± 52 lumens/μl. In comparison, the basal level of luciferase activity in Mv1Lu cells transfected with pmTG3.8-Luc and treated with 2.5 mM NaB was 14076 ± 703 lumens/μl. To evaluate the effects of BMP2 and BMP4 on transglutaminase promoter activity, Mv1Lu cells co-transfected with pmTG3.8-Luc and SV<sub>g</sub>-β-galactosidase were treated with 2.5 mM NaB or 2.5 mM NaB plus 2.5 mM BMP2 or 4% for 48 h. The results of this experiment (Fig. 2B) confirmed that BMP2 and BMP4 inhibited the transglutaminase activity of the mouse tissue transglutaminase promoter by 64 and 68%, respectively (p < 0.001; n = 5).

The basal activity of the pmTG1.8-CAT construct in Mv1Lu cells is higher than the pmTG3.8-Luc construct. Thus, we were able to examine BMP-mediated inhibition of the basal activity of the pmTG1.8-CAT construct without recourse to NaB treatment. BMP4 induces a dose-dependent inhibition in pmTG1.8-CAT activity in Mv1Lu cells with an IC<sub>50</sub> of 0.082 mM (Fig. 3A). Deletion of a proximal segment (−1094 to −90) of the pmTG1.8-CAT construct, pmTG1.8-Smal-CAT (Fig. 1A), resulted in a complete loss of the inhibitory activity for all concentrations of BMP4 (Fig. 3A). The level of reporter gene expression in cells transfected with pmTG1.8-Smal-CAT and treated with BMP4 for 48 h was 123% of control (Fig. 3B; p > 0.1; n = 6).

The stimulatory effect of TGF-β1 on transglutaminase expression depends on the integrity of a TRE located at −868. To determine if the same element contributed to the inhibitory effect of BMP4 on transglutaminase promoter activity, we compared the activity of the intact promoter (pmTG1.8-CAT) with the mutated promoter (pmTG1.8-ΔMut-CAT) in BMP4-treated Mv1Lu cells (Fig. 3B). In a series of experiments, the transglutaminase promoter activity in BMP4-treated Mv1Lu cells transfected with the wild-type promoter, pmTG1.8-CAT, was inhibited (48% of control, p < 0.001, n = 8). In contrast, BMP4 treatment of Mv1Lu cells transfected with the tissue transglutaminase promoter construct containing two mutated nucleotides (−872 and −874) within the core of the TRE, pmTG1.8-ΔMut-CAT, was unable to inhibit the activity of this construct (89% of control, p > 0.1, n = 6). BMP2 treatment was also ineffective at inhibiting pmTG1.8-ΔMut-CAT activity in Mv1Lu cells (data not shown). Thus, the TRE located at −868 of the tissue transglutaminase gene promoter is necessary for TGF-β1, BMP4, and BMP2 regulatory activities.
MC3T3 E1 Cells—Since inhibition of transcription for several genes is known to require the presence of the TRE sequence (23–28), we investigated whether our observation of this element mediating TGF-β1 induction and BMP2 and BMP4 inhibition of tissue transglutaminase promoter activity was specific for the Mv1Lu cell line. To examine this question, mouse calvarial pre-

**Fig. 1. Analysis of the mouse tissue transglutaminase gene promoter (mTgase).** A, the mouse transglutaminase promoter constructs linked to either a luciferase (Luc) or a CAT reporter gene that were used to identify the region of the 5'-flanking DNA mediating TGF-β or BMP responsiveness. *, HindIII sites at −1647 and −1287 were created by site-directed mutagenesis as described under “Experimental Procedures.” **, mutation of the identified region mediating cytokine responsiveness was accomplished by site-directed mutagenesis as described under “Experimental Procedures.” B, Mv1Lu cells were transfected with 1 μg of the reporter constructs and 0.1 μg of a normalization plasmid (SV-β-galactosidase or SV40-LucRen). After transfection, cells were treated with rhTGF-β1 at the indicated dose for 48 h. Control wells received no rhTGF-β1. Following treatment, cell extracts were analyzed for the indicated reporter and normalization genes as described under “Experimental Procedures.” Values represent the mean (LucFf or CAT)/(SV2-β-galactosidase or SV40-LucRen) ± S.D. of duplicate transfections from duplicate experiments, graphed as a percent of control values. C, normalized CAT activity (CAT/SV40-LucRen) of Mv1Lu cells transfected as described under “Experimental Procedures” with the indicated tissue transglutaminase promoter constructs and treated with TGF-β1 (0.2 nM) for 48 h. Values represent the mean ± S.D. of determinations from duplicate experiments conducted in duplicate. Groups marked by an asterisk are significantly different from the control group, p ≤ 0.05. D, comparison of TGF-β1-responsive elements for multiple genes. A consensus TRE (5'-GAGTGGTCC-3') was derived from a comparison of several genes (r-transin, h-Urokinase, h-Collagenase, human c-MYC, m-Elastase, and m-Mrp/Proliferin) whose expression is inhibited by TGF-β1 (29). *, pmTG1.8-mut-CAT.
osteoblasts (MC3T3 E1 cells) were transfected with the intact tissue transglutaminase gene promoter linked to a CAT reporter gene (pmTG1.8-CAT) or the tissue transglutaminase gene promoter containing the mutated TRE (pmTG1.8-Dmut-CAT) and treated with 0.2 nM TGF-β1, 2.5 nM BMP2, or 2.5 nM BMP4 for 48 h. In contrast to what we observed in Mv1Lu cells, the activity of the pmTG1.8-CAT promoter construct was inhibited by TGF-β1 to approximately 34% of control in MC3T3 E1 cells (Fig. 4). Consistent with our observations in Mv1Lu cells, both BMP2 and BMP4 inhibited the activity of pmTG1.8-CAT. However, as we observed in Mv1Lu cells, the response of all three cytokines was eliminated by mutation of two nucleotides contained within the core of the identified tissue transglutaminase TRE (pmTG1.8-Dmut-CAT, Fig. 4).

