BMP-1-mediated proteolytic processing of alternatively spliced isoforms of collagen type XI

Ryan J. MEDECK*, Sergio SOSA†, Nicholas MORRIS‡ and Julia Thom OXFORD*[1]

*Department of Biology, Boise State University, 1910 University Drive, Boise, ID 83725, U.S.A., †FibroGen Inc., 225 Gateway Blvd, South San Francisco, CA 94080, U.S.A., and ‡Robert W. Franz Cancer Research Center, 4805 NE Glisan, Portland, OR 97213, U.S.A.

Collagen type XI is a minor constituent of heterotypic collagen fibrils of developing cartilage and plays a regulatory role in fibril diameter. Collagen type XI is a heterotrimer composed of the α1, α2 and α3 chains. The mRNA encoding exons 6a, 6b and 8 of the α1 chain are expressed alternatively to generate six possible isoforms. The 6b-containing isoform has the most restricted distribution of all isoforms. It is first localized in the developing long bone, where mineralized tissue initially forms, and is later restricted to regions of cartilage that will be subsequently converted into bone. Bone morphogenetic protein 1 (BMP-1) and related proteins cleave procollagens I–III, V and VII, yielding triple-helical molecules that associate into collagen fibrils. The present study demonstrates that the α1 chain of collagen type XI can serve as a substrate for BMP-1. In addition, the efficiency with which BMP-1 processes different isoforms of the α1 chain varies. The amino acid sequence adjacent to the processing site influences the rate and extent of processing, as do sequences further away. Smaller fragments identified from cartilage extracts indicated that processing by BMP-1, in combination with other processing enzymes, generates small fragments of p6b-containing isoforms.

Key words: alternatively spliced isoform, BMP-1 (bone morphogenetic protein 1), collagen type XI.

INTRODUCTION

Collagen type XI is a component of the extracellular fibrillar network of cartilage. It belongs to the family of fibrillar collagens, which includes types I–III, V and XI [1]. It plays a role in the assembly of embryonic cartilage collagen fibrils, as the diameter of cartilage collagen fibrils is dependent on the relative ratio of collagen types II and XI [2]. The chondrodystrophic mouse (*cho*/*cho*) highlights the essential role of collagen type XI in fibril assembly and extracellular matrix organization during the early developmental stages of bone growth and cartilage formation. Mice exhibiting this disorder were shown to have an abnormality in the α1 chain of collagen type XI. The abnormality resulted in the deletion of a cytosine approx. 570 nucleotides downstream of the translation-initiation codon in the α1 chain of collagen type XI mRNA from *cho* homozygotes. The deletion caused a reading frameshift and introduced a premature stop codon [3].

Collagen type XI is initially synthesized as a procollagen, which is subsequently processed proteolytically at both the N- and C-termini [4]. Structurally, collagen type XI is a heterotrimer composed of three chains, namely α1, α2 and α3 [5]. The C-propeptide of the α1 chain of collagen type XI is homologous with other fibrillar collagens [6]. In addition to the characteristic minor triple helix, the α1 chain of collagen type XI contains a large non-triple-helical NTD (N-terminal domain). The Npp (N-propeptide) domain of NTD is conserved among collagens and other molecules, such as thrombospondin and laminins [7]. The α1 chain exists as a set of six isoforms that arise by alternative splicing of mRNA between exons 6a, 6b, 7 and 8 [8] encoding the corresponding protein regions p6a, p6b, p7 and p8 (Figure 1). This region gives rise to the variable region (vr) of the NTD of α1(XI). The variable region is the most unique region of α1(XI) collagen with respect to other collagen types. The Npp domain, encoded by exons 1–5, is removed during proteolytic processing [9], and is common to all isoforms of the α1(XI) collagen irrespective of the manner in which the mRNA is spliced. This domain may serve an essential role having a common function in all α1(XI) collagen isoforms. Initially, collagen type XI may function to nucleate the formation of new collagen fibrils. However, it may also play a role in limiting the lateral growth of collagen fibrils [10].

Bone morphogenetic protein 1 (BMP-1), a proteinase of the metzincin family [11], processes extracellular matrix molecules, collagenous and non-collagenous, and functions as a regulator in many biological events [12–15]. Other metzincins related to BMP-1 include mTLD (mammalian tolloid; an alternatively spliced form of the *bmp1* gene that encodes BMP-1) [16] and mTLL-1 and mTLL-2 (mTLD-like-1 and -2) [17].

BMP-1 is a cysteine-rich zinc peptidase and is involved in the processing of several procollagens to collagens [18]. BMP-1 processes the C-propeptides from procollagens I–III, whereas a separate enzyme processes the Npp [19]. Furthermore, BMP-1 is involved in the processing of pro-lysyl oxidase [20], probiglycan [21], the γ2 and α3 chains of laminin 5 [14] and chordin [22], all of which play definite roles in extracellular matrix assembly. The C- and N-propeptides of collagen type V can also be processed by BMP-1 [23]; however, the C-terminus may be processed by furin as well [24,25]. In addition, collagen type VII, a major component of the anchoring fibrils at the dermal–epidermal junction in the skin, has been identified as a substrate for BMP-1 [26].

Members of the enzyme family ADAMTS (**A** **D**isintegrin **A**nd **M**etalloproteinase with **T**hrombo**S**pondin motifs) have been implicated in proteolytic processing at the N-terminal site of collagen types I and II [27,28]. In addition, some ADAMTS share similarities with BMP-1, namely a zinc-binding motif in the metalloproteinase domain [29]. These enzymes are active metalloproteinases associated with the extracellular matrix; therefore they may be involved in collagen processing.

Abbreviations used: ADAMTS, **A** **D**isintegrin **A**nd **M**etalloproteinase with **T**hrombo**S**pondin motifs; BMP-1, bone morphogenetic protein 1; mTLD, mammalian tolloid; mTLL, mTLD-like; Npp, N-propeptide; NTD, N-terminal domain.

[1] To whom correspondence should be addressed (e-mail joxford@boisestate.edu).

