Bone morphogenetic protein-1 processes probiglycan

Received for publication, June 5, 2000, and in revised form, July 6, 2000
Published, JBC Papers in Press, July 14, 2000, DOI 10.1074/jbc.M004846200

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Bone morphogenetic protein-1 (BMP-1) is a metalloprotease that plays important roles in regulating the deposition of fibrous extracellular matrix in vertebrates, including provision of the procollagen C-proteinase activity that processes the major fibrillar collagens I–III. Biglycan, a small leucine-rich proteoglycan, is a nonfibillar extracellular matrix component with functions that include the positive regulation of bone formation. Biglycan is synthesized as a precursor with an NH²-terminal propeptide that is cleaved to yield the mature form found in vertebrate tissues. Here, we show that BMP-1 cleaves biglycan at a single site, removing the propeptide and producing a biglycan molecule with an NH² terminus identical to that of the mature form found in tissues. BMP-1-related enzyme activities in mammalian Tolloid and mammalian Tolloid-like 1 (mTLL-1) are shown to have low but detectable levels of probiglycan-cleaving activity. Comparison shows that wild type mouse embryo fibroblasts (MEFs) produce only fully processed biglycan, whereas MEFs derived from embryos homozygous null for the Bmp1 gene, which encodes both BMP-1 and mammalian Tolloid, produce predominantly unprocessed probiglycan, and MEFs homozygous null for both the Bmp1 gene and the mTLL-1 gene Tlll produce only unprocessed probiglycan. Thus, all detectable probiglycan-processing activity in MEFs is accounted for by the products of these two genes.

Bone morphogenetic protein-1 (BMP-1) is the prototype of a family of metalloproteases involved in morphogenesis in a broad range of species (1). BMP-1 and mammalian Tolloid (mTLD), a somewhat larger protein encoded by alternatively spliced RNAs of the Bmp1 gene (2), affect morphogenesis, at least in part, by providing the procollagen C-proteinase (PCP) activity that cleaves the C-propeptides of procollagens I–III to yield the major fibrous components of extracellular matrix (ECM) (3–6). These two proteases also contribute to the net deposition of insoluble ECM through proteolytic activation of lysyl oxidase (7), an enzyme necessary to the formation of covalent cross-links in collagen and elastic fibers. Two additional, genetically distinct, BMP-1/mTLD-related mammalian proteases have been described and designated mammalian Tolloid-like 1 (mTLL-1) and mTLL-2, due to domain structures identical to that of mTLL (5, 8). Although mTLL-1 has some PCP activity in vitro assays (5), the significance of this activity is unclear, as probiglycan processing appears unaffected in mTLL-1-deficient mice (9). PCP activity was not detected in vitro assays of mTLL-2 (5).

Recently, BMP-1/mTLL-related proteases Xenopus Xolloid (10) and zebrafish Tolloid (11) were shown to exert ventralizing effects during vertebrate embryogenesis by cleaving the secreted protein Chordin, which forms latent complexes with ventralizing TGF-ß-like molecules, such as BMPs 2 and 4 (12). BMP-1 and mTLL-1 are also capable of affecting dorsal-ventral patterning through cleavage of Chordin, whereas mTLD and mTLL-2 do not have detectable levels of this activity (5). In later development, BMP-1 and Chordin have been shown to have similar expression patterns in pre- and postnatal endochondral bone formation (5), whereas BMP-1 copurifies with TGF-ß-like BPs from osteogenic extracts of bone (13). Thus, BMP-1 and related proteases may serve to coordinate the deposition of ECM with the activation of certain TGF-ß-like BPs in early development and later in the development of bone and other tissues. Determining the extent of involvement of BMP-1 and related proteases in morphogenetic events will require identifying the range of substrates processed by each in vitro.

