Transforming Growth Factor-β Regulation of Bone Morphogenetic Protein-1/Procollagen C-proteinase and Related Proteins in Fibrogenic Cells and Keratinocytes*

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Transforming growth factor-β (TGF-β1) induces increased extracellular matrix deposition. Bone morphogenetic protein-1 (BMP-1) also plays key roles in regulating vertebrate matrix deposition; it is the procollagen C-proteinase (PCP) that processes procollagen types I–III, and it may also mediate biosynthetic processing of lysyl oxidase and laminin 5. Here we show that BMP-1 is itself up-regulated by TGF-β1 and that secreted BMP-1, induced by TGF-β1, is either processed to an active form or remains as unprocessed proenzyme, in a cell type-dependent manner. In MG-63 osteosarcoma cells, TGF-β1 elevated levels of BMP-1 mRNA ~7-fold and elevated levels of mRNA for mammalian tolloid (mTld), an alternatively spliced product of the BMP1 gene, to a lesser extent. Induction of RNA was dose- and time-dependent and cycloheximide-inhibitable. Secreted BMP-1 and mTld, induced by TGF-β1 in MG-63 and other fibrogenic cell cultures, were predominantly in forms in which pro-regions had been removed to yield activated enzyme. TGF-β1 treatment also induced procollagen N-proteinase activity in fibrogenic cultures, while expression of the procollagen C-proteinase enhancer (PCPE), a glycoprotein that stimulates PCP activity, was unaffected. In contrast to fibrogenic cells, keratinocytes lacked detectable PCPE under any culture conditions and were induced by TGF-β1 to secrete BMP-1 and mTld predominantly as unprocessed proenzymes.

Bone morphogenetic protein-1 (BMP-1) copurifies from osseogenic bone extracts with transforming growth factor-β (TGF-β)-like proteins BMP-2 through -7 (1). Thus, it was suggested that BMP-1, by structure an astacin-like protease, may function in morphogenesis by activating TGF-β-like molecules (1). Consistent with this possibility, BMP-1 has a domain structure similar to, but shorter than, that of tolloid, a Drosophila protein that appears to act in patterning of embryos by potentiating the activity of decapentaplegic, a TGF-β family member (2, 3). The mammalian BMP1 gene is now known to produce alternatively spliced mRNAs for BMP-1 and for a longer protein, mammalian tolloid (mTld), which has a domain structure identical to that of Drosophila tolloid (4).

Fibillar collagen types I–III are synthesized as procollagens, precursors containing N- and C-terminal propeptides that are cleaved extracellularly to yield mature triple helical monomers capable of associating into fibrils (for a review, see Ref. 5). Recently, BMP-1 was shown to be identical to procollagen C-proteinase (PCP) (6, 7), the activity that cleaves the C-propeptides of procollagen types I–III (8–10), and mTld has also been found to have PCP activity (7). Demonstration of PCP activity, however, does not preclude the possibility that BMP-1 and/or mTld may also activate TGF-β-like proteases. In fact, it is becoming increasingly apparent that products of the BMP1 gene play multiple roles in matrix deposition. These include proteolytic activation by fibrogenic cells of lysyl oxidase (11), an enzyme necessary to formation of covalent cross-links in fibillar collagens and elastin, and biosynthetic processing by keratinocytes of laminin 5 (12), a major basement membrane component of skin. PCP activity of BMP-1 is stimulated ~10-fold by the procollagen C-proteinase enhancer (PCPE), a glycoprotein that binds the type I procollagen C-propeptide (10). However, possible involvement of PCPE in other biological activities of BMP-1 and mTld has not been examined.

TGF-β1, prototype of the TGF-β superfamily, induces net increases in the deposition of insoluble matrix by cells. This is accomplished by effecting decreased production of proteases that degrade matrix and increased production of (i) inhibitors for such proteases, (ii) structural matrix components such as procollagen types I–III (13), and (iii) lysyl oxidase (14). Expression of the genes for the three polypeptide chains of laminin 5 is also up-regulated by TGF-β in keratinocytes (15). The induction by TGF-β1 of gene products involved in deposition of matrix in general, and of known and potential substrates of BMP-1/mTld in particular, prompted us to examine whether TGF-β1 also regulates BMP-1/mTld expression and/or the expression of PCPE. Here we document the effects of TGF-β1 on trophoresis; PBS, phosphate-buffered saline.

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§ The abbreviations used are: BMP-1, bone morphogenetic protein-1; TGF-β, transforming growth factor-β; mTld, mammalian tolloid; N-propeptide, amino-terminal propeptide; C-propeptide, carboxyl-terminal propeptide; pNα1(I), processing intermediate of procollagen type I chain; pCα1(I), processing intermediate of procollagen type I chain; Pα1(I), processing intermediate of procollagen type I chain; PBS, phosphate-buffered saline.