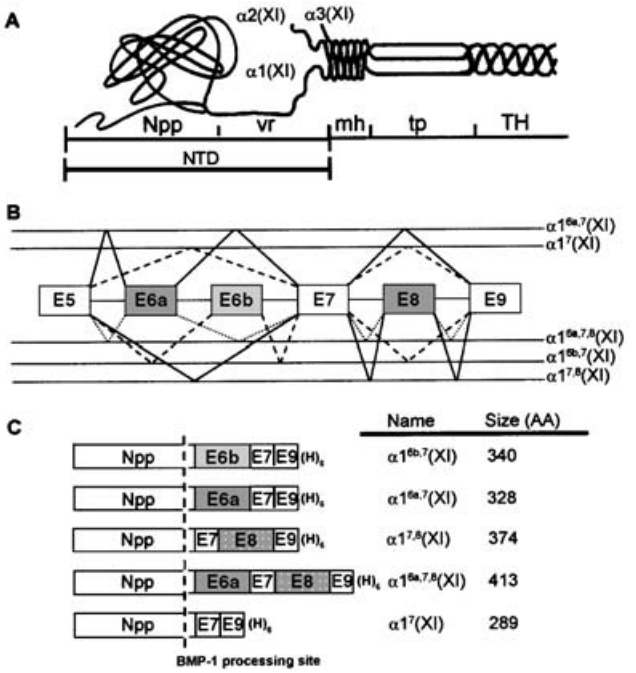


Figure 1 Schematic representation of the collagen type XI α1 chain

(**A**) The collagen type XI α1 chain structure is composed of an Npp, a variable region (vr), a minor helix (mh), a telopeptide (tp) and a major triple helix (TH). NTD refers to Npp + vr. (**B**) Alternative splicing occurs with exons 6a, 6b and 8 within the variable region of the α1(XI) chain, generating six possible isoforms. The five most prevalent isoforms are shown. (**C**) The composition, name and amino acid length of each isoform are given.

This demonstrates that the α1 chain of collagen type XI can serve as a substrate for BMP-1, as does the similar α1(V) collagen chain [24]. In addition, the rate and extent of proteolytic processing varies in an isoform-specific manner. Results demonstrate that sequences adjacent to the AAQA/QHIDE processing site identified previously [9,30] affected the rate of enzymic processing by BMP-1, as verified by comparing the rate and extent of processing among isoforms containing p6a, p6b or p7 sequences within the variable region. Absence or presence of the p8 sequence within the variable region influenced the processing by BMP-1 as well, even though a distance of 38–89 amino acids may separate p8 from the BMP-1 processing site, depending on the amino acid composition of the isoform. Isoform-specific variation in the extent of proteolytic processing *in vivo* was also confirmed by analysis of α1(XI) processing intermediates from foetal bovine cartilage. Finally, the present study demonstrated that fragments, resulting in part from BMP-1 processing, accumulated to detectable levels, suggesting that they are relatively long-lived and stable to further degradation. This new result is potentially significant in the light of the limited distribution of the p6b-containing isoform of α1(XI) found in developing skeletal structures. Ultimately, these events are expected to affect the organization of the extracellular matrix during development and the biophysical characteristics of the resulting tissue.

EXPERIMENTAL

Expression of rat recombinant protein in *Escherichia coli*

Isoforms of the collagen type XI α1 chain NTD from rat sequences were designed to contain the Npp domain, the putative BMP-1 processing site and the variable region terminating in a His_6 tag

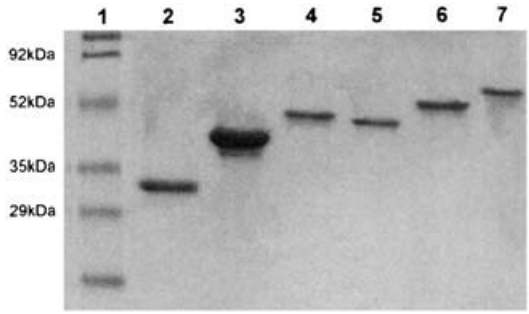


Figure 2 Recombinant protein expression of isoforms of the collagen type XI α1 chain

Coomassie Blue-stained SDS/polyacrylamide (13 %) gel, showing the isoform eluates used for *in vitro* assays. Lane 1, molecular-mass standards; lane 2, Npp; lane 3, $\alpha1^7$(XI); lane 4, $\alpha1^{6b,7}$(XI); lane 5, $\alpha1^{6a,7}$(XI); lane 6, $\alpha1^{7,8}$(XI); lane 7, $\alpha1^{6a,7,8}$(XI).

(Figure 1C). Recombinant NTD isoforms were expressed in BL21 DE3 *E. coli* cells (Novagen, Madison, WI, U.S.A.) using the pET11a bacterial expression vector (Stratagene, La Jolla, CA, U.S.A.). A recombinant protein expression was induced by the inclusion of 0.1 mM isopropyl-1-thio-D-galactopyranoside in the culture medium when cells had reached the mid-exponential phase of growth, as described previously [31].

Purification and refolding of rat recombinant proteins

Recombinant proteins were isolated by nickel-affinity chromatography from inclusion bodies. Proteins were unfolded in 20 mM Tris/HCl (pH 7.5), 300 mM NaCl, 5 mM imidazole, 6 M guanidine and 1 mM 2-mercaptoethanol and incubated for 30 min at 25 °C. Unfolded soluble protein was clarified by centrifugation at 10000 ***g*** for 25 min at 4 °C to remove the insoluble material. The supernatant was applied on to a Ni^{2+}-nitrilotriacetate–agarose column. The column was washed with 20 mM Tris/HCl (pH 7.5) buffered solution containing 6 M urea, 300 mM NaCl and 5 mM imidazole and refolded at decreasing concentrations of urea, then eluted from the column in 20 mM Tris/HCl (pH 7.5), 300 mM NaCl and 250 mM imidazole. Purified recombinant protein was characterized by SDS/PAGE (Figure 2). Disulphide-bonded cysteine pairs were verified by tryptic digestion followed by protein sequencing by MS of those bands present in the absence of 10 mM dithiothreitol, but not in samples treated with reducing agent. Refolded protein was characterized by CD in the far-UV range (260–180 nm) and was consistent with 10 % α-helix, 33 % β-sheet, 23 % β-turn and 34 % 'other' structures [31]. Protein concentration was determined by the Bradford assay [32]. Protein was stored in aliquots at − 20 °C in 20 % glycerol, 240 mM NaCl and 200 mM imidazole (pH 7.5) at a final protein concentration of 1.5 mg/ml.

Synthetic p6b peptide

A peptide comprising the 51-amino-acid sequence of p6b (5.8 kDa) was synthesized (Invitrogen, Carlsbad, CA, U.S.A.). The peptide was purified by HPLC and stored as aliquots at a concentration of 5 mg/ml in 5 % (v/v) acetic acid at − 80 °C.

In vitro BMP-1 digestion assay

Recombinant α1(XI) NTD isoforms and synthetic peptide were subjected to digestion by human recombinant BMP-1. Synthesis

in HEK-293 cells and subsequent purification to homogeneity were performed by S. Sosa (FibroGen). Recombinant isoform substrates were included in the digestion buffer (50 mM Tris/HCl, pH 7.5/150 mM NaCl/5 mM $CaCl_2$) at an initial concentration of 5 μmol. BMP-1 was included in the digestion assays at a concentration of 15 nmol. After incubation at 37 °C for a specific time period, reactions were terminated by rapid freezing. Samples were analysed by SDS/PAGE. Coomassie Blue-stained protein bands were quantified densitometrically using a Kodak ID Image Station within the linear range of detection.

Protein extraction from bovine cartilage

To obtain a source of collagen type XI, foetal bovine tissue was obtained from Gem Meat Packing (Garden City, ID, U.S.A.). Cartilage was obtained from the femoral head and homogenized in 50 mM Tris/HCl (pH 7.5), containing 1 M NaCl, 5 mM EDTA and 0.1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (Calbiochem, San Diego, CA, U.S.A.).