Vertebrate ECM largely comprises insoluble collagenous fibers and a hydrated interfibrillar network, predominantly composed of proteoglycans. Both large, aggregating and small, nonaggregating proteoglycans are thought to affect development and homeostasis through interactions with macromolecular structures of the ECM, growth factors, and cell surfaces (14). Biglycan and decorin are small, nonaggregating proteoglycans that contain either chondroitin sulfate or dermatan sulfate side chains and belong to the family of small leucine-rich proteoglycans (SLRPs) of the ECM. There are at least nine SLRPs, including lumican and fibromodulin, all of which possess a core protein with leucine rich repeat motifs flanked by cysteine-clusters (14). Biglycan and decorin, which show greater homology to each other than to other SLRPs, are widely distributed with overlapping but divergent patterns of expres-
sion in vertebrate connective tissues (15, 16). Creation of mice homozygous null for biglycan or decorin have shown the former to be a positive regulator of bone growth (17) and the latter to play a role in regulating type I collagen fibrillogenesis in skin and tendon (18). High levels of expression in preosteogenic cells and a pericellular distribution are consistent with a role for biglycan in osteoblast differentiation, whereas an association of decorin expression with tissues rich in fibrillar collagens is consistent with a role in fibrillogenesis (16). Although the molecular bases for the biological roles of biglycan and decorin are unclear, they may involve the demonstrated abilities of the two to interact with various collagens, other ECM proteins, and transforming growth factor-β (19–25).

Biglycan and decorin are unique among SLRPs in that they are synthesized as pro-forms containing N-propeptides of 21 and 14 residues, respectively, that are completely removed in most, but not all, connective tissues (26). Although the proteinase(s) responsible for these cleavage events has not been identified, the sites at which biglycan and prodecorin are processed in vivo have been determined (26–29), and the residues M(M/L)N-DEE and M(L/I)E-DE(A/G) found at the probiglycan and prodecorin sites, respectively, are conserved in various species (15, 30–38). The similarity of these sequences suggests that the same proteinase(s) may be responsible for processing of both proteins, and interestingly, these cleavage sites show similarities to the cleavage sites of the C-propeptides of procollagens I–III (3, 39, 40).

In the present study, we demonstrate that BMP-1 cleaves probiglycan at a single site, thus removing the N-propeptide to produce biglycan with an NH₂ terminus identical to that of mature biglycan isolated from tissues. Consistent with a physiological role for the processing of biglycan by BMP-1, expression domains of the two gene products are shown to be coincident in most developing tissues. A notable exception is postnatal articular cartilage, in which high levels of biglycan expression are not matched by detectable BMP-1 expression, and in which persistence of high levels of unprocessed probiglycan has previously been noted. The enzymes mTLD and mTLL-1 are also shown to have low levels of biglycan-processing activity. Moreover, whereas wild type mouse embryonic fibroblasts (MEFs) produce only mature biglycan, MEFs deficient for BMP-1 and mTLD are shown to produce predominantly unprocessed probiglycan, and MEFs deficient for the three enzymes BMP-1, mTLD, and mTLL-1 are totally devoid of biglycan-processing activity. Evidence that these propeptides are responsible for processing of biglycan in at least some tissues in vivo is discussed, as are the implications for ECM deposition and morphogenesis.

**EXPERIMENTAL PROCEDURES**

**Peptide Substrate Synthesis and Cleavage Assays—**Peptides incorporating the propeptide cleavage sites of decorin and biglycan (Fig. 1D) were synthesized by FMn (N-(9-fluorenylmethoxycarbonyl) chemistry using an ABI 431A synthesizer. The peptides contained a central region of sequences spanning the biglycan or decorin propeptide cleavage site and basic terminal regions to ensure solubility. A cysteine residue was added to carboxyl-terminals for conjugating peptides to ovalbumin. The peptides were coupled to lysine residues of ovalbumin using N-hydroxy-succinimidyl bromoacetate to yield solutions of conjugate in phosphate-buffered saline (about 4 mg/ml). Peptide-ovalbumin conjugates were then dialyzed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl₂, prior to analysis in cleavage assays.