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BMP-1, mTld, and PCPE expression in fibrogenic cells and keratinocytes and on levels of cleavage of type I procollagen C- and N-propeptides. Mechanisms for the post-translational regulation of BMP-1 and mTld activity are also noted, and implications of the various data for the regulation of matrix deposition are discussed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MG-63 human osteosarcoma cells were purchased from the American Type Culture Collection (Rockville, MD). MC3T3-E1 murine osteoblastic cells were obtained from Dr. Richard Wenslup (Children’s Hospital Research Foundation, Cincinnati, OH), and human AH1F neonatal foreskin fibroblasts and primary keratinocytes were obtained from Dr. Lynn Allen-Hoffman (University of Wisconsin, Madison, WI). MG-63, MC3T3-E1, and AH1F cells were maintained in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated (30 min, 55 °C) fetal calf serum. MC3T3-E1 cells were supplemented with 1% nonessential amino acids. For experiments in which RNA was prepared from MG-63 cells, media contained 0.1% heat-inactivated fetal calf serum. For experiments in which samples for immunoblots were prepared from MG-63, MC3T3, or AH1F cultures, media was serum-free unless otherwise indicated. Keratinocytes were maintained in 0.15 mM CaCl₂, 0.1 mM KGM BulletKit medium (Clonetics) with 30 μg/ml bovine pituitary extract. All cell types were cultured and maintained as described (10). PCPE antibodies, isolated from an IgG fraction by precipitation with protein A-Sepharose (Amersham Corp.), were radiolabeled to a specific activity of 3000 Ci/mmol. The antibodies were affinity-purified on columns of the mTld 1-18 peptide (Quality Controlled Biochemicals, Madison, WI). BMP-1/mTld prodomain antigen was purified from mice transfected with the plasmid encoding the BMP-1/mTld prodomain (Sigma). Western Blots—Protease inhibitors (2.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM α-phenanthroline), and proteases were precipitated by adding trichloroacetic acid to 10%. Pellets were washed twice with cold acetone and then washed twice with 75% ethanol, 12.5 mM Tris (pH 7.5), dried, and resuspended in SDS sample buffer with 5% β-mercaptoethanol. Cell layers were scraped into hot SDS sample buffer, as described (23). Media and cell layer samples, equivalent to 5 × 10⁶ fibrogenic cells or keratinocytes per lane, were subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore) by electroblotting for 1.5 h at 10 V, 190 mA, 10% methanol at 4 °C. Blots were incubated ~14 h with primary antibody diluted 1:5000 in PBS, 1% bovine serum albumin, 0.05% Tween-20. After washing three times with wash buffer (PBS, 0.05% Tween-20), blots were incubated 1 h with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) diluted 1:4000. Blots were then washed four times with wash buffer, incubated with SuperSignal CL-HRP substrate (Pierce), and exposed to film. Film images were obtained with a Kodak Disc Autoprobe (Kodak). To quantitate relative amounts of BMP-1, gel lanes were loaded with sample corresponding to the medium of 2.5 × 10⁶ untreated MG-63 cells or to the medium of 1.25 × 10⁶, 5.0 × 10⁶, or 2.5 × 10⁷ TGF-β1-treated MG-63 cells. After SDS-PAGE, a blot was prepared, treated with antibody to the BMP-1 C terminus (as above), and then incubated with 32P-labeled protein A (Amersham) for 1 h in PBS, 1% bovine serum albumin, 0.05% Tween-20, 1 mM dithiothreitol. The blot was washed 8 h with wash buffer and exposed to film, and the autoradiograph used as a template for excising radioactive BMP-1 bands, which were counted in an A 8000 cyclorad (Packard).

For digestion with peptide-N-glycosidase F, proteins were precipitated from media with trichloroacetic acid (as above); denatured in 0.5% SDS, 1% β-mercaptoethanol for 10 min at 100 °C; and incubated 1 h at 37 °C with 500 units of peptide-N-glycosidase F (New England Biolabs) in 50 mM sodium phosphate (pH 7.5), 0.1% SDS, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml soybean trypsin inhibitor, 10 mM benzamidine; and the reaction was stopped by adding 4 × SDS sample buffer and heating for 4 min at 100 °C.