To determine if the inhibitory effect of BMP4 on transglutaminase expression reflected a physiological effect on transglutaminase activity, we measured the transglutaminase activity in Mv1Lu and MC3T3 E1 cells treated with either TGF-β1 (0.2 nM), BMP2 (1.25 nM), or BMP4 (1.25 nM; Table I). To correct for any nonspecific changes in transcription resulting from BMP treatment, a separate set of Mv1Lu cells was pulse-labeled with [3H]uridine for 1 h prior to extract preparation. Neither BMP2 nor BMP4 produced a significant alteration in total transcription at 24 or 48 h (data not shown). The level of transglutaminase activity in untreated Mv1Lu cells (266 fmol/mg/h) was decreased 32% by BMP2 and 52% by BMP4 (Table I). Thus, the inhibition of endogenous transglutaminase activity by BMP2 and BMP4 paralleled our observations using the transfected transglutaminase promoter constructs. In MC3T3 E1 cells, TGF-β1, BMP2, and BMP4 treatments all inhibited the endogenous transglutaminase activity (Table I). Similar to our results with Mv1Lu cells, the degree of inhibition for each of these cytokines in MC3T3 E1 cells was similar to the inhibition we observed in our transfection studies.
Transglutaminase TGF-β1 Response Element

Since BMP2 and BMP4 inhibit tissue transglutaminase gene promoter activity in addition to inhibiting the endogenous tissue transglutaminase activity in both Mv1Lu and MC3T3 E1 cells, we investigated whether the effects of BMP2 or BMP4 could be explained by changes in the mRNA levels for tissue transglutaminase. The mRNA for mouse tissue transglutaminase and mouse 36B4 was determined by quantitative reverse transcriptase-PCR using MC3T3 E1 total RNA preparations as described under “Experimental Procedures.” The C\textsubscript{i} values for the sRNA for tissue transglutaminase and 36B4 were used to develop a linear regression equation for use in determining the mRNA levels of each of the samples (Fig. 5A). For both regression equations the correlation coefficient of the line fitting the standards was greater than 0.98. BMP2 and BMP4 significantly lowered the number of molecules of tissue transglutaminase per number of 36B4 molecules compared with control MC3T3 E1 cells.

Our results indicated that both the stimulatory effects of TGF-β1 and the inhibitory effects of BMP4 on the transglutaminase promoter activity are mediated through the same cis-regulatory element. To determine the interaction between TGF-β1 and BMP4, we investigated the effects of co-incubating the tissue transglutaminase gene promoter in the presence of both cytokines (Fig. 6). Mv1Lu cells were transfected with pmTG1.8-CAT and treated with TGF-β1 (0.08 or 0.8 nM) plus BMP4 (0.0, 0.625, or 3.75 nM) for 48 h. In the presence of 0.08 nM TGF-β1, BMP4 was able to significantly lower the TGF-β1 stimulation of pmTG1.8-CAT (p < 0.05). At 3.75 nM BMP4, the TGF-β1 treatment was no longer able to stimulate the pmTG1.8-CAT promoter to a value significantly greater than the control group (p > 0.05). However, using a higher dose of TGF-β1 (0.8 nM), BMP4 was unable to significantly reduce the TGF-β1 stimulation. The results of these experiments suggest a complex interaction between TGF-β1 and BMP4 to regulate the activity of the tissue transglutaminase gene promoter.

**DISCUSSION**

TGF-β superfamily members are important regulators of apoptosis in a number of tissues including liver, mammary gland, prostate gland, and the developing limb (31–38). In these tissues, the apoptotic processes are associated with a marked induction of tissue transglutaminase expression (1). A link between TGF-β superfamily members and tissue transglutaminase is suggested by the fact that TGF-β1 can induce tissue transglutaminase expression in apoptotic cells (14). The studies reported here were initiated to gain further insight into the molecular basis for TGF-β superfamily member regulation of tissue transglutaminase expression. The results we obtained indicate that BMP2 and BMP4, members of the TGF-β family of cytokines, inhibit the activity of the tissue transglutaminase gene promoter in both Mv1Lu and MC3T3 E1 cells. In contrast, our data suggest that TGF-β1 can directly increase the transcriptional activity of the tissue transglutaminase promoter in Mv1Lu cells and inhibit this transcriptional activity in MC3T3 E1 cells. Thus, the effect of TGF-β superfamily members on tissue transglutaminase promoter activity is specific for the cytokine and the cellular context.