Peptide cleavage assays employed recombinant BMP-1, prepared using a baculovirus expression system, as described (3). Only the peptide assays employed the baculovirus-generated material, whereas all subsequent cleavage assays with recombinant biglycan (see below) employed affinity-purified proteases produced in a mammalian expression system. Five μl of recombinant BMP-1 and 5 μl of peptide-ovalbu-

min conjugate were combined in a 40-μl volume of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl₂ and incubated at 37 °C for 16 h. Control incubations contained culture medium derived from native SF21 cells or cells infected with the wild type virus. Reactions were stopped by the addition of SDS-PAGE loading dye containing 2% β-mercaptoethanol and boiled for 5 min. Samples were subjected to SDS-PAGE on 10% acrylamide gels and electrophoresed to Sequi-Blot polyvinylidene difluoride membranes (Bio-Rad). The resulting peptide-ovalbumin conjugates were identified by staining with Coomasie Blue, and NH₂-terminal amino acid sequences were determined by automated Edman degradation on an ABI 473A protein sequencer.

**Probiglycan Expression Construct—**A cDNA encoding human probiglycan, except for the signal peptide, was generated by PCR using full-length human cDNA clone P16 (15) as template, and primers 5′-ACGTGACGTCAGTGCATCGCCCCCTTTTGGCAGAGCGGC-3′ (forward) and 5′-ACGTGACACTCAGGATCCTCAGGATGTTGCGC-3′ (reverse), corresponding to sequences 169–189 and 1278–1298, respectively, of the published human probiglycan sequence (15), plus an NheI or XhoI site, respectively, for cloning. The PCR employed Advantage cDNA polymerase mix (CLONTECH) and denaturation at 94 °C for 30 s, followed by 25 cycles of 94 °C for 10 s, 65 °C for 30 s, and 72 °C for 2 min and final extension at 72 °C/10 min. After digestion with NheI and XhoI, the 1129-base pair PCR product was inserted between the NheI and XhoI sites of expression vector pCEP-Pu/BM40s (41) downstream of, and in the same reading frame as, sequences encoding the BM40 signal peptide. Fidelity of the pCEP-Pu/BM40s-probiglycan expression vector was confirmed by DNA sequencing of the PCR insert and reactions on both strands.

**Expression of Recombinant Probiglycan—**293-EDNA human embryonic kidney cells (Invitrogen) were maintained in growth medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 1 mM t-glutamine, 0.1 mM nonessential amino acids, and 10% fetal bovine serum (HyClone). Cells at 90% confluence were transfected with 10 μg of rCEP-Pu/BM40s-probiglycan vector or empty pCEP-Pu/BM40s vector per 100-mm tissue culture dish, using LipofectAMINE according to the manufacturer’s instructions (Life Technologies, Inc.). After 48 h, cells were selected in growth medium containing 5 μg/ml puromycin (Sigma), and surviving cells were allowed to grow to confluent mass cultures in growth medium containing 5 μg/ml puromycin.

Medium was removed from cell cultures, which were then washed three times with phosphate-buffered saline and switched to serum-free Dulbecco’s modified Eagle’s medium containing 40 μg/ml soybean trypsin inhibitor (Sigma). After 24 h, conditioned medium was harvested, and protease inhibitors were added. Fresh serum-free medium was applied to the cells and similarly collected after an additional 24 h, and medium samples from 24 and 48 h harvest were pooled and centrifuged at 10,000 × g for 5 min, and 5 μl aliquots were electrophoresed on 4–15% acrylamide gradient gels. Western blot analyses of these materials and of purified samples described below involved transfers to polyvinylidene difluoride membranes, incubations of blots with antibodies, and washes, as described previously (42), using antibodies raised against a peptide (LPFFEQRGFWGCG) within the probiglycan N-propeptide (26), for both murine and human samples, or antibodies LF51, for human samples, and LF106, for murine samples, raised against peptides of murine (VPDLDSTVPTFSA) biglycan, respectively (15, 43). N-propeptide and LF51 antibodies were diluted 1:1000, whereas LF-106 and secondary antibodies of peroxidase-linked anti-rabbit Ig antisera were diluted 1:5000 (Amersham Pharmacia Biotech). Immunoreactive proteins were detected using SuperSignal peroxidase substrate (Pierce).