Immunoblot analysis

For verification of isoform identity and determination of the ratio of processed to unprocessed form for each of the isoforms of the α1(XI) collagen chain, proteins present in the cartilage were extracted in a buffer containing 1 M NaCl in the presence of proteinase inhibitors. Extracts were dialysed into 0.05 M Tris/HCl buffer (pH 7.5) containing 0.15 M NaCl, and separated on SDS/polyacrylamide gels, transferred to a PVDF membrane and processed as described previously [33] using antibodies to α1(XI) collagen. The following antibodies were used: (1) mouse monoclonal antibody to the α1(XI) carboxy telopeptide, (2) mouse monoclonal antibody to p8, (3) mouse monoclonal antibody to p6b and (4) rabbit polyclonal antibody to Npp [34]. Results were visualized using Western Lightning Chemiluminescence reagent (PerkinElmer LifeSciences, Boston, MA, U.S.A.).

Identification of low-molecular-mass polypeptide products related antigenically to p6b-containing isoforms of α1(XI)

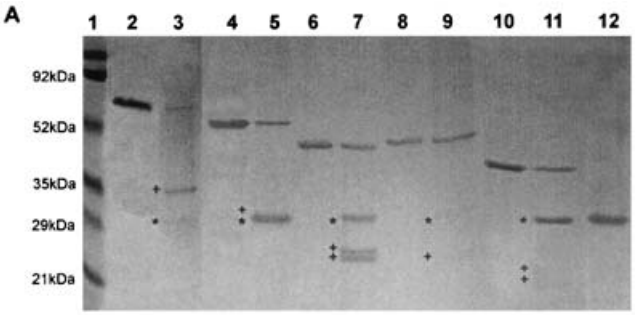
Protein extracted from foetal bovine cartilage was analysed on 13 % SDS/polyacrylamide gel, followed by immunoblotting using antibodies with specificity for the p6b region. The bands of interest were also subjected to collagenase digestion to diagnose the presence or absence of the minor helix. Antibodies to the Npp region and to the p7 sequence of NTD were also used for epitope-mapping of the bands of interest.

RESULTS

The rat recombinant collagen type XI α1 chain can serve as a substrate for BMP-1-mediated processing

Notable similarity exists between collagen types V and XI, including the predicted structure of the Npp domain and the amino acid sequence between the Npp domain and the variable region. Specifically, both contain the sequence AAQA/Q, which has been described as the BMP-1 processing site for α1(V) [24] as well as the site of processing for α1(XI) isoforms in cartilage from chicken [9]. These two pieces of evidence led to the prediction that BMP-1 is the enzyme responsible for proteolytic processing in the α1(XI) collagen chain as well.

Using a set of recombinant proteins that model the variation in the NTD of α1(XI) arising from alternative splicing, it was found



B

Band Identity	Calculated size (kDa)	Observed size (kDa)
$\alpha1^{6a,7,8}$(XI)	21.6	34.3
$\alpha1^{7,8}$(XI)	17.0	29.0
$\alpha1^{6b,7}$(XI)	13.0	23.2, 25.0
$\alpha1^{6a,7}$(XI)	11.9	25.1
$\alpha1^{7}$(XI)	7.4	20.2, 21.4
Npp	24.0	29.2

Figure 3 Collagen type XI recognition and proteolysis by BMP-1

Susceptibility to BMP-1 digestion at 37 °C after 8 h is demonstrated for the α1(XI) chain. (**A**) Coomassie Blue-stained SDS/polyacrylamide (13 %) gel. Lane 1, molecular-mass standards; lanes 2 and 3, $\alpha1^{6a,7,8}$(XI); lanes 4 and 5, $\alpha1^{7,8}$(XI); lanes 6 and 7, $\alpha1^{6b,7}$(XI); lanes 8 and 9, $\alpha1^{6a,7}$(XI); lanes 10 and 11, $\alpha1^{7}$(XI); lane 12, Npp. The basic nature of the $\alpha1^{6b,7}$(XI) isoform affects its migration on a gel, making it appear smaller than it really is. Lanes 3, 5, 7, 9 and 11 contain protein that has been incubated with BMP-1, whereas lanes 2, 4, 6, 8 and 10 contain control protein incubated in the absence of BMP-1. *, Npp; +, variable region fragments. (**B**) Expected and observed masses of the variable fragments.

that each of the isoforms can serve as a substrate for BMP-1 (Figure 3). After incubation with BMP-1 at 37 °C, all isoforms were converted from the full-length protein into fragments corresponding to the Npp domain and isoform-specific variable domains, which varied in apparent molecular mass from 20 to 35 kDa. Band identity was verified using isoform-specific antibodies and an antibody specific for the Npp domain (immunoblot results not shown).

Adjacent sequences influence the rate and extent of processing

Three possibilities exist for the adjacent sequence composition with respect to the BMP-1 processing site within the α1(XI) collagen chain; the sequence may arise from (1) exon 6b, (2) exon 6a or (3) exon 7 (Figure 1B). In each case, the amino acid sequence is unique as shown in Table 1. Exon 6b encodes a 51-amino-acid region rich in lysine residues, and has a theoretical pI > 11.0. In contrast, exon 6a encodes 39 amino acids with a prevalence of tyrosine residues and amino acids with negatively charged side chains at physiological pH, and it has a calculated pI of 3.6. Exon 7 encodes 31 amino acids with a theoretical pI of 4.0. A high degree of sequence conservation is observed when comparing the five different species shown in Table 1. Since these sequences are located in close proximity to the proteolytic processing site, the possibility that they might affect the rate or extent of proteolytic processing by BMP-1 was investigated.

Recombinant proteins that varied in amino acid composition adjacent to the BMP-1 processing site were incubated with BMP-1 at 37 °C for a time course of 8 h. Samples were analysed by

Table 1 Conserved amino acid sequence within the variable region

Comparison of amino acid sequence and pI of the variable region. Sequences immediately adjacent to the BMP-1 processing site, namely p6a, p6b and p7, are shown. Comparison of available amino acid sequences (bovine, human, mouse, chicken and rat) were used to generate consensus sequences. Values of pI represent the average of all available sequences. Amino acid composition and the calculated pI are unique. –, non-conserved amino acids.