**In Vitro Procollagen Cleavage Assays**—32P-labeled procollagen substrate prepared from chick embryo tendon cultures (8) was kindly provided by Dr. Darwin Prockop (Allegheny University, Philadelphia, PA). Media tested for PCP activity contained 1 μM each phenylmethysulfonyl fluoride, α-phenanthroline, and N-ethylmaleimide; was concentrated 50-fold in Centriprep concentrators (Amicon); and washed twice with 0.125 M Tris-HCl, 0.1 mM NaCl, 5 mM CaCl₂, 5 μl of concentrated medium was added to 0.125 μl of substrate in 5 μl of 0.1 mM Tris-HCl, 0.1 mM NaCl, 5 mM CaCl₂, 0.02% Brij 35 (pH 7.6) and incubated for 4 h at 35 °C. Reactions were stopped by adding 5 μl of 3 × SDS sample buffer and heating for 4 min at 100 °C prior to SDS-PAGE on nonreduced 6% gels. Gels were fixed in 20% MeOH, placed in Amplify (Amersham) for 30 min, dried, and exposed to film. To quantitate the amount of cleavage, the area under each band was calculated for each lane of an autoradiogram by comparing signal remaining in the pro-γ band (procollagen in which cleavage has not occurred in any of the three PCP cleavage sites) to total signal (pro-γ + pro-β + pro-α + pNα1 + α1 + pNα2 + α2 + free C-propeptide bands) (see Fig. 5). The percentages of cleavage achieved with media from cultures treated with TGF-β1, ascorbate, or TGF-β1 plus ascorbate were then compared with the percentages of cleavage achieved with media from untreated controls to calculate -fold increases in PCP activity. Signals on autoradiograms were quantitated with an IS1000 digital imaging system (Alpha Innovex).

**R Nas protection Assays**—To generate BMP-1/mTld-specific riboprobes, a 1619-bp Apal-EagI fragment was excised from a baculovirus transfer vector containing full-length human BMP-1 cDNA (6). The fragment, extended from nuclease 615 of the BMP-1 cDNA sequence (1) to an EagI site in the vector polylinker, was excised by the SphI restriction enzyme and ligated into pBluescript KS+ (Stratagene) Apal and EagI sites and linearized at a HindII site at nucleotide 1655 of the BMP-1 sequence (1). This template produced a 617-base riboprobe of which 575 bases are BMP-1 specific and 481 bases are specific for alternatively spliced mTld RNA. For PCP-specific riboprobes, a 328-bp fragment of PCP cDNA clone pBluescript KT3 (16) was excised with EcoRI and SacII and inserted between the pBluescript KS+ plasmid vector pEcoRI and SacII sites. This template, linearized at the pBluescript XhoI site, produced a 391-base riboprobe, 329 bases of which correspond to nucleotides 557–885 of the human PCP cDNA sequence (16). Control riboprobes were generated with template pTRI-b-actin-Human (Ambion). Uniformly 32P-labeled riboprobes were gener-
Regulation of BMP-1 by TGF-β

RESULTS

TGF-β Selectively Increases Levels of BMP-1 and mTld, but Not PCPE, mRNA in MG-63 Cells—To determine whether levels of PCPE, BMP-1, and/or mTld mRNA are influenced by treatment with TGF-β, just-confluent MG-63 cultures were treated for 24 h with 2 ng/ml TGF-β1. Levels of the ∼3.0-kb BMP-1 mRNA and ∼5.5- and ∼4.1-kb mTld mRNAs (4) were all increased by TGF-β1 treatment (Fig. 1). Interestingly, mRNAs for the two proteins did not increase to the same extent, with BMP-1 mRNA increasing ∼7-fold and both mTld mRNA forms increasing only ∼4-fold. By comparison, levels of the 4.8- and 5.8-kb pro-α(1) collagen mRNAs (25) increased ∼9-fold, while those of the β-actin mRNA control did not change. In contrast to the induction of BMP-1 and mTld mRNA, levels of PCPE mRNA were not increased by TGF-β1.

Incubation of MG-63 cells with TGF-β concentrations ranging from 0.01 to 10 ng/ml showed induction of BMP-1 and mTld mRNAs to be dose-dependent (Fig. 2A), with substantial increases first noted at 0.5 ng/ml and maximum induction at 2 ng/ml. Induction of mTld and BMP-1 mRNAs was somewhat less at 10 ng/ml than at 2 ng/ml, possibly due to the toxicity of TGF-β1 at higher concentrations (26). When kinetics of induction of BMP-1/mTld mRNAs were examined by incubating MG-63 cultures in 2 ng/ml TGF-β1 for varying times (Fig. 2B), substantial increases first occurred at 12 h, with maximal levels attained at 24 h post-treatment. Thus, induction was delayed compared with induction of RNAs encoding a variety of extracellular matrix proteins, which generally occurs within 3–5 h of TGF-β treatment of various cell types (13). This delay suggested that induction of BMP-1/mTld mRNAs may occur secondarily to an earlier event triggered by TGF-β1, a possibility supported by experiments in which treatment of confluent cultures with cycloheximide prior to TGF-β1-treatment showed protein synthesis to be required prior to induction of BMP-1/mTld mRNAs (Fig. 2C).