**Purification of Recombinant Probiglycan—**Conditioned medium
samples were applied to a 1.5 \times 3\text{-}cm DEAE-cellulose column (DE52, Whatman) pre-equilibrated with Buffer A (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 1 mM N-ethylmaleimide, 1 mM p-aminobenzoic acid, and 0.1 mM phenylmethylsulfonyl fluoride). The column was washed in Buffer A, and bound proteins were eluted in a linear gradient of Buffer A made 250–0 mM NaCl. The proteoglycan form of probiglycan was eluted at \(\sim 350\) mM NaCl and fractions containing this form were dialyzed into 50 mM Tris-HCl, pH 7.5, 150 mM NaCl.

In Vitro Enzyme Assays—Proteases used for cleavage assays of recombinant probiglycan were recombimant human BMP-1, mTLD, mTLL-1, and mTLL-2, with COOH-terminal Flag-tags, purified on anti-Flag affinity columns, and quantitated as described (5). To optimize comparison of activities, the various proteases were prepared and purified using identical conditions, and each enzyme preparation was pure to the extent that only a single band of appropriate size was detectable on zinc-stained SDS-PAGE gels (not shown). Approximately 500 ng of purified probiglycan and 15 ng of purified Flag-tagged BMP-1, mTLD, mTLL-1, or mTLL-2 were combined in a 50-\(\mu\)l volume of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3 mM CaCl\(_2\), and incubated 15 h at 37 °C. Subsequently, 10 \(\mu\)l of a solution containing 100 mM Tris-HCl, pH 8.0, 240 mM sodium acetate, 25 mM EDTA, and 0.02 units of protease-free chondroitinase ABC (Seikagaku Corp.) was added to each assay, and samples were incubated an additional 4 h at 37 °C. Reactions were stopped by the addition of 10× SDS-PAGE sample buffer. Proteins were separated on 7.5% SDS-PAGE gels containing 2% w/v acrylamide, and proteins were transferred to nitrocellulose for 1 h, and centrifuged, and precipitates were dissolved in SDS-PAGE sample buffer. Proteins were resolved by SDS-PAGE on a 10% acrylamide gel and electrotransferred to a Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad). Proteins were identified by staining with 0.1% Amido Black in 10% acetic acid, and NH\(_2\)-terminal amino acid sequences were determined by automated Edman degradation on an Applied Biosystems Procise 494 HT protein sequencing system at the Harvard University Microchemistry Facility.

Reverse Transcription (RT)-PCR of Mouse Embryo Fibrolast RNA—RNA was isolated from \(-5 \times 10^5\) MEFs using TRIZol reagent (Life Technologies, Inc.). RT of 500 ng of RNA was performed using SuperScript II reverse transcriptase (Life Technologies, Inc.). The reported cleavage sites at which BMP-1 cleaves the C-propeptides of probiglycan I–III (3, 39, 40), Chordin (5), and prolysyl oxidase (7), with the sites at which the N-propeptides of probiglycan and prodecorin are removed in vivo (26–29) are similar to the cleavage sites at which BMP-1 has previously been shown to process procollagens I–III, Chordin, and prolysyl oxidase, particularly in that each site contains an aspartate residue at the P1 position and tyrosine and/or methionine residues NH\(_2\)-terminal to the cleavage site, usually in the P3 or P2 position (Fig. 1A). In an initial assay to determine whether BMP-1 was capable of recognizing and cleaving the probiglycan and prodecorin, synthetic peptides spanning the two cleavage sites were prepared (Fig. 1B). The peptides were coupled to ovalbumin via their carboxyl-terminal residues, so that following internal cleavage the peptide bearing the new amino terminal would remain coupled and could be readily purified by SDS-PAGE. Analysis of these products by NH\(_2\)-terminal amino acid sequencing revealed that in both cases, baculovirus-generated recombinant BMP-1 (3) cleaved at the predicted sites, without the occurrence of cleavages at any additional sites.