Exon	Amino acid sequence	pI
6a	Y–PED––EYDYEYG–––YKE–ESVTE–P–––EET–AQTE	3.6
6b	KKK––––KK–RT––T–SK–K––––T––KSEK––SKKKK–––A––––KLGV–	11.0
7	ANIVDDFQ–YNYGTME–YQTE–PR–VSG–NE	4.1

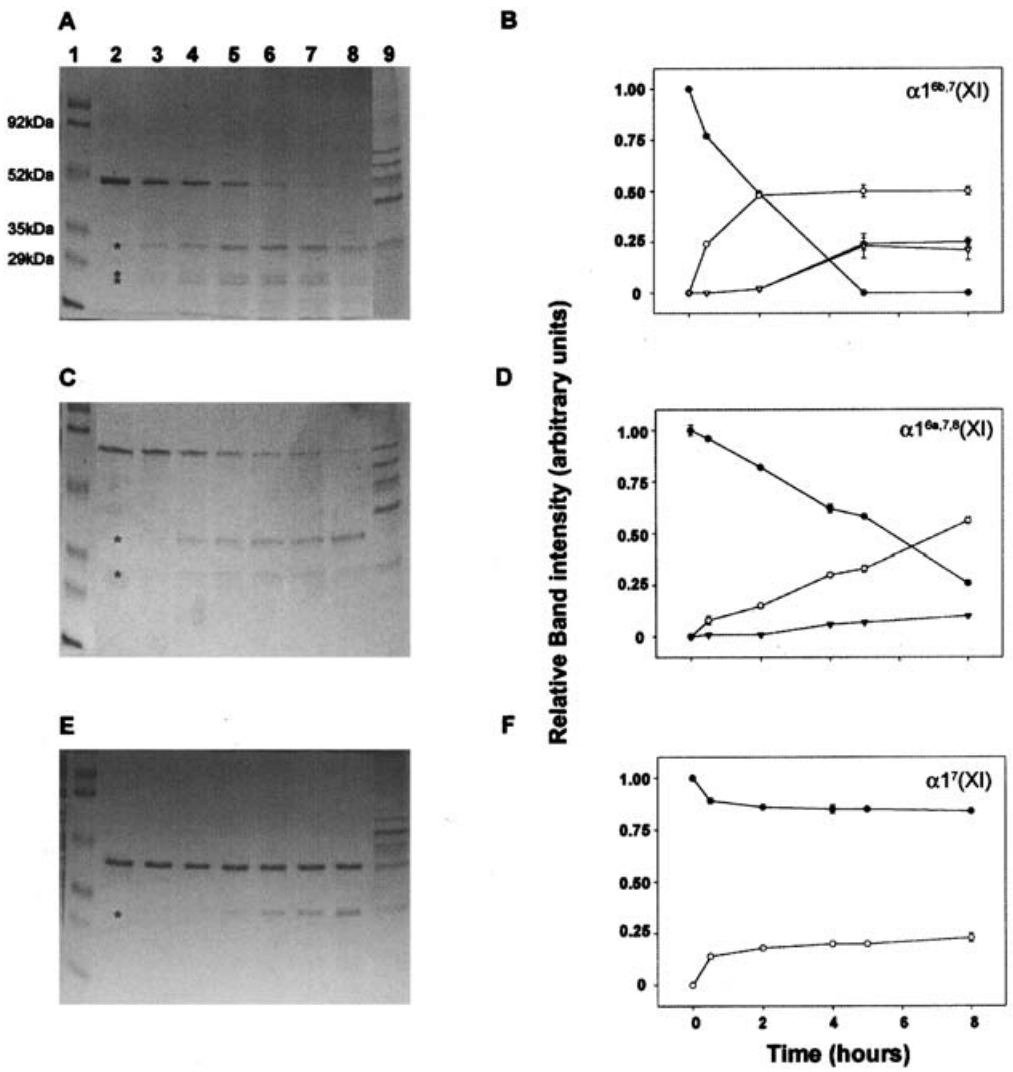


Figure 4 Rate of processing of type XI α1 chain isoforms by BMP-1

Recombinant isoforms most prevalent during vertebrate development, containing variable exons (either exon 6a, 6b or 8), were incubated for a total of 8 h with and without BMP-1. Coomassie Blue-stained SDS/polyacrylamide (13 %) gels [12 % in (**A**)], showing isoform-specific processing with BMP-1 (**A**, **C**, **E**). Lanes 1, SDS/polyacrylamide gel molecular-mass standards; lanes 2, 0 min digestion; lanes 3, 30 min digestion; lanes 4, 1 h digestion; lanes 5, 2 h digestion; lanes 6, 4 h digestion; lanes 7, 5 h digestion; lanes 8, 8 h digestion; lanes 9, collagen type XI α1 NTD molecular-mass standard. *Products of proteolytic processing. (**A**, **B**) $\alpha 1^{6b,7}$(XI); (**C**, **D**) $\alpha 1^{6a,7,8}$(XI); (**E**, **F**) $\alpha 1^{7}$(XI). Controls for each isoform without BMP-1 were run in parallel with the BMP-1-treated isoform (results not shown). Rate of processing is plotted in (**B**, **D**, **F**) corresponding to a specific isoform. ○, Npp; ●, NTD; ▼, variable region (vr); △, vr′.

SDS/PAGE and the intensity of bands was determined densitometrically. The extent of proteolytic processing of the three isoforms was determined at specific time points (Figure 4). The presence of p6b immediately adjacent to the processing site had a positive effect on the rate of processing, whereas the presence of p6a was associated with a lower rate.

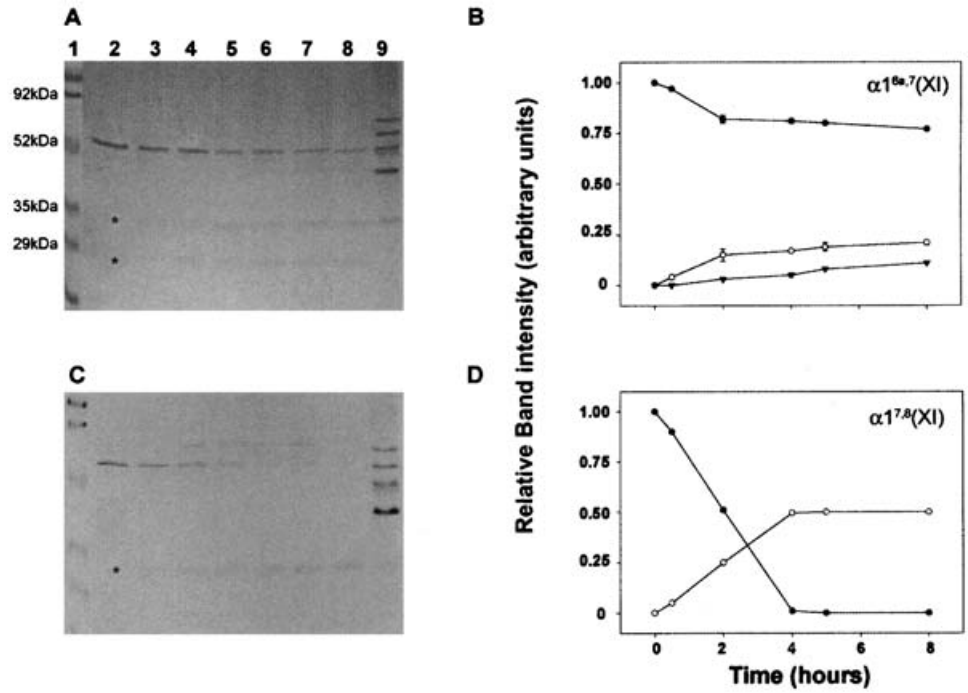


Figure 5 Rate of processing of $\alpha1^{6a,7}$ and $\alpha1^{7,8}$ isoforms by BMP-1

