Processing of Type II Procollagen Amino Propeptide by Matrix Metalloproteinases*

Received for publication, June 13, 2001, and in revised form, November 6, 2001
Published, JBC Papers in Press, November 8, 2001, DOI 10.1074/jbc.M105485200

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In many embryonic tissues, type IIA procollagen is synthesized and deposited into the extracellular matrix containing the NH₂-propeptide, the cysteine-rich domain of which binds to bone morphogenetic proteins. To investigate whether matrix metalloproteinases (MMPs) synthesized during development and disease can cleave the NH₂-terminus of type II procollagens, we tested eight types of enzymes. Recombinant trimeric type IIA collagen NH₂-propeptide encoded by exons 1–8 fused to the lectin domain of rat surfactant protein D was used as a substrate. The latter allowed trimerization of the propeptide domain and permitted isolation by saccharide affinity chromatography. Although MMPs 1, 2, and 8 did not show cleavage, MMPs 3, 7, 9, 13, and 14 cleaved the recombinant protein both at the telopeptide region and at the procollagen N-proteinase cleavage site. MMPs 7 and 13 demonstrated other cleavage sites in the type II collagen-specific region of the N-propeptide; MMP-7 had another cleavage site close to the COOH terminus of the cysteine-rich domain. To prove that an MMP can cleave the native type IIA procollagen in situ, we demonstrated that MMP-7 removes the NH₂-propeptide from collagen fibrils in the extracellular matrix of fetal cartilage and identified the cleavage products. Because the N-proteinase and telopeptidase cleavage sites are present in both type IIA and type IIB procollagens and the telopeptide cleavage site is retained in the mature collagen fibril, this processing could be important to type IIB procollagen and to mature collagen fibrils as well.

Fibrillar collagen types I, II, III, and V are synthesized with globular propeptides at both the COOH and NH₂ termini that are generally removed prior to formation of fibrils. In contrast, there are certain instances in which the collagen fibril retains the NH₂-propeptide, primarily types I and III in embryonic skin (1) and type IIA during skeletal development. We have shown that the type IIA form of type II procollagen retains the NH₂-propeptide when synthesized during chondrogenesis and in other embryonic tissues where type II collagen is made, such as notochord, aorta, kidney, and skin (2–4). Type IIA pN procollagen is also found in fetal and adult vitreous humor (5, 6). Processed type I NH₂-propeptide has been isolated from bovine bone (7) and the type II NH₂-propeptide from embryonic chicken cartilage (8). In addition, the NH₂-propeptides of type I, IIA, and III have been found in serum (9–11) and used as markers for metabolic activity of the collagen-synthesizing tissues. In type I, and presumably type II and type III procollagens, the NH₂-propeptide is removed from the procollagen by a specific N-proteinase characterized by Prockop and colleagues (12). Eyre and colleagues (13) demonstrated that MMP-3 (stromelysin) can cleave type II collagen at two locations in the amino telopeptide just a few amino acids COOH-terminal of the N-proteinase cleavage site, thereby acting as a telopeptidase, suggesting the possibility that MMP-3 also can release the propeptide region. The ability of other MMPs to cleave this region is not known.

We have shown recently that the cysteine-rich domain of type IIA procollagen NH₂-propeptide binds to members of the TGF-β superfamily, TGF-β1 and BMP-2, thereby predicting an important function for the NH₂-propeptide in the extracellular matrix (3). Cleavage of the NH₂-propeptide in the extracellular matrix could have significant effects on the binding or presentation of the BMPs. Support for this hypothesis comes from BMP-binding proteins that are involved in dorsal-ventral patterning: chordin in Xenopus and sog in Drosophila. Chordin and sog are homologues containing the same cysteine-rich domain as the collagen NH₂-propeptide and bind to BMP-4 and decapentaplegic, respectively (14, 15). Binding of these BMPs to chordin or sog inhibits interaction of the BMP with its cellular receptors. Furthermore, enzymic cleavage of chordin by the Xenopus enzyme xolloid and sog by the Drosophila enzyme tolloid serves to activate the bound BMP (14, 15).