Expression and Characterization of Recombinant Probiglycan—The reported cleavage sites at which probiglycan and prodecorin are proteolytically processed in vivo (26–29) are similar to the cleavage sites at which BMP-1 has previously been shown to process procollagens I–III, Chordin, and prolysyl oxidase, particularly in that each site contains an aspartate residue at the P1 position and tyrosine and/or methionine residues NH\(_2\)-terminal to the cleavage site, usually in the P3 or P2 position (Fig. 1A). In an initial assay to determine whether BMP-1 was capable of recognizing and cleaving the probiglycan and prodecorin, synthetic peptides spanning the two cleavage sites were prepared (Fig. 1B). The peptides were coupled to ovalbumin via their carboxyl-terminal residues, so that following internal cleavage the peptide bearing the new amino terminal would remain coupled and could be readily purified by SDS-PAGE. Analysis of these products by NH\(_2\)-terminal amino acid sequencing revealed that in both cases, baculovirus-generated recombinant BMP-1 (3) cleaved at the predicted sites, without the occurrence of cleavages at any additional sites.
with the expression construct or with an empty pCEP-Pu/BM40s vector were examined by Western blot for secreted biglycan. As can be seen (Fig. 2A), antibodies specific for probiglycan N-propeptide sequences detected a heterogeneous smear centered around 100 kDa and a discrete band of approximately 50 kDa. These two forms are larger than the 36-kDa band, which approximates the size of mature biglycan, may represent endogenous biglycan produced by 293-EBNA cells, all of which is processed to the mature form, whereas the 36-kDa band may represent a proteolytic fragment of the endogenous biglycan. However, levels of neither the ~47-kDa nor the 36-kDa form seem appreciably increased in cultures of transfected 293-EBNA cells producing large amounts of recombinant probiglycan (Fig. 2B). Thus, either the cells lack the capacity to process additional probiglycan into the ~47- and ~35-kDa forms, or these forms represent nonbiglycan proteins that cross-react with the biglycan antibodies. In either case, neither the ~47-kDa nor the ~35-kDa species co-purified in detectable quantities with the recombinant biglycan isolated by DEAE-chromatography and used in the enzyme cleavage assays described below.

**BMP-1 Efficiently Processes the N-propeptide of Recombinant Probiglycan at the Physiological Site**—To determine whether the N-propeptide of probiglycan might be processed by BMP-1 and related mammalian enzymes, recombinant probiglycan was purified from conditioned media of untransfected 293-EBNA cells (not shown). Thus, the ~47-kDa band, which approximates the size of mature biglycan, may represent endogenous biglycan produced by 293-EBNA cells, all of which is processed to the mature form, whereas the 36-kDa band may represent a proteolytic fragment of the endogenous biglycan. However, levels of neither the ~47-kDa nor the 36-kDa form seem appreciably increased in cultures of transfected 293-EBNA cells producing large amounts of recombinant probiglycan (Fig. 2B). Thus, either the cells lack the capacity to process additional probiglycan into the ~47- and ~35-kDa forms, or these forms represent nonbiglycan proteins that cross-react with the biglycan antibodies. In either case, neither the ~47-kDa nor the ~35-kDa species co-purified in detectable quantities with the recombinant biglycan isolated by DEAE-chromatography and used in the enzyme cleavage assays described below.