Recombinant isoforms less prevalent during vertebrate development: (**A**, **B**) $\alpha1^{6a,7}$(XI); (**C**, **D**) $\alpha1^{7,8}$(XI). Samples were incubated for a total of 8 h with and without BMP-1. Coomassie Blue-stained SDS/polyacrylamide (13 %) gel, showing isoform-specific processing with (**A**, **C**) BMP-1. Lanes 1, SDS/polyacrylamide gel molecular-mass standards; lanes 2, 0 min digestion; lanes 3, 30 min digestion; lanes 4, 1 h digestion; lanes 5, 2 h digestion; lanes 6, 4 h digestion; lanes 7, 5 h digestion; lanes 8, 8 h digestion; lanes 9, collagen $\alpha1$ XI NTD molecular-mass standard. *Products of proteolytic processing. Controls for each isoform without BMP-1 were run in parallel with the BMP-1-treated isoform (results not shown). (**B**, **D**) Rate of processing corresponding to a specific isoform. ○, Npp; ●, NTD; ▼, variable region.

Sequence at a distance affects the efficiency of proteolytic processing by BMP-1

In addition to the site immediately adjacent to the proteolytic processing site, there is an additional site that undergoes alternative splicing during mRNA processing giving rise to an additional variation in this region of $\alpha1$(XI) collagen chains. Exon 8 can be included or excluded (Figure 1B). To address the possibility that the presence or absence of p8 could alter the efficiency of BMP-1-mediated proteolytic processing of $\alpha1$(XI) chains, similar *in vitro* processing assays with two additional isoforms (Figure 5) were performed. A comparison of isoform $\alpha1^{6a,7}$ (Figures 5A and 5B) with isoform $\alpha1^{6a,7,8}$ (Figures 4C and 4D) demonstrated that the presence of p8 resulted in an enhancement of proteolytic processing at the BMP-1 processing site. Similarly, a comparison of isoform $\alpha1^{7}$ (Figures 4E and 4F) with isoform $\alpha1^{7,8}$ (Figures 5C and 5D) showed an enhancement, owing to the presence of p8 as well. A comparison of the results presented in Figures 4 and 5 is summarized in Table 2.

Table 2 Extent of processing of collagen type XI $\alpha1$ chain isoforms by BMP-1

The presence of p8 enhances BMP-1 processing. The extent of processing of each isoform of the collagen type XI $\alpha1$ chain by BMP-1 after 8 h incubation in the presence of BMP-1 is shown. Isoforms $\alpha1^{7}$(XI) and $\alpha1^{6a,7}$(XI) are processed more completely when the isoform contains a p8 sequence.

Isoform	% processed*
$\alpha1^{6b,7}$	100
$\alpha1^{7}$	18
$\alpha1^{7,8}$	100
$\alpha1^{6a,7}$	23
$\alpha1^{6a,7,8}$	75

* After 8 h at 37 °C with BMP-1.

Protein extracted from bovine cartilage demonstrates isoform-specific variation in the extent of proteolytic processing

Using antibodies specific for the regions encoded by exons 6b, 7 and 8, as well as migration on SDS/polyacrylamide gels, a difference in the ratio of precursor to processed form of $\alpha1$(XI) chain isoforms was demonstrated. Since different antibodies were used for the detection of each isoform, the intensity of the bands could not be used to quantify the relative abundance of distinct $\alpha1$(XI) isoforms within a sample. However, the relative intensities could be used to compare the abundance of the same $\alpha1$(XI) isoform at different positions in a given lane on a gel. Steady-state levels of $\alpha1^{7,8}$ are approx. 50 % of $\alpha1^{6a,7,8}$ (Figures 6A and 6D). With an immunoblot showing total $\alpha1$(XI) chains serving as the reference (Figure 6A), > 80 % of $\alpha1^{6b,7}$ steady-state levels were proteolytically processed (Figure 6C). Approximately 65 % of $\alpha1^{6a,7,8}$ steady-state levels were found in the processed state, whereas approx. 50 % of $\alpha1^{7,8}$ retained the Npp domain (Figure 6D). Most of the $\alpha1$(XI) collagen that retained the Npp domain corresponded to the $\alpha1^{7}$ isoform (Figure 6A, lanes 1 and 4).