**BMPs—**During limb development, tissue transglutaminase is expressed in the same regions (interdigital webs and apical ectodermal ridge) as BMP2 and BMP4 (16, 30, 31). This observation prompted us to examine whether these TGF-β superfamily members could regulate the activity of the tissue transglutaminase promoter. The results of our studies indicate that both BMP2 and BMP4 can negatively regulate the tissue transglutaminase gene promoter activity. This inhibition was discernible in both Mv1Lu and MC3T3 E1 cells, suggesting a ubiquitous activity for these cytokines on tissue transglutaminase activity. In both cell lines, the BMP-mediated inhibition of the tissue transglutaminase gene promoter required the presence of a canonical TRE located 868 bp upstream of the transcription start site. Thus, the effects of BMPs on transglutaminase expression are quite similar to those of retinoids (8) and TNFα (12), both of which regulate tissue transglutaminase expression via activation of specific cis-regulatory elements embedded within the promoter of the gene. For retinoids, the regulation is mediated via a complex retinoid response element located 1703 bp upstream of the transcription start site (8). For TNFα, regulation is mediated through an NF-κB site located in the proximal 5′-flanking DNA (~1338 bp) (12). Thus, factors such as BMPs, retinoids, and TNFα that can regulate apoptosis in different cell types control tissue transglutaminase expression via distinct cis-regulatory elements embedded within the promoter of the gene.

**TGF-β1—** Our studies with TGF-β1 were prompted by the observations that TGF-β1 increases both the activity and mRNA levels for tissue transglutaminase in NHEK cells (14, 15) and that tissue transglutaminase, in turn, cooperates to promote the conversion of latent TGF-β1 to its active form (17, 18). This positive cooperation appears to be restricted to the
TGF-β1-transglutaminase interaction since our studies with other members of the TGF-β superfamily, specifically BMP2 and BMP4, demonstrated a negative regulation of the tissue transglutaminase gene promoter.

Although there are many enzymes that are known to be activated in apoptotic cells, there are very few genes whose expression is induced during the process of apoptosis. P21/WAF1 is a cytoplasmic enzyme that inhibits cyclin-dependent kinase activity in apoptotic cells (39). TGF-β1 induces p21/WAF1 expression via activation of a 10-bp Sp-1/Sp-3 binding motif in the proximal promoter (40). This motif is completely distinct from the TRE we have identified as mediating the activation of the tissue transglutaminase promoter. The reciprocal activity of TGF-β1 and BMPs on gene expression is not restricted to tissue transglutaminase gene expression. Alkaline phosphatase expression in osteoblastic cells (41) and expression of an early response gene, XFD-1, in Xenopus (42) are also reciprocally regulated by TGF-β1 and BMP2 and BMP4. However, the cis-regulatory elements mediating the reciprocal regulation of alkaline phosphatase and XFD-1 are not yet known, whereas in the transglutaminase promoter the same cis-regulatory element mediates both effects. Thus, TGF-β1 and BMPs either promote the association of different trans-acting factors with the transglutaminase TRE or the activity of the TRE-binding protein(s) varies depending on which cytokine is activating the cell.

The pluripotent activity of TGF-β1 is extended by our observations that treatment of MC3T3 E1 cells with TGF-β1 inhibits the activity of the tissue transglutaminase gene promoter and that this inhibitory activity is eliminated by mutation of the tissue transglutaminase TRE. The mechanisms underlying the multiple, cell-specific effects of TGF-β family members is unknown; however, the necessity of an intact TRE within the tissue transglutaminase gene promoter appears to be a common link. The activity mediated by the tissue transglutaminase TRE appears to be cell type- (i.e. Mv1Lu versus MC3T3E1) and cytokine-specific (i.e. TGF-β1 versus BMP2 and BMP4). The specificity of this tissue transglutaminase TRE in addition to its necessity for both TGF-β1 and BMP2 and BMP4 activity suggests that the regulatory activity is determined by the signaling molecules for TGF-β family members, Smad and Mad like proteins (Smads). The signals for activated TGF-β1 or BMP cell-surface receptors are transmitted through phosphorylation of the Smad proteins, resulting in their nuclear accumulation (43–45). Recently, regulation of nuclear localization of the Smad proteins was demonstrated to be mediated through a separate set of Smad proteins that inhibit the nuclear accumulation (46–48).

TGF-β1 suppresses the expression of proteases such as elastase (28), collagenase (23), and stromelysin (23) that degrade the extracellular matrix. TGF-β1 also induces the expression of proteins such as plasminogen activator inhibitor-1 (PAI-1) (22), collagen 1A2 (21), and transglutaminase (14, 21) that stabilize extracellular matrix. Transglutaminase-mediated cross-link-
Transglutaminase TGF-β1 Response Element

The induction or repression of tissue transglutaminase by TGF-β family members may involve the interaction of different trans-acting factors with a common cis-regulatory region within the tissue transglutaminase gene promoter.

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