**FIG. 3. Cleavage assays of recombinant probiglycan incubated separately with BMP-1 and related mammalian proteases.** Western blot analyses using antibodies specific for sequences within the probiglycan N-propeptide (A) or antibodies specific for sequences within mature biglycan (B) were employed to monitor processing of probiglycan by BMP-1 and related mammalian proteases mTLD, mTLL-1, and mTLL-2. Molecular masses (in kDa) are indicated for protein standards. Pro and Mat denote the positions of pro- and mature forms of biglycan, respectively.
A Product, or Products, of the Bmp1 Gene Is Responsible for the Majority of Probiglycan Processing in MEFs—We have previously described mice with null alleles for the Bmp1 gene, which encodes BMP-1 and mTLD (6). Mice homozygous null for the Bmp1 gene are perinatal lethal, with defects in fibrillogenesis and diminished procollagen processing, but with some residual PCP activity, presumably provided through functional substitution by mTLL-1 and/or other proteases with related activity. To ascertain whether MEFs from Bmp1+/–, Bmp1−/–, and Bmp1−/− embryos have diminished ability to process probiglycan, MEFs were derived from Bmp1+/+, Bmp1+/−, and Bmp1−/− 13.5-days postconception (dpc) embryo littermates, as described (6), and the conditioned media of confluent cultures of each type of MEFs were examined by Western blot for secreted pro- and mature forms of biglycan. As can be seen (Fig. 5A), antibodies specific for probiglycan N-propeptide sequences detected a ~50-kDa probiglycan band, subsequent to treatment of conditioned media samples with chondroitinase ABC, in the culture medium of Bmp1+/− MEFs but not in the culture media of Bmp1+/+ or Bmp1−/− MEFs. Conversely, analysis of chondroitinase ABC-treated conditioned medium samples with antibodies specific for sequences within mature biglycan (top panels) or antibodies specific for sequences within mature biglycan (bottom panels) were employed to monitor processing of probiglycan in cultures of Bmp1+/+, Bmp1−/−, and +/+ MEFs (A) or MEFs, with genotypes as shown, derived from the embryos of Bmp1+/−; Tll1−/+ double heterozygote matings (B). Pro and Mat denote the positions of pro- and mature forms of biglycan, respectively.

Although the majority of probiglycan remains uncleaved in Bmp1+/− MEF cultures (Fig. 5A), significant residual process-
ing remains. Because mTLL-1 was found to have some probiglycan-processing activity in in vitro assays (Fig. 3), we sought to determine whether the mTLL-1 gene, Tll1, is expressed in MEF cells isolated from 13.5-dpc embryos, such that mTLL-1 would be properly situated to supply some portion of the residual probiglycan processing activity observed in Bmp1−/− MEFs. It was also of interest to determine whether the mTLL-2 gene, Tll2, is expressed in MEF cultures. Toward these ends, a series of RT-PCR analyses were performed on RNA isolated from Bmp1 +/+, +/-, and −/− MEFs to gauge the expression of RNA for mTLL-1, mTLL-2, and the two products of the Bmp1 gene, BMP-1 and mTLD. As can be seen (Fig. 6), expression of mTLL-1 RNA is readily detectable in Bmp1 +/+, +/−, and −/− MEFs, whereas PCR products corresponding to both wild type and mutant Bmp1 alleles are detectable in the Bmp1+/− MEF sample, and only products corresponding to the Bmp1 mutant allele are detectable in the Bmp1−/− MEF sample.