The type IIA pN-procollagen containing the cysteine-rich domain is deposited in the extracellular matrix providing potential localization of BMP in the insoluble compartment of the extracellular matrix. In analogy to cleavage of chordin and sog, the NH₂-propeptide could be liberated by cleavage from the pN-procollagen. Cleavage of the procollagen in different domains would theoretically produce two different types of fragments. If cleavage takes place at the telopeptide the trimeric NH₂-propeptide would be released; if cleavage takes place near the BMP-binding domain, monomeric cysteine rich domains could be liberated. Likely candidates for extracellular cleavage of pN procollagen are the MMPs and astacin proteinases such as tolloid and BMP-1 (procollagen C-proteinase). The purpose of this study was to determine the cleavage specificity of the potentially relevant MMPs. We tested MMPs 1, 2, 3, 7, 8, 9, 13, and 14 and found that MMPs 3, 7, 8, 9, 13, and 14 were capable of specific cleavage of the type IIA procollagen NH₂-propeptide at various sites. All enzymes cleave in domains common to both type IIA and type IIB procollagen, whereas MMPs 7 and 13 also...
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In preliminary studies, we observed that recombinant propeptides of type IIA procollagen expressed in prokaryotic or eukaryotic cells are secreted as monomers. To study the cleavage of a trimeric propeptide domain, we utilized a previously characterized chimera consisting of the propeptide domain of IIA procollagen linked to a surfactant protein D (SPD) sequence that encodes a contiguous coiled-coil trimerization domain and a C-type lectin domain (16). The recombinant protein is secreted as a trimeric protein with a triple-helical minor helix and can be readily isolated by saccharide affinity chromatography on maltosyl-agarose.

EXPERIMENTAL PROCEDURES

Preparation of the Substrates—Triple-helical type II procollagen N-propeptide was prepared as a fusion protein with the neck region and lectin domain of rat SPD (Fig. 1, C and D). PCR overlap extension was used to create IIA-SPD cDNA in pGEM-3Z (17, 18). The generated construct was excised from pGEM-3Z and ligated to create IIA-SPD cDNA in pGEM-3Z (Fig. 1D). Following the detection of exon 2-encoded region of type IIA procollagen was confirmed (data not shown). The neck region and carbohydrate recognition domain in IIA-SPD was detected by SPD (Fig. 1C). The detection of exon 2-encoded region of type IIA procollagen was confirmed (data not shown). The neck region and carbohydrate recognition domain in IIA-SPD was detected by SPD (Fig. 1C). The detection of exon 2-encoded region of type IIA procollagen was confirmed (data not shown). The neck region and carbohydrate recognition domain in IIA-SPD was detected by SPD (Fig. 1C). The detection of exon 2-encoded region of type IIA procollagen was confirmed (data not shown). The neck region and carbohydrate recognition domain in IIA-SPD was detected by SPD (Fig. 1C).

Preparation of the Substrates—Triple-helical type II procollagen N-propeptide was prepared as a fusion protein with the neck region and carbohydrate recognition domain of rat SPD (Fig. 1, C and D). PCR overlap extension was used to create IIA-SPD cDNA in pGEM-3Z (17, 18). The generated construct was excised from pGEM-3Z and ligated into the multiple cloning site of pE14 (18) with this vector, several transient expression plasmids of Chinese hamster ovary-KI cells were established using methionine sulfoximine as a selectable marker. To obtain IIA-SPD, the clones were cultured in glutamine-free Glasgow's minimum essential medium (Sigma) containing 10% dialyzed fetal calf serum (HyClone, Logan, UT) and 50 μg/ml ascorbic acid (Sigma) with 100–1000 μg/ml methionine sulfoximine (Sigma). The purification of IIA-SPD followed the method previously used for recombinant rat SPD (18). Briefly, medium from cultured cells was dialyzed against Tris-buffered saline, pH 7.5, containing 10 mM EDTA and 0.2 mM phenylmethylsulfonyl fluoride. Following recalcification, IIA-SPD was isolated by affinity chromatography on maltosyl-agarose (Bio-Rad).

MMP Cleavage—Recombinant human MMPs 1 (collagenase-1), 3 (stromelysin-1), 7 (matrilysin), 8 (neuropilin collagenase, collagenase-2), and 14 (catalytic domain, MT1-MMP) were purchased from Chemicon International (Temecula, CA). Recombinant human MMPs 2 (gelatinase A) and 9 (gelatinase B) were provided by Dr. Robert Senior (Washington University, St. Louis, MO). Purified rat MMP-13 (collagenase-3) was obtained from Dr. John Jeffrey (Albany Medical College, Albany, NY). Except for MMP-7 lacking propeptide domain and the catalytic domain of MMP-14, all the MMPs were activated by the incubation with 1 mM 4-aminophenylmercuric acetate for 1–24 h at 37 °C.

For cleavage experiments, IIA-SPD and recombinant rat SPD were dialyzed against MMP cleavage buffer (0.2 M NaCl, 10 mM CaCl2, 50 mM Tris, pH 7.5) and adjusted to a final concentration of 100 ng/μl. Cleavage was performed by incubating the substrates with each MMP at 37 °C for 24 h or as indicated, at an enzyme to substrate ratio of 1:50 w/w (for MMPs 1, 2, 3, 8, and 14) or 1:100 (for MMPs 7, 9, and 13). The ratios were determined by the efficiency of cleavage for the respective enzymes. The cleavage was then terminated by the addition of EDTA to a final concentration of 20 mM.

