Type IIA Procollagen Containing the Cysteine-rich Amino Propeptide Is Deposited in the Extracellular Matrix of Prechondrogenic Tissue and Binds to TGF-β1 and BMP-2

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Abstract. Type II procollagen is expressed as two splice forms. One form, type IIB, is synthesized by chondrocytes and is the major extracellular matrix component of cartilage. The other form, type IIA, contains an additional 69 amino acid cysteine-rich domain in the NH₂-propeptide and is synthesized by chondrogenic mesenchyme and perichondrium. We have hypothesized that the additional protein domain of type IIA procollagen plays a role in chondrogenesis. The present study was designed to determine the localization of the type IIA NH₂-propeptide and its function during chondrogenesis. Immunofluorescence histochemistry using antibodies to three domains of the type IIA procollagen molecule was used to localize the NH₂-propeptide, fibrillar domain, and COOH-propeptides of the type IIA procollagen molecule during chondrogenesis in a developing human long bone (stage XXI). Before chondrogenesis, type IIA procollagen was synthesized by chondroprogenitor cells and deposited in the extracellular matrix. Immunoelectron microscopy revealed type IIA procollagen fibrils labeled with antibodies to NH₂-propeptide at ~70 nm interval suggesting that the NH₂-propeptide remains attached to the collagen molecule in the extracellular matrix. As differentiation proceeds, the cells switch synthesis from type IIA to IIB procollagen, and the newly synthesized type IIB collagen displaces the type IIA procollagen into the interterritorial matrix. To initiate studies on the function of type IIA procollagen, binding was tested between recombinant NH₂-propeptide and various growth factors known to be involved in chondrogenesis. A solid phase binding assay showed no reaction with bFGF or IGF-1, however, binding was observed with TGF-β1 and BMP-2, both known to induce endochondral bone formation. BMP-2, but not IGF-1, coimmunoprecipitated with type IIA NH₂-propeptide. Recombinant type IIA NH₂-propeptide and type IIA procollagen from media coimmunoprecipitated with BMP-2 while recombinant type IIB NH₂-propeptide and all other forms of type II procollagens and mature collagen did not react with BMP-2. Taken together, these results suggest that the NH₂-propeptide of type IIA procollagen could function in the extracellular matrix distribution of bone morphogenetic proteins in chondrogenic tissue.

Key words: type IIA procollagen • bone morphogenetic proteins • chondrogenesis • collagen NH₂-propeptide • skeletal patterning

Long bones and many other components of the skeleton are formed through endochondral ossification, a process wherein bone is laid down on cartilaginous anlagen. The ultimate pattern of these bones is determined by the location and extent of cartilage formation, i.e., during chondrogenesis. In 1986, Thorogood and colleagues first suggested that type II collagen, the characteristic structural collagen of cartilage, plays a role in induction of chondrogenesis (Thorogood et al., 1986; Wood et al., 1991). Epithelial cell-derived type II collagen or associated components of the extracellular matrix (ECM) were proposed to provide a template that mediates the differentiation and patterning of the cartilaginous neurocranium by chondrogenic mesenchyme. Evidence for this hypothesis came from a number of sources including the presence of immunodetectable type II collagen in neuroepithelial and chondrogenic tissues at sites of future chondrogenesis in chicken (Thorogood et al., 1986), mouse (Wood et al., 1991).
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lagen NH2 contain multiple copies of a domain homologous to collagen (Cheah et al., 1991; Wu et al., 1986; Fouser et al., 1991); however, a definitive function has been proven. The present study was designed to explore the function of type II NH2-propeptide in chondrogenesis. The hypothesis tested was that type II NH2-propeptide is present in the ECM and can function to bind growth factors or cytokines. Of particular interest was whether type II NH2-propeptide could bind to members of the TGF-β superfamily in order to regulate the availability of the morphogen in prechondrogenic mesenchyme in a manner similar to the function of sog and chordin in dorsal-ventral patterning. If so, a direct mechanistic connection would be established between the patterning of the body axis and the patterning of the skeleton. We show that during chondrogenesis in the limb, type II is synthesized as a procollagen retaining the cysteine-rich amino propeptide, and it is incorporated into fibrils and deposited into the ECM of precartilaginous mesenchyme. Furthermore, the NH2-propeptide binds to members of the TGF-β superfamily, namely TGF-β1 and BMP-2. We propose that this interaction could potentially localize the factors capable of inducing chondrogenesis. These findings suggest a novel function for the collagen NH2-propeptide and begin to establish a mechanistic paradigm for the regulation of pattern formation in basic body plan and the skeleton.

Materials and Methods

Tissues

Tissues used in this study were stage XXI human fetal limbs, 50-d gestation, provided by the Central Laboratory for Human Embryology (University of Washington, Seattle, WA). Tissues were frozen in OCT compound (Miles Laboratories Inc.) and sectioned with cryostat. The sections (8–10 μm) were stored at −70°C until used.