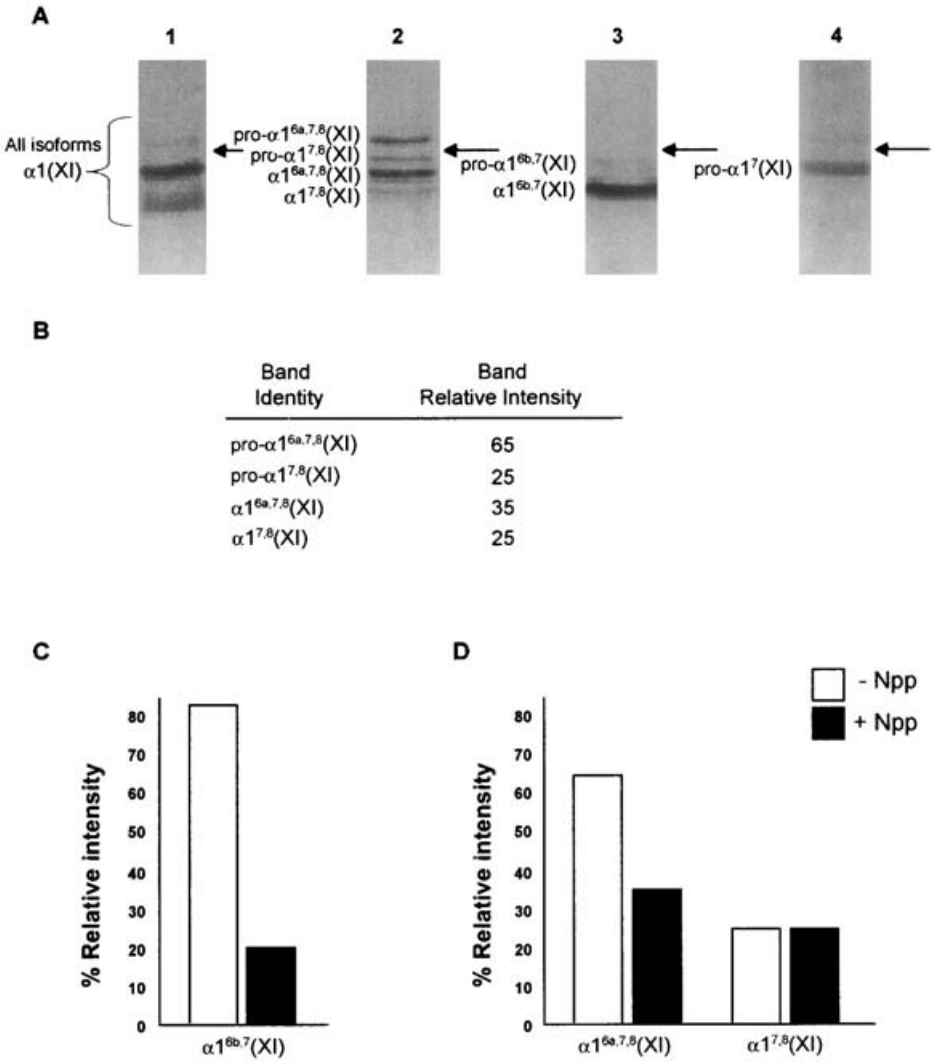


Figure 6 Processing of isoforms *in vivo*

Intact triple-helical collagen molecules with and without Npp were resolved on an SDS/polyacrylamide (5 %) gel. (**A**) Protein extracted from bovine cartilage was transferred on to a PVDF membrane. Lane 1, mouse monoclonal antibody to the α1(XI) carboxy telopeptide; lane 2, mouse monoclonal antibody to p8; lane 3, mouse monoclonal antibody to p6b; lane 4, rabbit polyclonal antibody to Npp. Band identity was deduced by migration on gel in conjunction with antigenicity. Arrows indicate position of the 200 kDa molecular-mass standard. (**B**) Relative band intensities of the precursor and processed forms of the $\alpha 1^{6a,7,8}$(XI) and $\alpha 1^{6a,7}$(XI) isoforms. (**C**) Quantification of relative amounts of precursor and processed $\alpha 1^{6b,7}$(XI). (**D**) Quantification of relative amounts of precursor and processed $\alpha 1^{6a,7,8}$(XI) and $\alpha 1^{6a,7}$(XI). $n = 3$, S.E.M. = 0.00078 for $\alpha 1^{6b,7}$(XI), 0.00069 for $\alpha 1^{6a,7,8}$(XI), and 0.000041 for $\alpha 1^{6a,7}$(XI).

An additional site of proteolytic processing within the $\alpha 1^{6b,7}$ isoform

After analysis of the protein that was extracted from cartilage, two smaller bands (17 and 21 kDa) were detected with an antibody specific for the p6b sequence of the variable region by SDS/PAGE and subsequent immunoblot analysis (Figure 7A). These bands exhibited sensitivity to collagenase, indicating the presence of the minor helix. The presence of these bands is consistent with processing within the amino telopeptide. An antibody raised against the first 20 amino acids of p6b recognized the higher-molecular-mass band, whereas an antibody raised against the last 20 amino acids of p6b recognized both bands equally well, indicating that the difference between the two bands was due to the presence or absence of a portion of the p6b sequence (Figure 7B). The mass difference between the bands, in combination with loss of epitope, suggested an additional processing site within the p6b sequence. However, BMP-1 did not recognize a synthetic p6b peptide as a substrate (results not shown).

DISCUSSION

Isoform-specific processing by BMP-1 is determined by the composition of the variable region

In the present study, it was shown that BMP-1 could process all isoforms of the collagen type XI α1 chain. This finding is consistent with results from a previous study demonstrating that the same site was used for the processing of all isoforms in chicken [30], and demonstrates that this common site can be recognized by BMP-1. It was also demonstrated that isoforms differ in the extent and rate of proteolytic processing by BMP-1. Both amino acid sequences adjacent to the processing site as well as sequences further away affect the efficiency of processing by BMP-1.

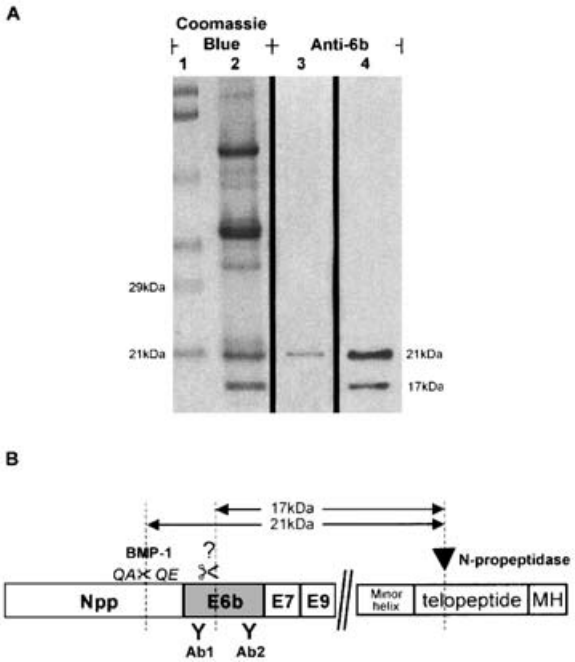


Figure 7 Production of smaller polypeptides in bovine cartilage from proteolytic processing

Protein was extracted from bovine cartilage and separated on SDS/polyacrylamide (13 %) gel. Isoform-specific antibodies were used in immunoblotting to detect α1(XI)-related peptides. (**A**) Coomassie Blue-stained SDS/polyacrylamide (13 %) gel, showing molecular-mass standards (lane 1) and total protein from bovine cartilage tissue extract (lane 2). Anti-6b antibodies were used in immunoblotting; lane 3, first 20 amino acids of p6b sequence; lane 4, last 20 amino acids of p6b sequence. (**B**) Diagram of the deduced $\alpha 1^{6b,7}$(XI) cleavage site and proposed schematic representation of how the 21 and 17 kDa protein fragments arise. The BMP-1 cleavage site producing the Npp fragment is indicated (by scissors and italicized recognition sequence). The other scissor with '?' indicates proposed cleavage sites within the p6b sequence. ▼, N-propeptidase, an enzyme that has been proposed to process proteolytically within the telopeptide region of collagens; MH, the major helix. Antibodies Ab1 and Ab2 correspond to lanes 3 and 4 of (**A**) respectively.

The highly charged nature of p6a and p6b specify the chemical nature of the variable region according to the isoform identity. This could in turn modulate biological activity of the α1(XI) collagen chain. Lying adjacent to the proteolytic processing site, the sequences could influence the rate of removal of the large Npp, and in this way affect the morphology of the resulting fibril. We decided to evaluate the impact of the three possible sequences that can be found adjacent to the BMP-1 processing site on the rate and extent of proteolytic processing. Amino acid sequences encoded by exons 6a, 6b and 7 can be located next to the BMP-1 recognition site, depending on the identity of the isoform. Results indicate that p6b, which is basic in nature and possesses relatively low sequence complexity owing to clusters of lysine and arginine residues, enhanced the rate of processing by BMP-1, relative to p7 and p6a, both of which being acidic in nature.