Levels of Secreted BMP-1 and mTld, but Not PCPE, are Up-regulated by TGF-β1 in Fibrogenic Cell Cultures—To determine whether induction of steady state levels of BMP-1 and mTld mRNA in MG-63 cells was paralleled by increases in the cognate proteins, levels of BMP-1 and mTld protein secreted into culture media were examined by Western blots. Antibodies specific for the BMP-1 C terminus detected bands of ∼88 and ∼77 kDa, both of which were strongly up-regulated by TGF-β1 (Fig. 3A). Antibody to the mTld C terminus detected a ∼130-kDa band that was up-regulated by TGF-β1 to a lesser extent than the anti-BMP-1-reactive bands. The relative extent of induction of BMP-1- and mTld-specific bands, thus appeared similar to the relative extent of induction of the cognate mRNAs.

To quantitate the induction of secreted BMP-1, Western blots were incubated with antibody to the BMP-1 C terminus and then with 125I-protein A, to allow subsequent excision and quantitating of counts in BMP-1 bands. This method, which can provide good quantitation of relative amounts of proteins (27), showed levels of the 88-kDa BMP-1 band to be ∼8-fold higher in TGF-β1-treated MG63 cultures than in untreated cultures (not shown). Thus, the level of induction of secreted BMP-1 was similar to the level of induction of cognate mRNA. The 77-kDa band was not detected on these blots (Fig. 3D, lane 3) and was determined to be unrelated to BMP-1 (see below).

Ascorbate may stimulate production of type I procollagen at transcriptional and posttranscriptional levels (28–30). Since we were interested in mechanisms that might contribute to co-expression of BMP-1 and type I procollagen, we examined whether levels of BMP-1 were also up-regulated by ascorbate. Although the intensity of BMP-1 and mTld protein bands did not appreciably increase in the presence of ascorbate alone, intensities seemed somewhat higher in the presence of TGF-β1.
plus ascorbate than in the presence of TGF-β1 alone (Fig. 3A). In contrast to BMP-1 and mTld, levels of secreted PCPE did not increase in MG-63 cultures treated with TGF-β1, ascorbate, or ascorbate plus TGF-β1 (Fig. 3A). Thus, as with BMP-1 and mTld, levels of secreted PCPE paralleled levels of cognate mRNA.

To ascertain whether results like those obtained with MG-63 cells were common to other fibrogenic cells, analyses were done on MC3T3-E1 osteoblastic mouse cells and human dermal fibroblasts (Fig. 3, B and C). Results obtained with these cells were similar to those observed with MG-63 cells in that levels of secreted BMP-1 and mTld were elevated in the presence of TGF-β1 and appeared to be slightly more elevated in the presence of TGF-β1 plus ascorbate. As with MG-63 cells, levels of PCPE were not dramatically changed in a consistent way by the addition of TGF-β1 and/or ascorbate.

Some Western blots using BMP-1 C terminus antibody detected a ~77-kDa band, in addition to the ~88-kDa BMP-1 band, in MG-63 media (Fig. 3, A and D, lane 2). This band, without counterpart in MC3T3-E1 or fibroblast cultures, was not always detected in MG-63 cultures treated with TGF-β1 or TGF-β1 plus ascorbate and was absent upon culturing in the presence of 0.1% serum (Fig. 3D, lane 1) or when protein A was used instead of secondary antibody. Thin and thick arrows denote 101-kDa latent and 88-kDa processed forms of BMP-1, respectively. Arrowheads denote the 77-kDa artifactual band. SDS-PAGE gels for mTld, BMP-1, and PCPE blots were 7.5, 10, and 12% polyacrylamide, respectively.

### Fig. 3. Secreted BMP-1 and mTld, but not PCPE, are induced by TGF-β1 treatment of fibrogenic cells. Media from MG-63 (A), MC3T3-E1 (B), and AH1F fibroblast (C) cultures were examined by immunoblotting for secreted BMP-1, mTld, and PCPE. Lanes labeled C, T, A, and TA are from untreated control cultures or cultures treated with TGF-β1, ascorbate, or ascorbate plus TGF-β1, respectively. D, variability in detection of a 77-kDa band is demonstrated in MG-63 cells grown in the presence of 0.1% heat-inactivated serum (lane 1) or in the absence of serum (lanes 2 and 3). Antibodies to the C termini of mTld and BMP-1 or to full-length PCPE were primary antibodies for the various blots. For the blot in D, lane 3, 125I-labeled protein A was used instead of secondary antibody. Thin and thick arrows denote 101-kDa latent and 88-kDa processed forms of BMP-1, respectively. Arrowheads denote the 77-kDa artifactual band. SDS-PAGE gels for mTld, BMP-1, and PCPE blots were 7.5, 10, and 12% polyacrylamide, respectively.