In Situ Hybridization

Probes specific for type II and IIIB procollagen were used. A 207-bp cDNA, H-IIA, encoding exon 2 of human collagen type II (II) was used to detect type II procollagen mRNA. Primers (5' primer, 5'-CGT-GAATTCAGAGGACTCGAGCTTGGTGTG-3'; 3' primer, 5'-GATT-GGATCCCGCGAAGGGTCGACTTGGCAGAT-3') that flank the exon 2 splice site were used to amplify a 207-bp fragment with EcoRI and BamHI restriction sites from 54-d human fetal embryonic tissue total RNA by using reverse transcription-polymerase chain reaction (RT-PCR), and cloned into pGEM-3zf (+) expression vector (Promega Corp.). This construct was used to generate antisense and sense riboprobes by in vitro transcription for in situ hybridization. Antisense 35S-labeled RNA probe was transcribed by SP6 RNA polymerase on EcoRI linearized DNA template. Sense RNA probe was transcribed by T7 RNA polymerase on EcoRI linearized DNA template linearized with BamHI. For detecting human type IIB procollagen mRNA, an oligonucleotide probe was used containing 12 nucleotides of exons 1 and 12 nucleotides of exon 3, 5'-CTCCGTGTTGC-GGAATTCAGAGGACTCGAGCTTGGTGTG-3' (Ryan and Sandell, 1990). The probe was labeled with 5'-(α-thiol-35S)-ATP (New England Nuclear) using terminal deoxynucleotidyl transferase. In situ hybridization was performed as described previously (Sandell et al., 1991; Wicic, 1993).
Antibodies

Three antibodies were used for immunohistochemistry of type II procollagen, and another two were used to detect BMP-2 and type I collagen by ELISA and Western blots. Rabbit antiserum against recombinant human type IIA-GST (IIA) only recognizes the exon 2 domain of type II procollagen (Oganesian et al., 1997). Rabbit antiserum IIC reacts with bovine COOH-terminal propeptide of type II collagen (provided by Dr. A. Robin Poole) and rat antiserum against bovine type II collagen, IIF (provided by Dr. M. Cremer), recognizes the triple-helical domain of type II collagen. Preimmune sera from the rabbit producing anti-type II procollagen antibodies and non-immune rat serum (Jackson Immunoresearch Laboratories, Inc.) were used as controls. A single-human IgG, pAb mAb (GIBCO BRL) was used to demarcate the periphery of chondrocytes.

TGF-β1 antibodies were obtained from Santa Cruz Biotechnology. They are specific for active TGF-β1. 1:100 dilutions were mounted in fluorescent mounting medium (Vector Laboratories).

Immunofluorescence Staining

Frozen sections (8–10 μm) mounted on polylysine coated slides (Fisher Scientific Co.) were fixed in 4% paraformaldehyde for 10 min at room temperature, and incubated with hyaluronidase (1 mg/ml) for 30 min at 37°C. Sections were blocked in PBS containing 10% (vol/vol) normal donkey serum (blocking buffer, Jackson ImmunoResearch Laboratories, Inc.) for 1 h at 37°C. 1:1 primary antibodies were diluted in PBS containing normal donkey serum (1% vol/vol). A 1:5000 dilution of secondary antibody (Jackson Immunoresearch Laboratories, Inc.) was applied and detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.). After 24 h of culture, the medium was adjusted to 5 mM EDTA and 1 mM N-ethylmaleimide. Proteins were precipitated by the addition of 300 μg/ml of ammonium sulfate which was stirred overnight at 4°C.

Cell Cultures

RJC 31 C5.18 cells were maintained in α-MEM supplemented with 10% heat-inactivated FCS (Gibco/BRL, etc., 1989). The cells were labeled after the last medium change for 24 h in serum-free α-MEM (5 ml/dish) supplemented with 50 μg/ml ascorbate and 50 μg/ml β-aminopropionitrile fumarate and containing 25 μCi/ml of [3H]proline (>20 Ci/mmol, Amersham Corp.) and 50 μCi/ml of [35S]sulfate (1071 Ci/mmol, Amersham Corp.). After 24 h of culture, the medium was adjusted to 5 mM EDTA and 1 mM N-ethylmaleimide. Proteins were precipitated by the addition of 300 μg/ml of ammonium sulfate which was stirred overnight at 4°C.

Immunolectron Microscopy

Images were collected on a Biorad MRC 600 scanning laser confocal microscope mounted on a Nikon Optiphot. Data were collected using either a Nikon 20×/0.50 or a 40×/0.70 NA objective. The Biorad A 1A-2 cubes were used with an Ar laser producing excitation at 514 nm and collecting emission at 520–560 nm (green) and >600 nm (red). Optical sections were ~2 μm with the 20× objective and 1 μm with the 40× objective. Full frame (768 × 512) 8-bit images were collected for analysis and overlaid in 24-bit RGB using Adobe Photoshop.