Analysis of the Cleaved Products—The cleaved products of IIA-SPD were analyzed by silver staining and Western blotting. After the incubation with MMPs, the substrates were loaded onto 4–20% SDS-polyacrylamide gel (Gradipore, North Ryde, New South Wales, Australia) and electrophoresed under reducing conditions with 0.1M dithiothreitol as for gel staining. The primary antibodies used were at the concentrations of 1:1000 (IIA-SPD antisera) or 1:500 (anti-IIE3–8) in 1% dry milk in PBS. After washing in PBS, the membranes were incubated with appropriate secondary antibodies coupled to hors eradish peroxidase at the concentration of 1:2000 in 1% dry milk in PBS at room temperature for 1 h. The analyses were accomplished by chemiluminescence on autoradiography films (ISO-MAX, Scimitar, St. Louis, MO), using enhanced chemiluminescent reagents for horseradish peroxidase (SuperSignal™ West Pico, Pierce).

NH2-terminal amino acid sequencing was performed to determine the cleavage sites. Cleaved fragments were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene membrane (Sequoi-Blot PVDF membrane, Bio-Rad). Protein bands were visualized by staining with Coomassie Brilliant Blue R-250, excised, then sequenced with an ABI 473A protein sequencer equipped with the model 610A data analysis software. For the bands with multiple sequences, initial and repetitive yields were used to assign identity of the cleavage site.

Cleavage of Native Type IIA Procollagen by MMP-7 in Tissue Sections—Tissues from a 9-week gestation human fetus (provided by the Central Laboratory for Human Embryology, University of Washington, Seattle, WA) were frozen in OCT compound (Miles Laboratories, Elkhart, IN), and 10-μm-thick sections were prepared with a cryostat and placed on a polylysine coated glass slides (Fischer Scientific, Pittsburgh, PA). After initial preliminary experiments to establish enzyme to substrate levels, time of digestion, and washing conditions, 12 serial sections were subjected to MMP-7 digestion. They were divided into three sets of four serial sections. In each set, three sections were incubated with 50 μl of MMP cleavage buffer containing 5, 10, or 20 ng/μl MMP-7. The other one was treated with the same buffer containing both 10 ng/μl MMP-7 and 20 mM EDTA and served as a control (EDTA control). After the incubation at 37 °C for 24 h, the sections were washed in PBS containing 0.05% Triton X-100 at room temperature for 60 min to remove the cleaved fragments.

Immunofluorescence Staining—After MMP-7 treatment, the sections were washed in PBS and fixed in 4% paraformaldehyde PBS solution for 10 min at room temperature. They were then treated with 0.2% hyaluronidase (type III, Sigma) in PBS for 30 min at 37 °C, and blocked with 1% (v/v) normal donkey serum in PBS for 1 h at room temperature. Double immunostaining was performed. To detect type IIA NH2-propeptide, antiserum was used at a dilution of 1:400. To detect the major triple-helical domain, rat antiserum against bovine type II collagen (a gift from Dr. Michael Cremer, University of Tennessee, Memphis, TN) was used at the concentration of 1:40.

The sections were incubated at 4 °C overnight in PBS containing the designated sera with 1% (v/v) normal donkey serum. Two other sections were incubated with normal rabbit and rat sera at the same concentrations and served for the normal sera control. After washing in PBS, the sections were incubated sequentially with secondary antibodies for the respective primary antibodies: cyanine 3-conjugated donkey anti-rabbit IgG F(ab)2 fragment with a dilution of 1:200 and FITC-conjugated donkey anti-rat IgG F(ab)2 fragment with a dilution of 1:100. Finally, the sections were washed in PBS and mounted in fluorescent mounting medium (DAKO, Carpenteria, CA). All the normal sera and the secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Because MMP-7 does not cleave fibrillar type II collagen (data not shown), cleavage by MMP-7 in the exon 2–8-encoded region of native type IIA procollagen was evaluated quantitatively by the fluorescence intensity. Immunoreactivity for the exon 2-encoded region in NH2-propeptide was compared with that for the major triple-helical domain of type II collagen (Fig. 1A). Fluorescence intensity was measured by confocal laser-scanning microscopy. The images for quantification were