### Fig. 4. Cleavage of endogenous type I procollagen C- and N-propeptides in MG-63 cultures. MG-63 media (A) or cell layers (B) were examined by Western blot analysis using primary antibodies directed against the pro-α1(I) N-propeptide (parts a), α1(I) C-telopeptide (parts b) or pro-α1(I) C-propeptide (parts c). Lanes C, T, A, and TA are from untreated control cultures or from cultures treated with TGF-β1, ascorbate, or ascorbate plus TGF-β1, respectively. SDS-PAGE gels were 7.5% polyacrylamide.

pNα1(I) chains in media of untreated cells or, surprisingly, in media of cells treated with TGF-β1 alone, although the latter contained relatively high levels of procollagen (Fig. 4A), BMP-1, and mTld (Fig. 3A). In contrast, pNα1(I) chains were found in media of ascorbate-treated cells, in a pro-α1(I):pNα1(I) ratio of ~1.0:0.3, and at higher levels in media of cells treated with TGF-β1 plus ascorbate, with a pro-α1(I):pNα1(I) ratio of ~1.0:0.8 (Fig. 4A, part a).

Pro-α1(I) C-telopeptide antibody, capable of detecting α1(I), pNα1(I), and pCa1(I) chains (Fig. 4A, part b), revealed relative levels of pro-α1(I) and pNα1(I) chains in the various media, similar to those detected by the first antibody. In addition, pCa1(I) and mature α1(I) chains were detected in media of cells treated with TGF-β1 plus ascorbate. In the latter sample, the ratio of unprocessed pro-α1(I) chains to chains from which the C-propeptide had been cleaved (pNα1(I) + α1(I)) was ~1.0:2.7, a value ~10-fold greater than the pro-α1(I):pNα1(I) ratio (~1.0:0.3) detected in the ascorbate-treated sample by the same antibody. Thus, although cultures treated with TGF-β1 plus ascorbate contained substantially more substrate than cultures treated with ascorbate alone, a greater fraction had undergone removal of C-propeptides, indicating significantly increased PCP activity. A diffuse band, about the size of pCa1(I), faintly detected by C-telopeptide antibody in samples treated with TGF-β1 alone (Fig. 4A, part b) was not detected by C-propeptide-specific antibody (see below) and probably represents partially degraded pro-α1(I) chains.

The appearance of quantities of pCa1(I) and α1(I) chains only in media of MG-63 cells treated with TGF-β1 plus ascorbate, indicated that procollagen N-proteinase (PnP) activity is also elevated under these conditions. Highlighting this induction, pro-α1(I) C-propeptide antibody detected pCa1(I) chains only in media of cells treated with TGF-β1 plus ascorbate (Fig. 4A, part c). Since the N-propeptides of procollagens I and III...
are cleaved by two different PNP enzymes (31, 32), and since MG-63 cells produce procollagen III (33). Western blots using antibody against the type III procollagen C-propeptide were performed and showed that procollagen III PNP activity is also elevated in the presence of TGF-β1 plus ascorbate (not shown).

Since procollagen processing may help regulate incorporation of monomers into fibrils (34), levels of processed α1(I) chains incorporated into MG-63 cell layers were also examined. Consistent with results obtained from media, C-telopeptide antibody found high levels of α1(I) chains in cell layers treated with TGF-β1 plus ascorbate, while untreated cell layers or cell layers treated with TGF-β1 alone did not contain detectable α1(I) (Fig. 4B, part b). Cell layers treated with ascorbate or with ascorbate plus TGF-β were enriched for α1(I) chains compared with media from the same cultures (Fig. 4A and B, parts b). This enrichment may reflect preferential incorporation of mature monomers into growing fibrils, rather than increased processing in cell layers compared with media. Consistent with this likelihood, pNα1(I), but not pCo1(I), chains were found in cell layers treated with ascorbate or with TGF-β1 plus ascorbate (Fig. 4B, parts a, b, and c), appearing to confirm results previously obtained with in vitro fibrillogensis systems (34), suggesting that pNα1(I) but not pCo1(I) chains are incorporated into growing fibrils. Unprocessed pro-α1(I) in MG-63 cell layers (Fig. 4B) was likely intracellular, since procollagen is not thought to be incorporated into fibrils (34). As a result, a diffuse band about the size of pCo1(I), detected in some cell layers by C-telopeptide and N-propeptide antibodies but not by C-propeptide antibody, probably represents partially degraded pro-α1(I) chains.