Quite surprising was the finding that the amino acid sequence p8, located at a distance from the processing site, also affected the rate and extent of proteolytic processing by BMP-1. The amino acid composition of p8 is also acidic in nature; however, unlike the inhibitory effect of p6a and p7 immediately adjacent to the processing site, the presence of p8 at a distance increased the rate and extent of processing by BMP-1. This apparent contradiction may be explained in part by results from previous structural studies [31]. CD studies indicate that the variable region is a distinct domain with a lack of periodic structure. Secondary-structure predictions and conserved domain searches found no known structural elements. However, in the presence of p8, an additional globular domain was visible by rotary shadowing of the purified recombinant $\alpha 1^{6a,7,8}$ isoform [31]. It is possible that the presence of p8 overcomes the inhibitory effect of p7 or p6a alone by allowing the variable region to adopt structural features that are favourable to proteolytic processing by BMP-1 more so than in the absence of p8.

Working model of proteolytic processing of the α1(XI) collagen chain within collagen fibril

The α1 chain of collagen type XI is required for assembly of the extracellular matrix of cartilage [3,35]. Proteolytic processing removes the Npp, and *in vitro* studies have shown that this domain is removed slowly, relative to collagen type II processing or the processing of the α2 chain of collagen type XI [36]. Proteolytic processing by BMP-1 at the site determined by Rousseau et al. [9] for the $\alpha 1^{7}$ isoform, analogous to the BMP-1 processing site for the α1(V) collagen chain [24], would result in the retention of the entire variable region and the minor helix. Fragments generated from the p6b-containing isoform of α1(XI) collagen were detected. They are consistent with proteolytic processing at the amino telopeptide region, within the p6b sequence, and within the QA QE site.

Ultrastructural analysis has confirmed that the variable region is an integral part of the collagen fibril, as p6b and p8 epitopes have been localized at the fibril surface [37]. However, these results indicate that although this is true, some proportion of the variable region, along with the minor helix, may be removed. In addition to influencing the rate and extent of processing, the functional significance of the isoforms may reside in their ability to mediate interactions between the collagen fibril and other molecules of the extracellular matrix. Regulated removal of this domain could then be a mechanism for the regulation of specific molecular interactions.

Possible role for products of processing

These studies demonstrated that smaller fragments arising from the $\alpha 1^{6b,7}$ isoform are detectable within a cartilage tissue as soluble polypeptides. These fragments were visible by Coomassie Blue staining of proteins extracted in 1 M NaCl at physiological pH in the absence of denaturing reagents such as guanidinium chloride. The presence of small, highly charged polypeptides within the extracellular matrix cartilage is an intriguing finding, in the light of the highly restricted location of the $\alpha 1^{6b,7}$ isoform of α1(XI) collagen chain in developing long bones. This isoform is restricted to the region immediately adjacent to the perichondrium under the newly forming bony collar at the diaphysis [38]. The enzyme-mediated generation of small, highly charged polypeptides at this distinct location within the developing skeleton may contribute to early events in bone formation.

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REFERENCES

1 Yoshioka, H. and Ramirez, F. (1990) Pro-α1(XI) collagen. Structure of the amino-terminal propeptide and expression of the gene in tumor cell lines. J. Biol. Chem. **265**, 6423–6426

2 Eikenberry, E. F., Mendler, M., Burgin, R., Winterhalter, K. H. and Bruckner, P. (1982) Fibrillar organization in cartilage. In Articular Cartilage and Osteoarthritis (Kuettner, K.E., Schleyerbach, R., Peyron, J. G. and Hascall, V. C., eds.), pp. 133–148, Raven Press, New York

3 Li, Y., Lacerda, D. A., Warman, M. L., Beier, D. R., Yoshioka, H., Ninomiya, Y., Oxford, J. T., Morris, N. P., Andrikopoulos, K. and Ramirez, F. (1995) A fibrillar collagen gene, *Col11a1*, is essential for skeletal morphogenesis. Cell (Cambridge, Mass.) **80**, 423–430

4 Sussman, M. D., Ogle, R. C. and Balian, G. (1984) Biosynthesis and processing of collagens in different cartilagenous tissues. J. Orthop. Res. **2**, 134–142

5 Eyre, D. E. and Wu, J. J. (1987) The type XI or $1\alpha2\alpha3\alpha$ collagen. In Structure and Function of Collagen Types (Mayne, R. and Burgeson, R. E., eds.), pp. 261–309, Academic Press, Orlando, FL

6 Bernard, M., Yoshioka, H., Rodriguez, E., van der Rest, M., Kimura, T., Ninomiya, Y., Olsen, B. R. and Ramirez, F. (1988) Cloning and sequencing of pro-α1(XI) collagen cDNA demonstrates that type XI belongs to the fibrillar class of collagens and reveals that the expression of the gene is not restricted to cartilagenous tissue. J. Biol. Chem. **263**, 17159–17166

7 Beckmann, G., Hanke, J., Bork, P. and Reich, J. G. (1998) Merging extracellular domains: fold prediction for laminin G-like and amino-terminal thrombospondin-like modules based on homology to pentraxins. J. Mol. Biol. **275**, 725–730

8 Oxford, J. T., Doege, K. J. and Morris, N. P. (1995) Alternative exon splicing within the amino-terminal nontriple-helical domain of the rat pro-α1(XI) collagen chain generates multiple forms of the mRNA transcript which exhibit tissue-dependent variation. J. Biol. Chem. **270**, 9478–9485

9 Rousseau, J. C., Farjanel, J., Boutillon, M. M., Hartmann, D. J., van der Rest, M. and Moradi-Ameli, M. (1996) Processing of type XI collagen. Determination of the matrix forms of the α1(XI) chain. J. Biol. Chem. **271**, 23743–23748

10 Blaschke, U. K., Eikenberry, E. F., Hulmes, D. J., Galla, H. J. and Bruckner, P. (2000) Collagen XI nucleates self-assembly and limits lateral growth of cartilage fibrils. J. Biol. Chem. **275**, 10370–10378

11 Bode, W., Gomis-Rüth, F. X. and Stöcker, W. (1993) Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. FEBS Lett. **331**, 134–140

12 Kessler, E., Takahara, K., Biniaminv, L., Brusel, M. and Greenspan, D. S. (1996) Bone morphogenetic protein-1: the type I procollagen C-proteinase. Science **271**, 360–362

13 Borel, A., Eichenberger, D., Farjanel, J., Kessler, E., Gleyzal, C., Hulmes, D. J., Sommer, P. and Font, B. (2001) Lysyl oxidase-like protein from bovine aorta. Isolation and maturation to an active form by bone morphogenetic protein-1. J. Biol. Chem. **276**, 48944–48949

14 Amano, S., Scott, I. C., Takahara, K., Koch, M., Champliaud, M. F., Gerecke, D. R., Hudson, D. L., Nishiyama, T., Lee, S., Greenspan, D. S. et al. (2000) Bone morphogenetic protein 1 is an extracellular processing enzyme of the laminin $5\gamma2$ chain. J. Biol. Chem. **275**, 22728–22735