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MMP Cleavage of Recombinant Rat SPD and IIA-SPD—Under reducing conditions, recombinant rat SPD and IIA-SPD (Fig. 1, C and D) appeared as bands of 43 and 47 kDa, respectively (Fig. 2). None of the MMPs tested showed detectable cleavage of recombinant rat SPD under the conditions used in this study (Fig. 2A). IIA-SPD was efficiently cleaved by MMPs 3, 7, 9, 13, and 14, whereas MMPs 1, 2, and 8 did not show significant cleavage (Fig. 2B). Because rat SPD was resistant to MMP cleavage, we inferred that cleavage of IIA-SPD occurred within the procollagen domain. Accordingly, our subsequent experiments focused on the cleavage of IIA-SPD by MMPs 3, 7, 9, 13, and 14. The cleavage patterns for these MMPs were similar (Fig. 2B). Two bands of ~17 and 20 kDa were common to all, and these MMPs also generated an additional band of ~25 kDa. MMPs 3 and 13 generated components of higher apparent molecular mass, between 34 and 30 kDa.

Next, the time course of the cleavage was studied for all the MMPs. The results for MMPs 9 and 14 are shown in Fig. 3. MMP-9 digested IIA-SPD completely at 24 h (Fig. 3A), whereas MMP-14 did not cleave the protein completely even at 48 h (Fig. 3B). In the cleavage by MMP-9, a faint band at 32 kDa was seen only for the first 1 h of incubation, but all the other bands appeared at 0.5 h and remained unchanged up to 48 h. The pattern of cleavage by MMP-14 was consistent throughout the incubation period. For both of these enzymes, the fragment at 20 kDa was likely derived from a preferred cleavage site, as well as from the action of contaminating proteinases.

Western Blot Analysis and Amino Acid Sequencing of the Cleaved Fragments—All the major bands visualized by gel staining were recognized by at least one of the three antibodies used in this study, confirming they were cleavage products of IIA-SPD. For each MMP, these bands were numbered sequentially according to their molecular sizes.

Four major bands were identified following cleavage with MMP-3 (Fig. 4A). In Western blot analysis, bands 1 and 2 were recognized by both IIA antiserum and anti-IEE3-8, whereas bands 3 and 4 were detected only by SPD antiserum. From the results, MMP-3 was inferred to cleave IIA-SPD at two sites between the epitopes for anti-IEE3-8 and those for SPD anti-
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serum (Fig. 1D), generating two pairs of fragments. Subsequently, bands 3 and 4 were subjected to amino acid sequencing as they contained the epitopes for the SPD antiserum. The results of microsequencing showed that each of the bands contained two fragments cleaved at consecutive sites (Table I). The analysis of band 3 revealed two cleavage sites at Ala156-Gln157, which is also the procollagen N-proteinase cleavage site (20). Band 4 contained two fragments cleaved at Asp86-Ile87 and Asp89-Ile90 at an equal ratio. The analysis of band 5 revealed the cleavage at Gln157-Met158 and that of band 6 was another site in N-telopeptide region at Gly172-Val173. MMP-9 cleavage produced three bands (Fig. 4C). Among them, band 1 was detected by both IIA antiserum and anti-IIIE3–8, and bands 2 and 3 were visualized only by SPD antiserum (Fig. 4C). The pattern was similar to that with MMP-3, and the cleavage sites with MMP-9 were considered to be same or close to those with MMP-3. The lower two bands recognized by SPD antiserum were subjected to amino acid sequencing (Table I). The analysis of band 2 presented two consecutive cleavage sites at Ala156-Gln157 and Gln157-Met158 in an equal amount. These are the same cleavage sites recognized by MMP-3. Band 3 with MMP-9 contained two consecutive sites in N-telopeptide region at Val173-Met174 and Met174-Gln175 at the ratio of 3:1.

MMP-13 cleavage produced five bands, and the result of immunostaining was the same as that obtained for bands 2–6 generated by MMP-7 cleavage (Fig. 4D) (Table I). Among the five bands, bands 1, 4, and 5 were analyzed by amino acid sequence (Table I). The cleavage sites for band 1 were the same as obtained for band 2 following cleavage with MMP-7: two fragments at Asp86-Ile87 and Asp89-Ile90 were present at a 1:1 ratio. The analysis of band 4 revealed one cleavage site at Gln157-Met158, and that of band 5 revealed another site in N-telopeptide region at Val173-Met174.

Four fragments were observed by the cleavage with MMP-14 (Fig. 4E). Western blotting showed the cleavage pattern was same as that produced by MMP-3; bands 1 and 2 were recognized by anti-IIIE3–8 and SPD antiserum, and bands 3 and 4 were detected only by SPD antiserum. Microsequencing was performed on bands 3 and 4. Band 3 contained one fragment cleaved at Ala156-Gln157 and another cleavage site in N-telopeptide region at Gly172-Val173 was determined in band 4 (Table I).