PCP and PNP Activity against Exogenous Procollagen Substrate Is Increased in Media of TGF-β1-treated MG-63 Cultures—It seemed paradoxical that although secreted BMP-1 and mTld were elevated to only slightly higher levels in MG-63 cultures treated with TGF-β1 plus ascorbate than in cultures treated with TGF-β1 alone (Fig. 3A), processing of endogenous procollagen was detectable only in the former (Fig. 4). Ascorbate is a necessary cofactor for enzymic hydroxylation of collagen prolyl residues, leading to a more stable triple helix (35). Thus, to examine whether differences in endogenous substrate might contribute to differences observed in levels of processing, MG-63 media were assayed for PCP and PNP activity using exogenous radiolabeled procollagen substrate. As with endogenous substrate, PCP and PNP activities against exogenous substrate were highest in media of cells treated with TGF-β1 plus ascorbate, as evidenced by the generation of mature α-chains, processing intermediates, and free C-propeptides (Fig. 5). However, in contrast to results with endogenous substrate, PCP and PNP activities against exogenous substrate were increased in media of cells treated with TGF-β1 alone, to levels approaching those of cultures treated with TGF-β1 plus ascorbate. Thus, in seven independent experiments, one of which is shown in Fig. 5, the mean increase of PCP activity against exogenous substrate was 2.4 ± 0.2-fold for cultures treated with TGF-β1 plus ascorbate and 1.7 ± 0.1-fold for cultures treated with TGF-β1 alone (values are mean ± S.E.). PCP activity in media of cultures treated with ascorbate alone was virtually unchanged, 1.0 ± 0.1-fold, compared with untreated controls. These results suggest that observed differences in processing of endogenous substrate in cultures treated with TGF-β1 alone, compared with cultures treated with TGF-β1 plus ascorbate, were at least partly due to differences in the hydroxylation state and conformation of the substrate.

Most BMP-1 and mTld Secreted by Fibrogenic Cells in Response to TGF-β1 Is Processed to Mature, Active Forms—Proteases of the astacin family are synthesized as proenzymes with N-terminal proregions that must be removed for activation (36, 37). Thus, the lesser induction of PCP activity against exogenous substrate (~1.7-fold for TGF-β1-treated cultures), relative to levels of induction of secreted BMP-1 detected by quantitative Western blots (~8-fold for TGF-β1-treated cultures), might have been due to secretion of most BMP-1 and mTld as inactive precursors. To ascertain whether this was the case, antibody to proregion sequences common to BMP-1 and mTld was prepared and used to examine immunoblots of MG-63 medium samples. This antibody did not recognize the major 88-kDa BMP-1 band but did recognize a ~101-kDa band that also appears as a minor band on immunoblots using the BMP-1 C terminus antibody (Figs. 6A and 3A). Thus, although a small proportion is unprocessed, the majority of TGF-β1-induced BMP-1 secreted by the fibrogenic cells is in the processed active form. In addition, the proregion antibody did not recognize the ~130-kDa band detected by mTld C terminus antibody but did recognize a ~143-kDa band (Fig. 6A). Thus, the majority of mTld secreted by TGF-β-treated fibrogenic cells is also processed to the mature form, while a small proportion, at levels undetectable with the mTld C terminus antibody, is unprocessed. Interestingly, in repeated experiments of the type shown in Fig. 6A, media of MG-63 cells treated with TGF-β1 alone consistently had lesser amounts of activated, and greater amounts of unprocessed, BMP-1 and mTld than did cultures treated with TGF-β1 plus ascorbate. Thus, additional processing of BMP-1 and mTld into mature forms seems to occur in the presence of ascorbate, perhaps accounting for the higher levels of PCP activity against exogenous substrate in media of cultures treated with TGF-β1 plus ascorbate than in media of cultures treated with TGF-β1 alone.

Previously, recombinant human BMP-1 produced in a baculovirus system by this laboratory was shown to have high PCP activity and to be processed to the mature form (6). Thus, BMP-1 secreted by TGF-β1-treated fibrogenic cells should have the same electrophoretic mobility as the recombinant BMP-1, if in an active processed form. Electrophoretic mobilities of BMP-1 from TGF-β1-treated fibroblast media and from the baculovirus system were compared after treatment of samples with peptide-N-glycosidase F to control for mobility differences due to differences in Asn-linked glycosylation between insect
and mammalian cells (6, 38). Upon removal of Asn-linked carbohydrates, the major form of BMP-1 secreted by TGF-1 \(-\)-treated fibroblasts had the same mobility as recombinant active BMP-1 (Fig. 6B), bolstering the conclusion that most BMP-1 secreted by fibrogenic cells in response to TGF-1 \(-\)-is processed to the mature form.