15 Suzuki, N., Labosky, P. A., Furuta, Y., Hargett, L., Dunn, R., Fogo, A. B., Takahara, K., Peters, D. M., Greenspan, D. S. and Hogan, B. L. (1996) Failure of ventral body wall closure in mouse embryos lacking a procollagen C-proteinase encoded by *Bmp1*, a mammalian gene related to *Drosophila* tolloid. Development **122**, 3587–3595

16 Takahara, K., Lyons, G. E. and Greenspan, D. S. (1994) Bone morphogenetic protein-1 and a mammalian tolloid homologue (mTld) are encoded by alternatively spliced transcripts which are differentially expressed in some tissues. J. Biol. Chem. **269**, 32572–32578

17 Scott, I. C., Blitz, I. L., Pappano, W. N., Imamura, Y., Clark, T. G., Steiglitz, B. M., Thomas, C. L., Mass, S. A., Takahara, K., Cho, K. W. Y. et al. (1999) Mammalian BMP-1/Tolloid-related metalloproteinases, including novel family member mammalian Tolloid-like 2, have differential enzymatic activities and distributions of expression relevant to patterning and skeletogenesis. Dev. Biol. **213**, 283–300

18 Prockop, D. J., Sieron, A. L. and Li, S. W. (1998) Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. Matrix Biol. **16**, 399–408

19 Colige, A., Li, S. W., Sieron, A. L., Nusgens, B. V., Prockop, D. J. and Lapeire, C. M. (1997) cDNA cloning and expression of bovine procollagen I N-proteinase: a new member of the superfamily of zinc-metalloproteinases with binding sites for cells and other matrix components. Proc. Natl. Acad. Sci. U.S.A. **94**, 2374–2379

20 Uzel, M. I., Scott, I. C., Babakhanlou-Chase, H., Palamakumbura, A. H., Pappano, W. N., Hong, H. H., Greenspan, D. S. and Trackman, P. C. (2001) Multiple bone morphogenetic protein 1-related mammalian metalloproteinases process pro-lysyl oxidase at the correct physiological site and control lysyl oxidase activation in mouse embryo fibroblast cultures. J. Biol. Chem. **276**, 22537–22543

21 Scott, I. C., Imamura, Y., Pannano, W. N., Troedel, J. M., Recklies, A. D., Roughley, P. J. and Greenspan, D. S. (2000) Bone morphogenetic protein-1 processes probiglycan. J. Biol. Chem. **275**, 30504–30511

22 Wardle, F. C., Welch, J. V. and Dale, L. (1999) Bone morphogenetic protein 1 regulates dorsal–ventral patterning in early *Xenopus* embryos by degrading chordin, a BMP4 antagonist. Mech. Dev. **86**, 75–85

23 Kessler, E., Fichard, A., Chanut-Delalande, H., Brusel, M. and Ruggiero, F. (2001) Bone morphogenetic protein-1 (BMP-1) mediates C-terminal processing of procollagen V homotrimer. J. Biol. Chem. **276**, 27051–27057

24 Imamura, Y., Steiglitz, B. M. and Greenspan, D. S. (1998) Bone morphogenetic protein-1 processes the NH_2-terminal propeptide, and a furin-like proprotein convertase processes the COOH-terminal propeptide of pro-α1(V) collagen. J. Biol. Chem. **273**, 27511–27517

25 Unsold, C., Pappano, W. N., Imamura, Y., Steiglitz, B. M. and Greenspan, D. S. (2002) Biosynthetic processing of the pro-α1(V)2pro-α2(V) collagen heterotrimer by bone morphogenetic protein-1 and furin-like proprotein convertases. J. Biol. Chem. **277**, 5596–5602

26 Rattenholl, A., Pappano, W. N., Koch, M., Keene, D. R., Kadler, K. E., Sasaki, T., Timpl, R., Burgeson, R. E., Greenspan, D. S. and Bruckner-Tuderman, L. (2002) Proteinases of the bone morphogenetic protein-1 family convert procollagen VII to mature anchoring fibril collagen. J. Biol. Chem. **277**, 26372–26378

27 Li, S. W., Arita, M., Fertala, A., Bao, Y., Kopen, G. C., Langsjo, T. K., Hyttinen, M. M., Helminen, H. J. and Prockop, D. J. (2001) Transgenic mice with inactive alleles for procollagen N-proteinase (ADAMTS-2) develop fragile skin and male sterility. Biochem. J. **355**, 271–278

28 Fernandes, R. J., Hirohata, S., Engle, J. M., Colige, A., Cohn, D. H., Eyre, D. R. and Apte, S. S. (2001) Procollagen II amino propeptide processing by ADAMTS-3. Insights on dermatosparaxis. J. Biol. Chem. **276**, 31502–31509

29 Kuno, K., Terashima, Y. and Matsushima, K. (1999) ADAMTS-1 is an active metalloproteinase associated with the extracellular matrix. J. Biol. Chem. **274**, 18821–18826

30 Moradi-Ameli, M., de Chassey, B., Farjanel, J. and van der Rest, M. (1998) Different splice variants of cartilage α1(XI) collagen chain undergo uniform amino-terminal processing. Matrix Biol. **17**, 393–396

31 Gregory, K. E., Oxford, J. T., Chen, Y., Gambee, J. E., Gygi, S. P., Aebersold, R., Neame, P. J., Mechling, D. E., Bachinger, H. P. and Morris, N. P. (2000) Structural organization of distinct domains within the non-collagenous N-terminal region of collagen type XI. J. Biol. Chem. **275**, 11498–11506

32 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. **72**, 248–254

33 Jin, Y. and Cerletti, N. (1992) Western blotting of transforming growth factor β2. Optimization of the electrophoretic transfer. Appl. Theor. Electrophor. **3**, 85–90

34 Davies, G. B., Oxford, J. T., Hausefus, L. W., Smoody, B. F. and Morris, N. P. (1998) Temporal and spatial expression of alternative splice-forms of the α1(XI) collagen gene in fetal rat cartilage. Dev. Dyn. **213**, 12–26

35 Seegmiller, R., Fraser, F. C. and Sheldon, H. (1971) A new chondrodystrophic mutant in mice. Electron microscopy of normal and abnormal chondrogenesis. J. Cell Biol. **48**, 580–593

36 Thom, J. R. and Morris, N. P. (1991) Biosynthesis and proteolytic processing of type XI collagen in embryonic chick sterna. J. Biol. Chem. **266**, 7262–7269

37 Keene, D. R., Oxford, J. T. and Morris, N. P. (1995) Ultrastructural localization of collagen types II, IX, and XI in the growth plate of human rib and fetal bovine epiphyseal cartilage: type XI collagen is restricted to thin fibrils. J. Histochem. Cytochem. **10**, 967–979

38 Morris, N. P., Oxford, J. T., Davies, G. B., Smoody, B. F. and Keene, D. R. (2000) Developmentally regulated alternative splicing of the α1(XI) collagen chain: spatial and temporal segregation of isoforms in the cartilage of fetal rat long bones. J. Histochem. Cytochem. **48**, 725–741

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