Cleavage sites recognized by all MMPs tested are shown in the amino acid sequence of type IIA procollagen (Fig. 5). As noted, all the MMP cleavage sites are distributed in the non-Gly-X-Y portions of type IIA procollagen domain in IIA-SPD, and the exon 2-encoded region contains only one cleavage site by MMP-7. MMPs 3, 9, and 14 cleave this NH2-propeptide domain at Ala156-Gln157 which is also the procollagen N-proteinase cleavage site. MMPs 7 and 13 did not cleave this site but cleaved one residue downstream. All the enzymes have at least one cleavage site in N-telopeptide region downstream of

substrate was analyzed at 0, 0.5, 1, 2, 4, 8, 24, and 48 h after the start of incubation, and the reaction was terminated by addition of EDTA at a final concentration of 20 mm. The samples were separated on 4–20% Tris/glycine gels under reducing conditions with dithiothreitol. The bands were visualized by silver staining.

FIG. 2. Cleavage of recombinant rat SPD and IIA-SPD by MMPs. Recombinant rat SPD (A) and IIA-SPD (B) in MMP cleavage buffer at the concentration of 100 ng/μl were incubated with MMPs 1, 2, 3, 7, 8, 9, 13, or 14 at the enzyme to substrate ratio of 1:100 and 1:50, respectively. The digested substrate was analyzed at 0, 0.5, 1, 2, 4, 8, 24, and 48 h after the start of reaction. The digested substrate was analyzed at 0, 0.5, 1, 2, 4, 8, 24, and 48 h after the start of incubation, and the reaction was terminated by addition of EDTA at a final concentration of 20 mm. The samples were separated on 4–20% Tris/glycine gels under reducing conditions with dithiothreitol, and the bands were visualized by silver staining. The samples incubated with the enzymes and 20 mm EDTA at 37 °C for 24 h were loaded in each lane and separated on 20% SDS-polyacrylamide gel under reducing conditions with dithiothreitol. The bands were visualized by silver staining.

FIG. 3. Sequential cleavage of IIA-SPD by MMPs 9 and 14. IIA-SPD in MMP cleavage buffer (100 ng/μl) was incubated at 37 °C for 24 h with MMP-9 (A) or the catalytic domain of MMP-14 (B) at the enzyme to substrate ratios of 1:100 and 1:50, respectively. The digested substrate was analyzed at 0, 0.5, 1, 2, 4, 8, 24, and 48 h after the start of incubation, and the reaction was terminated by addition of EDTA at a final concentration of 20 mm. The samples were separated on 4–20% Tris/glycine gels under reducing conditions with dithiothreitol, and the bands were visualized by silver staining. The samples incubated with the enzymes and 20 mm EDTA at 37 °C for 48 h are shown together.

with the previously reported result on cleavage of the mature form of native bovine type II collagen (13).

Digestion by MMP-7 produced six bands as seen by gel staining (Fig. 4B), and bands 3–6 showed the same pattern of immunostaining as that with MMP-3, i.e. bands 3 and 4 were detected by both IIA antiserum and anti-IIIE3–8, and bands 5 and 6 were recognized only by SPD antiserum. Therefore, MMP-7 appears to have two cleavage sites at or near the sites of cleavage for MMP-3. In contrast, the top two bands were unique for MMP-7. Band 1 was recognized by anti-IIIE3–8 and SPD antiserum but not by IIA antiserum, and the cleavage for this band should occur between the epitopes for IIA antiserum and those for anti-IIIE3–8 (Fig. 1D). Band 2 was recognized only by SPD antiserum, and the cleavage between anti-IIIE3–8 recognition sites and the SPD antiserum recognition sites could generate this band, upstream of the MMP-3 cleavage sites. Subsequently, bands 1, 2, 5, and 6 were subjected to amino acid sequencing (Table I). The results showed that band 1 contained one fragment cleaved at Asp86-Ile87 in the exon 2-encoded domain at Val87, Arg88, Ile87 at an equal ratio. The analysis of band 5 revealed the cleavage at Gln157-Met158 and that of band 6 showed another site in N-telopeptide region at Gln170-Leu171.

MMP-9 cleavage produced three bands (Fig. 4C). Among them, band 1 was detected by both IIA antiserum and anti-IIIE3–8, and bands 2 and 3 were visualized only by SPD antiserum (Fig. 4C). The pattern was similar to that with MMP-3, and the cleavage sites with MMP-9 were considered to be same or close to those with MMP-3. The lower two bands recognized by SPD antiserum were subjected to amino acid sequencing (Table I). The analysis of band 2 presented two consecutive cleavage sites at Ala156-Gln157 and Gln157-Met158 in an equal amount. These are the same cleavage sites recognized by MMP-3. Band 3 with MMP-9 contained two consecutive sites in N-telopeptide region at Val173-Met174 and Met174-Gln175 at the ratio of 3:1.