The Major Secreted Product of the BMP1 Gene in TGF-1 \(-\)-treated Human Keratinocytes Is Unprocessed mTld—It was of interest to determine whether the patterns of expression and regulation of BMP-1 and mTld described above are also found in non-fibrogenic cells. Keratinocytes were chosen for study, since they do not produce readily detectable fibrillar collagens but do appear to utilize BMP-1 and/or mTld for processing to contain higher levels of BMP-1 RNA (Fig. 7C). Thus, secretion of predominantly mTld by keratinocytes and predominantly BMP-1 by MG-63 cells is reflected, at least in part, in levels of cognate RNA transcripts. RNase protection assays of 20 \(\mu\)g of total RNA from TGF-1 \(-\)-treated MG-63 cells (\(M\)) or keratinocytes (\(K\)). Protected fragments were electrophoresed on 6% sequencing gels. The arrows denote 575-, 481-, 329-, and 245-nucleotide protected fragments specific for BMP-1, mTld, PCPE, and the \(\beta\)-actin control, respectively.

DISCUSSION

The necessary action of BMP-1 and mTld in processing of matrix components (6, 7, 12) and lysyl oxidase (11) implies that these proteins play key roles in controlling the deposition of matrix in developmental and homeostatic processes. Previously, however, mechanisms for regulating functional expression of these key proteins have not been explored. In this study we have demonstrated that TGF-1 \(-\) elevates levels of BMP-1 and mTld in fibrogenic cells and keratinocytes. PNP activity against procollagens I and III was also elevated by TGF-1 \(-\) in fibrogenic cells. Thus, in addition to increasing the deposition of matrix through induction of matrix components, lysyl oxidase, and metalloprotease inhibitors, TGF-1 also influences

![Figure 6](image1.png)

**Fig. 6.** Most BMP-1 and mTld proteins secreted by fibrogenic cells in response to TGF-1 \(-\) are processed to mature, active forms. A, duplicate lanes of samples from MG-63 media of untreated control cultures (lane \(C\)), cultures treated with TGF-1 \(-\) (lane \(T\)), ascorbate (lane \(A\)), or ascorbate plus TGF-1 \(-\) (lane \(TA\)), were electrophoresed on the same SDS-PAGE gel and assayed on separate immunoblots with antibody to the mTld C terminus (mTld-C), the BMP-1/mTld proregion (Proregion), or the BMP-1 C terminus (BMP-C). Thin arrows denote 143- and 101-kDa latent forms of mTld and BMP-1, respectively. Thick arrows denote the 130- and 88-kDa processed forms of mTld and BMP-1, respectively. B, medium samples from AH1F fibroblasts (AH) or from a baculovirus system producing recombinant BMP-1 (6) (B) were treated (\(+\)) or untreated (\(-\)) with peptide-N-glycosidase F (PNGase F) prior to SDS-PAGE and immunoblotting with antibody to the BMP-1 C terminus. SDS-PAGE gels were 7.5% (A) and 10% (B) polyacrylamide.

![Figure 7](image2.png)

**Fig. 7.** Keratinocytes produce predominantly unprocessed mTld in response to TGF-1 \(-\) and do not produce detectable PCPE. For panels A and B, sample loaded in each lane corresponds to medium of 5 \(\times\) 10\(^5\) cells. A and B, samples from keratinocyte media of untreated control cultures (lane \(C\)) or cultures treated with TGF-1 \(-\) (lane \(T\)), ascorbate (lane \(A\)), or ascorbate plus TGF-1 \(-\) (lane \(TA\)) or are from MG-63 medium of cultures treated with TGF-1 \(-\) (T\(^*\)). The immunoblot in panel A was with antibody to PCPE. The large immunoblot in panel B was with antibody to the BMP-1/mTld proregion; the small immunoblot was with antibody to the BMP-1 C terminus. The two blots in \(B\) are from different lanes of the same gel. Thin arrows denote the 143- and 101-kDa latent forms of mTld and BMP-1, respectively. The thick arrow denotes 88-kDa processed BMP-1. C, autoradiograms of RNase protection assays of 20 \(\mu\)g of total RNA from TGF-1 \(-\)-treated MG-63 cells (\(M\)) or keratinocytes (\(K\)). Protected fragments were electrophoresed on 6% sequencing gels. The arrows denote 575-, 481-, 329-, and 245-nucleotide protected fragments specific for BMP-1, mTld, PCPE, and the \(\beta\)-actin control, respectively.
The induction of BMP-1 and mTld by TGF-β1, described here, is particularly intriguing in the context of previous suggestions that BMP-1- and tolloid-like proteins may activate TGF-β-like molecules (1–3). TGF-β1 is itself secreted as a latent form (40), and the possibility, therefore, exists of a positive feedback loop in which BMP-1 and/or mTld activate, and are in return induced by, TGF-β1. There is a precedent for such positive feedback loops in matrix deposition, since the effects of TGF-β1 on matrix deposition are amplified and prolonged through autoinduction by TGF-β1 of its own expression (41). Preliminary studies with recombinant proteins suggest that BMP-1 may indeed be capable of directly activating the TGF-β1 small latent complex. 