*mTLL-1 Provides the Residual Probiglycan-Processing Activity in Bmp-1-null MEFs*—Because the Bmp1 gene and the Tll1 gene, which encodes mTLL-1, map to different chromosomes (8), we were able to generate embryos homozygous null at both loci through matings of Bmp1+/−;Tll1+/− double heterozygotes. Thus, a comparison could be made of probiglycan processing in MEF cultures derived from wild type, Bmp1−/−, Tll1−/−, and Bmp1−/−;Tll1−/− doubly null embryos (Fig. 5B). Despite the low but detectable levels of probiglycan processing shown by recombinant mTLL-1 in vitro (Fig. 3B), processing of probiglycan to mature biglycan appeared to be complete in cultures of Tll1−/− MEFs (Fig. 5B). This is similar to our previous finding that type I procollagen is processed to similar extents in cultures of wild type and Tll1−/− MEFs, despite evidence of mTLL-1 PCP activity (5, 9). Nevertheless, analysis found probiglycan to be totally unprocessed in cultures of MEFs from Bmp1−/−;Tll1−/− doubly null embryos (Fig. 5B). The most straightforward interpretation of these results is that although the more robust probiglycan-processing activity of BMP-1, combined with that of mTLD, seem sufficient to fully compensate for loss of mTLL-1 activity in Tll1−/− MEFs, mTLL-1 provides the residual probiglycan-processing activity observed in Bmp1−/− MEFs. Thus, products of the related Bmp1 and Tll1 genes appear to provide all detectable probiglycan-processing activity in mouse embryonic fibroblasts.

**Overlapping Expression Domains of BMP-1/mTLD and Biglycan RNA in 15.5-dpc Mouse Embryos**—Because BMP-1 can correctly and efficiently process probiglycan in vitro and because products of the Bmp1 gene seem responsible for the preponderance of probiglycan processing in MEFs, it was of interest to obtain insights regarding the possible codistribution of expression of the Bmp1 gene and the probiglycan gene Bgn, in vivo. As can be seen in serial sagittal sections of a 15.5-dpc mouse embryo (Fig. 7), both genes are broadly co-expressed throughout mesenchymal tissues, with particularly high signals for both found in developing bones, as is particularly evident in cross sections of the developing clavicle, basioccipital bone, vertebrae, and bones of the hind limb. It has previously been noted (26) that unprocessed probiglycan is detectable in some tissues, and particularly in articular cartilage. To ascertain whether there might be a correlation between the distributions of expression of Bmp1 and Bgn, and the previous finding of unprocessed probiglycan in articular cartilage (26), expression patterns of the two genes were compared in serial sections of the femoral growth plate of a 21-day-old mouse (Fig. 8). As can be seen, both Bmp1 and Bgn are expressed at high levels in the epiphyseal and metaphyseal centers of ossification and in the area of ossification corresponding...
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Previously, we have speculated that products of the Bmp1 and Tll1 genes might be capable of functional substitution for each other in vivo, with mTLL-1 partially compensating for loss of BMP-1 and mTLD in Bmp1–/– embryos, and with BMP-1 and mTLD partially compensating for loss of mTLL-1 in Tll1–/– embryos (6, 8, 9). Similarly, we have speculated that mTLL-1 might supply the residual procollagen C-proteinase activity found in Bmp1–/– MEFs (6), despite the observation that MEFs cultured from Tll1–/– embryos show levels of procollagen processing activity indistinguishable from those of wild type MEFs (9). The observation in the current study that mTLL-1 provides the residual probiglycan-processing activity observed in Bmp1–/– MEF cultures is the first demonstration of functional substitution by products of the two genes in a biological system and is consistent with the possibility that the product of the Tll1 gene can compensate for a deficiency in the products of the Bmp1 gene, and vice versa, in at least some tissues. Nevertheless, observations that BMP-1 has higher levels of PCP activity (5) and probiglycan-processing activity, in vitro, than either mTLD or mTLL-1, and that Tll1–/– MEFs have levels of PCP (6) and probiglycan-processing activities indistinguishable from those of wild type MEFs, suggest that BMP-1 may normally be the major protease responsible for provision of both activities in vivo.