MMP-13 cleavage produced five bands, and the result of immunostaining was the same as that obtained for bands 2–6 generated by MMP-7 cleavage (Fig. 4D) (Table I). Among the five bands, bands 1, 4, and 5 were analyzed by amino acid sequence (Table I). The cleavage sites for band 1 were the same as obtained for band 2 following cleavage with MMP-7: two fragments at Asp86-Ile87 and Asp89-Ile90 were present at a 1:1 ratio. The analysis of band 4 revealed one cleavage site at Gln157-Met158, and that of band 5 revealed another site in N-telopeptide region at Val173-Met174.

Four fragments were observed by the cleavage with MMP-14 (Fig. 4E). Western blotting showed the cleavage pattern was same as that produced by MMP-3; bands 1 and 2 were recognized by anti-IIIE3–8 and SPD antiserum, and bands 3 and 4 were detected only by SPD antiserum. Microsequencing was performed on bands 3 and 4. Band 3 contained one fragment cleaved at Ala156-Gln157 and another cleavage site in N-telopeptide region at Gly172-Val173 was determined in band 4 (Table I).

Cleavage sites recognized by all MMPs tested are shown in the amino acid sequence of type IIA procollagen (Fig. 5). As noted, all the MMP cleavage sites are distributed in the non-Gly-X-Y portions of type IIA procollagen domain in IIA-SPD, and the exon 2-encoded region contains only one cleavage site by MMP-7. MMPs 3, 9, and 14 cleave this NH2-propeptide domain at Ala156-Gln157 which is also the procollagen N-proteinase cleavage site. MMPs 7 and 13 did not cleave this site but cleaved one residue downstream. All the enzymes have at least one cleavage site in N-telopeptide region downstream of
Lys165, which is a known cross-link site for the type II collagen fibril (21, 22).

Cleavage of NH₂-propeptide in Tissue by MMP—To determine whether the NH₂-propeptide can be cleaved by MMPs in native tissue, frozen tissue sections and fresh tissues from human fetuses were subjected to MMP-7 treatment. MMP-7 was chosen because it can cleave type IIA procollagen efficiently in NH₂-propeptide and N-telopeptide regions, whereas it does not cleave fibrillar domain of type II collagen. Fetal tissue was chosen because type IIA procollagen is expressed at a high level during chondrogenesis (3).

In the experiment with tissue sections, sections treated with normal sera instead of the primary antibodies showed virtually no fluorescence for both cyanine 3 and FITC (Table II), and the specificity of immunofluorescence staining was confirmed. Because type IIA procollagen is found at high levels at the perichondrium and periphery of the developing cartilage, the change in the fluorescence by MMP-7 treatment was evaluated in this area. On the EDTA control sections, strong fluorescence of cyanine 3 bound to IIA antiserum was observed at the periphery of the cartilage, where type IIA procollagen reacts with both antibodies. Fluorescence of FITC bound to the antibody against major triple-helical domain of type II collagen was more evident in the central area where primarily type IIB procollagen is found (Fig. 6, A and B). The intensities of red and green fluorescence were measured in arbitrary sets and the change in fluorescence was evaluated by their ratio as described under “Experimental Procedures.” The tissues were treated with the three graded concentrations of MMP-7. Dose-dependent removal of the NH₂-propeptide domain was observed in the peripheral region as indicated by a decrease in the ratio of red to green fluorescence (Fig. 6, C and D; Table II).

FIG. 4. Detection of MMP cleavage products of IIA-SPD by Western blotting. IIA-SPD was incubated with each of MMPs 3, 7, 9, 13, or 14 at 37 °C for 24 h as for gel staining and was separated by SDS-polyacrylamide gel electrophoresis under reduced conditions with dithiothreitol (for anti-III3–8 and SPD antiserum) or unreduced conditions (for IIA antiserum). The samples were then transferred to supported nitrocellulose membranes, and the bands were detected by three kinds of antibodies that recognize specific sites of IIA-SPD (Fig. 1D). Results with MMPs 3, 7, 9, 13, and 14 are shown in panels A, B, C, D, and E, respectively. In these panels, all the major bands visualized by gel staining were numbered sequentially according to their molecular sizes for their reference, and the correspondence of the bands are shown by gray lines. The result of immunostaining was displayed in a box placed right in each panel, with the schematic of the antibody recognition sites.
The amino acid sequences determined in the MMP cleaved fragments of IIA-SPD

The glutamine residue at the NH₂-terminal of the secreted form of type IIA procollagen was numbered as the first amino acid. The ratio of two sequences detected from one band is shown in parentheses. Sequences from the chimeric part of rat SP-D are underlined. *, sequence from the previously reported cleavage site (13).