Removal of the proregion from astacin-like proenzymes is thought necessary for the production of active forms of these enzymes (37). Thus, persistence or removal of the proregion represents another potential control point for regulating BMP-1 and mTld activities. In the present study, the degree of processing of secreted BMP-1 and mTld is shown to be cell type-specific, with predominantly processed forms produced by TGF-β1-treated fibrogenic cells and predominantly unprocessed forms produced by TGF-β1-treated keratinocytes. Clearly, the production of large amounts of activated BMP-1 and mTld would aid fibrogenic cells in their highly specialized roles of producing large quantities of fibrillar collagen matrix, especially in response to TGF-β. It is less clear why keratinocytes produce predominantly unprocessed, inactive forms of mTld and BMP-1 in response to TGF-β. Consistent with this finding, however, is the observation that processing of γ2, the laminin 5 chain cleaved by mTld and/or BMP-1, is delayed following secretion by cultured keratinocytes (12). Thus, extracellular processing of mTld and BMP-1 may be a rate-limiting step in the deposition of keratinocyte extracellular matrix, of which laminin 5 is a major component (42, 43). In vivo, such processing may be regulated by epithelial-mesenchymal interactions that influence the production of matrix by basal keratinocytes (44). Keratinocytes were found not to produce detectable amounts of PCP, suggesting that PCP may not play a role in laminin 5 processing. However, the possibility that, in vivo, PCP may be provided by dermal fibroblasts for this purpose has not been precluded.

Interestingly, the relative amounts of BMP-1 and mTld produced by cells were also found to be cell type-specific: fibrogenic cells produced relatively large amounts of BMP-1, while keratinocytes produced predominantly mTld, at both RNA and protein levels. The possible functional significance for the production of differing ratios of BMP-1 and mTld by different cell types remains to be determined, however, since a functional difference has yet to be discerned for these two protein products of the same gene.

Previously, the low levels of PCP activity detectable in tissues and in cell culture systems have led to suggestions that the enzyme is either secreted as an inactive precursor or co-expressed with an endogenous inhibitor (8). Either of these possibilities might have explained the discrepancy observed in the present study between the induction by TGF-β1 in MG-63 cultures of an ∼8-fold increase in secreted BMP-1 and a ∼2-fold increase in medium PCP activity against exogenous substrate. However, since secreted BMP-1 and mTld were both found predominantly as processed forms, this leaves the interesting possibility of an endogenously produced inhibitor. Prevously, localized control over the activities of various proteins, including degradative metalloproteases such as astromelysins and collagenases, has been shown to involve not only the processing of proenzymes to mature forms but also the balance between levels of activated enzyme and levels of specific inhibitors co-expressed by the same cell types (45). Studies to determine the possible existence of inhibitors for BMP-1, mTld, and related proteases (46) seem warranted by results presented in the current study. Clearly, such inhibitors, should they exist, could play roles in morphogenetic processes as important as those of the proteases with which they interact.

A final point of interest relates to the observed processing of endogenous procollagen in MG-63 cultures treated with TGF-β1 plus ascorbate and the absence of such processing in cultures treated with TGF-β1 alone. Both cultures secreted high levels of similarly processed BMP-1 and mTld. Moreover, the TGF-β1 plus ascorbate-treated cultures had only slightly higher PCP activity against exogenous substrate, corresponding to a slightly higher ratio of activated to unprocessed BMP-1. One interpretation of these data is that fully hydroxylated procollagen produced by ascorbate-treated cultures, or supplied as exogenous substrate, is much better substrate for BMP-1 than is underhydroxylated procollagen produced in the absence of ascorbate. Since underhydroxylated procollagen is not likely to be in a compact triple helical form at 37 °C (35), these data might indicate some conformational requirement for the cleavage of procollagen by PCP. However, this would be in contrast to an earlier report (8) that found PCP to cleave heat-denatured procollagen with about the same efficiency as native procollagen.

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