In a previous study, immunohistochemical analysis of aorta with an antibody specific for the probiglycan N-propeptide found intense intracellular staining but an absence of extracellular staining, suggesting that processing of the propeptide occurs prior to secretion (47). In contrast, the finding of secreted, unprocessed probiglycan in the ECM of tissues such as articular cartilage (26) has suggested that processing of the N-propeptide may occur extracellularly. It is clear that BMP-1, mTLD, and mTLL-1 are secreted proteases (3, 5, 39, 40, 42), whereas both BMP-1 and mTLD have been shown to have slightly basic pH optima (39, 40), suggesting that they operate most efficiently in the extracellular milieu. Thus, the finding in the present report that all MEF probiglycan-processing activity is provided by products of the Bmp1 and Tll1 genes suggests that processing of probiglycan to its mature form occurs extracellularly. Interestingly, however, although the cleavage site for removal of the decorin N-propeptide is similar to a number of previously characterized sites utilized by BMP-1 and related enzymes, and although we have shown that BMP-1 is capable of recognizing and cleaving the decorin site in a synthetic peptide, pulse-chase experiments have previously suggested that the N-propeptide of decorin is removed intracellularly, in the Golgi apparatus, prior to elongation of glycosaminoglycan chains (48). Thus, it is possible that decorin is not processed by BMP-1 and/or related enzymes but that it is instead processed by different proteases than is probiglycan. In support of the possibility that processing of decorin differs from that of probiglycan, our attempts to produce recombinant decorin, using full-length human decorin cDNA and the same pCEP-Pu/BM40s vector/293-EBNA cell expression system used to efficiently produce unprocessed probiglycan, resulted in the detection of only processed mature decorin in the media of transfected 293-EBNA cultures. Nevertheless, because 293-EBNA cells produce low levels of endogenous BMP-1,3 it remains possible that decorin is cleaved by the same enzymes as is probiglycan but at a much higher rate. Efforts to examine processing of decorin

3 I. C. Scott, Y. Imamura, and D. S. Greenspan, unpublished observations.
in MEF cultures were inconclusive, due to the small size difference between pro- and mature forms of decorin and the absence of an antibody that recognizes the murine decorin N-propeptide. If, however, prodecorin is indeed processed by the same enzymes as biglycan, and if prodecorin is in fact processed in the Golgi, then it is implied that BMP-1 and related proteases are capable of processing some substrates in tissues to be extremely rapid, consistent with the studies that removal of the N-propeptide induces conformational changes in amino acid residues, but previous antibody studies also suggest the absence of an antibody that recognizes the murine decorin N-propeptide. If, however, prodecorin is indeed processed by related proteases are capable of processing some substrates in tissues to be extremely rapid (49, 50), consistent with the possibility that cleavage occurs coincident with secretion and in a pericellular environment. The latter environment might well include the trans Golgi compartment, in which the processing of some portion of procollagens, probiglycan, and prodecorin by BMP-1 and related proteases may occur in vivo.

Cleavage of the N-propeptide not only changes the primary structure of biglycan, by removing the NH$_2$-terminal 21 amino acid residues, but previous antibody studies also suggest that removal of the N-propeptide induces conformational changes that affect the availability of epitopes in both the NH$_2$-terminal (47) and COOH-terminal (26) portions of the mature biglycan molecule. Therefore, removal of the N-propeptide, through cleavage by BMP-1 and related enzymes, is likely to have significant effects on the properties of biglycan, which, in turn, are likely to affect its interactions with other molecules. Interestingly, biglycan has been shown to bind TGF-$eta$ (24) and may also interact with various collagen types (21, 22, 44), whereas BMP-1, mTLD, and mTLL-1 are involved in the formation of collagenous ECM and in modulating the activity of certain TGF-$eta$-like molecules (3–6, 9). Thus, the interaction of these proteases with biglycan would conform to the previously suggested roles of these molecules. In particular, biglycan has been shown to play an important role in the formation of bone (24) and may also interact with various collagen types (21, 22, 44), whereas BMP-1 and mTLD have also been implicated in bone morphogenesis (5, 6, 13). Thus, the interplay of these molecules may normally be of particular importance in the proper formation of this tissue.

Acknowledgments—We are grateful to Dr. Brigid L.M. Hogan for provision of heterozygous Bmp1-null mice, to Dr. Larry W. Fisher for providing biglycan cDNAs and anti-biglycan antibody LF-51, and to Satoshi S. Kinoshita for excellent technical assistance.

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