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<th>Enzyme</th>
<th>MMP-3</th>
<th>MMP-7</th>
<th>MMP-9</th>
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<td>Band 1</td>
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<tr>
<td>Band 5</td>
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FIG. 5. The distribution of MMP cleavage sites in type IIA procollagen domain of IIA-SPD. MMP cleavage sites determined in this study are shown by arrows in the amino acid sequence of exon 2–8-encoded region of type IIA procollagen, together with the procollagen N-proteinase cleavage site. The paired arrows indicate the cleavage sites determined together in a single band. Except one site between Asp⁶⁷ and Leu⁶⁸ by MMP-7, all the cleavage sites were in the interrupted portion and N-telopeptide region, and no cleavage sites existed between Ala₁₆⁹ and Gln₁₇⁵ in N-telopeptide region. In this figure, the N-proteinase cleavage site, and all of them had at least one cleavage site same site or one residue to the COOH-terminal side of the procollagen domain of IIA-SPD.

The change of immunofluorescence intensity for exon 2-encoded region in IIA NH₂-propeptide and the fibrillar domain of type II collagen by MMP-7 treatment

Table II

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Fluorescence intensity (arbitrary unit)</th>
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<td>74.3 ± 14.5 99.7 ± 12.0 0.74 ± 0.09</td>
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<tr>
<td>MMP-7 10 ng/µl</td>
<td>67.9 ± 12.5 107.8 ± 11.8 0.63 ± 0.09</td>
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<tr>
<td>MMP-7 20 ng/µl</td>
<td>58.5 ± 16.1 101.0 ± 12.8 0.58 ± 0.13</td>
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<tr>
<td>EDTA control</td>
<td>124.6 ± 13.2 120.2 ± 14.1 1.04 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Normal sera</td>
<td>7.1 ± 2.2 1.7 ± 0.7</td>
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Upon treatment with the MMP-7, the reactivity to the NH₂-propeptide antibody decreased, whereas reactivity to the fibrillar domain remained the same, strongly suggesting removal of the NH₂-propeptide.

Cleavage products by MMP-7 were analyzed by Western blotting using anti-IIIE3-8 (Fig. 7A). Before cleavage, a single band was observed at 20 kDa. As the cleavage progressed, other bands of higher molecular mass were released from the tissue, and five bands were recognized in 24-h sample. Two of them, bands 2 and 4, were also recognized by IIA antisera, indicating that they contain exon 2-encoded region (Fig. 7B).
The NH2-propeptide of type IIA procollagen is present in the extracellular matrix of developing tissues including cartilage (2, 3). Electron microscopy has identified IIA procollagen fibrils in both developing cartilage and in adult vitreous humor (3, 6). There is increasing evidence that the NH2-propeptide of type IIA procollagen may have growth factor-regulating activity. Recombinant IIA propeptides have been shown to bind TGF-β and type IIB procollagen may have growth factor-regulating activity. As these cleavage sites were defined using recombinant protein as substrate, we further demonstrated the ability of one MMP, MMP-7, to cleave type IIB NH2-propeptide and mature type II collagen as well. When cleavage occurs in the N-telopeptide region (site D), the NH2-propeptide is removed as an intact triple-helical trimer. Indeed, we have shown by biophysical methods that MMP-9 liberates trimeric triple-helical NH2-propeptide. The cleavage at this site suggests that, even when these MMPs work on the mature collagen, the lysine cross-link would be released and the collagen fibril would be more susceptible to disruption, in analogy to the cleavage of the telopeptide by MMP-3 discovered by Wu and Eyre (25). In fact, degradation by MMP-3 was shown to change the composition and physical properties of the cartilage (26), and a similar phenomenon could be expected from digestion with the other MMPs described here. Cleavage at the N-proteinase cleavage site (site C) would produce a similar trimeric peptide without disturbing the minor triple helix or cross-link site. Therefore, cleavage at sites C or D would produce a triple-helical trimeric NH2-propeptide that could retain the ability to bind to BMPs. By contrast, cleavage at site A would release monomeric cysteine-rich domains that, in analogy to chordin, would liberate BMPs to bind to their receptors (14). Similarly, cleavage at site B is predicted to release the NH2-propeptide in a monomeric form that could have reduced growth factor binding efficiency. These functional studies are in process.

Our studies strongly suggest that the NH2-propeptide can be removed from the fibrillar collagen domain by enzymes other than the type I collagen N-proteinase. The possibility of proteinase redundancy has been suggested from at least two previous studies. First, cartilage structure was not affected by a genetic inactivating mutation of N-proteinase (dermatosparaxis or Ehlers-Danlos syndrome type VIIC (27). Second, fibril-
logenesis in cartilage was apparently unaffected following knock-out of ADAMTS-2 (type I collagen N-proteinase) (28). In addition, other proteinases have been shown to cleave the propeptide or telopeptide. For example, Wu and colleagues (21) showed that MMP-3 (stromelysin) could cleave intact type II collagen fibrils from mature extracellular matrix. The localization of type II procollagen processing has not been determined; however, it is thought to occur during the process of secretion from the cell as described for type I procollagen (29). Interestingly, MMP-14, shown here to act as both an N-proteinase and a telopeptidase, is a membrane-bound enzyme that could be well positioned for processing procollagen.

In tissues where type II collagen is a major constituent of the extracellular matrix, it is likely that processing of this molecule by MMPs is an important mechanism during development. In particular, the IIA splice variant of type II procollagen is the form expressed in developing cartilage by pre-chondrocytes (30, 31); thus, processing of the IIA NH2-propeptide may be important in regulating growth factor activity. Among the MMPs shown to cleave the IIA NH2-propeptide in this study, MMPs 3, 9, and 14 were shown to be expressed during the early stages of cartilage development (32–34). Cleavage within the N-telopeptide of type IIA procollagen by MMPs could also occur in the hypertrophic site of the developing skeleton. Recent evidence suggests that cleavage of type IIA procollagen NH2-propeptide could play a role in remodeling of the growth plate. Type IIA procollagen has been localized in the hypertrophic region of the growth plate (3, 35), and MMPs 9 and 13 appear to play major roles in the cartilage to bone transition at the growth plate (33, 36, 37). MMP-14 has been shown to be expressed in the hypertrophic chondrocyte (34), by which cartilage is gradually replaced by bone and IIA NH2-propeptide is removed (3). Therefore, the cleavage by MMPs of the NH2-propeptide potentially bearing MMPs could be important in angiogenesis or osteogenesis at the cartilage-bone interphase. The expression of MMPs is, of course, not enough to say the enzymes are actually active in the developing skeleton. MMPs are synthesized as inactive pro-forms that require subsequent activation in the matrix. In addition, another level of MMP regulation exists in the form of tissue inhibitors of metalloproteinases. Because the in situ activity of MMPs during skeletal development is not well defined, further studies are necessary to verify that the NH2-propeptide is indeed cleaved by MMPs in the developing embryo. Demonstration of active MMP cleavage with tissue extracts or detection of the predicted cleavage sites on tissue sections would provide sound evidence to compliment the findings of the present study.

In osteoarthritic cartilage, but not in normal adult articular cartilage, type IIA procollagen is synthesized and deposited around the cells (35, 38). MMPs 3, 9, 13, and 14 are also known to be elevated with most of the matrix degrading activity attributed to MMP-13 (39). MMP-7 also plays a role in OA (40) and could contribute to the liberation of the NH2-propeptide. In studies designed to test whether free NH2-propeptide is present in the serum, the type IIA-specific antibody was used in an enzyme-linked immunosorbent assay to detect NH2-propeptide (10).

The NH2-propeptides of fibrillar procollagens types I, II, and III are thought to be processed similarly; however, type II procollagen may possess intrinsic qualities that make the processing of the NH2-propeptide different from processing of type I procollagen. First, type II procollagen is a homotrimer of three identical α chains, whereas type I procollagen contains the cysteine-rich domain only in the α(I) chain, not in the α(II) chain; therefore, the trimeric NH2-propeptide may be different in structure. Second, type II procollagen contains a triple-helical domain (minor helix) in the NH2-propeptide that is almost twice as long as the type I minor helix (Figs. 1B and 5), allowing for further extension from or through the fibril. Third, the type II propeptide minor helix contains a 5-amino acid interruption that provides cleavage sites for MMPs 7 and 13. Finally, the amino acid sequence of the telopeptide of type I procollagen is different from type II procollagen and does not contain the enzyme cleavage sites for MMPs reported here. For N-proteinase activity, type I procollagen apparently requires a hairpin loop at the telopeptide (41); however, the three-dimensional structure of type II procollagen NH2-propeptide cleavage sites has not been reported.

It seems increasingly clear that functional changes occur within microenvironments of the extracellular matrix that can dramatically affect both matrix and cell function. Cleavage of the type II collagen fibril by MMPs could allow swelling of the tissue and permit new molecules to be incorporated into the matrix. Removal of the NH2-propeptide could have profound effects both functionally and structurally. The growth factor could be redistributed either in an inactive or active state, new molecules could be attracted to the collagen, or lateral association of adjacent collagen fibrils could result in significant changes in matrix architecture.

REFERENCES

Enzymic Processing of the Fibrillar Collagen N-propeptide