High resolution images were collected on a DeltaVision SA 3.1 wide-field deconvolution optical sectioning device (Applied Precision, Inc.) mounted on an Olympus IMT-2 microscope. Data were collected using either a Nikon 60×/1.4 or 100×/1.4 NA objective using oil with an i.r. = 1.515. Hoechst dye 33258 (1 μg/ml, Calbiochem-Novabiochem Corp.) was used for fluorescent nuclear stain for 10 min at room temperature. A fter washing, sections were mounted in fluorescent mounting medium (Vector Laboratories, Inc.) for 30 min at room temperature. Hoechst dye 33258 (1 μg/ml, Calbiochem-Novabiochem Corp.) was used for fluorescent nuclear stain for 10 min at room temperature. A fter washing, sections were mounted in fluorescent mounting medium (Vector Laboratories, Inc.) and viewed on a Nikon O ptophot using D M 445 (for Hoechst dye), D M 510 (for FITC), and D M 580 (for Cy3/3 filter cubes. Normal rabbit and rabbit serum were used as control instead of primary antibodies.

Microscopy

Images were collected on a Biorad MRC 600 scanning laser confocal microscope mounted on a Nikon O ptophot. Data were collected using either a Nikon 20×/0.50 or a 40×/0.70 NA objective. The Biorad A 1A-2 cubes were used with an Ar laser producing excitation at 514 nm and collecting emission at 520–560 nm (green) and >600 nm (red). Optical sections were ~2 μm with the 20× objective and 1 μm with the 40× objective. Full frame (768 × 512) 8-bit images were collected for analysis and overlaid in 24-bit RGB using Adobe Photoshop.

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Expression of Recombinant Human Type IIIB Collagen NH2-propeptide

RT-PCR was carried out to amplify a 315-bp fragment encoding the entire common domain of the type II collagen NH2-propeptide from exon 3 (beginning of minor helix) through exon 8 (beginning of the major helix) from 54-d human fetal embryonic tissue total RNA. The forward 35-mer primer was 5'-AATGGATCCCAACCAGGACCAAAGGGACAGA-3' (exon 1) and 5'-GATGGA-GAACCTGGTGACCTGGTG-3' (exon 7), were used to amplify type IIA and IIB cDNA fragments which are 457 and 253 bp, respectively. PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide.

To identify type II procollagens, the proteins collected from culture medium were separated on 5% SDS-polyacrylamide gel and then analyzed by Western blotting. Three antibodies, rabbit anti-IIA + GST (IIA) at 1:1,000 dilution, rat anti-IIA at 1:500, and rabbit anti-IIC at 1:1,000, were used. An n-tetra- and –rat IgG conjugated with HRP (Jackson Immunoresearch Laboratories, Inc.) were applied and detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.). Pepsin solubilized chick type II collagen (Sigma Chemical Co.) was used to indicate the migration of the type II collagen α chain.

Cartilage, samples were first exposed to chondroitinase ABC (Sigma Chemical Co.), 290 U/ml PBS for 2 h at 37°C, followed by rinsing in PBS. After a substantial wash in PBS, the samples were immersed in goat anti-rabbit 5-nm secondary gold conjugate (Amersham Corp.) diluted 1:3 in BSA, pH 7.8, overnight at 4°C. The samples were washed, fixed in aldehydes containing in 0.1% (wt/vol) tannic acid for 60 min followed by 1% OsO4 for 120 min, then dehydrated and embedded in Spurr’s epoxy.

Further details about the localization of type IIA-GST (IIA) propeptide within the fibrils, cartilage containing perichondrium from the same fetus was sheared in 0.2 M ammonium bicarbonate, pH 7.6, using an Omni International 2000 homogenizer. The homogenate was washed three times with resuspension in PBS and centrifugation at 600 g for 5 min. The resulting homogenate was either directly deposited onto carbon coated grids and stained with 3% phosphotungstic acid, pH 7.0, labeled only with primary antibody (1:5 in PBS) before staining, or labeled with primary antibody followed by secondary antibody 5-nm gold conjugate (1:3 in BSA) before staining.

Immunoprecipitation

60 nM recombinant human type IIA procollagen NH2-propeptide (rhIIA-GST, exon 2-GST fusion protein; Oganesian et al., 1997), 60 nM human

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IGF-1 (R&D Systems), or 15 nM human BMP-2 (Genetics Institute) was incubated for 1 h at room temperature in 1 ml of PBS containing 1 mM CaCl$_2$, 3 mM MgCl$_2$, and 1 mg/ml BSA. 10 µl rabbit antisera against NH$_2$-propeptide or preimmune serum was added to the samples and incubated for 2 h at 4°C. 20 µl of protein A-Sepharose beads (Pharmacia Biotech) were added and incubated for 3 h. Beads were pelleted for 1 min and precipitated immune complexes were washed five times with 1 ml PBS, pH 7.2, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and once with 1 ml of 10 mM Tris-HCl, pH 6.8. The samples were resuspended in 40 µl Laemmli sample buffer (without DTT), boiled for 5 min, electrophoresed through SDS polyacrylamide gels under nonreducing conditions, and electroblotted onto PVDF membranes. The membranes were blocked with 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20 containing 3% BSA, and incubated in the same buffer for 1 h at room temperature with primary antibody, anti-human BMP monoclonal or anti-human IGF-1 monoclonal (Austral Biologicals), both at a dilution of 1:500. A nti–mouse secondary antibodies were used and detected by Western blue stabilized substrate for alkaline phosphatase (Promega Corp.).

For comparison of binding to IIA and IIB procollagens, recombinant proteins for type IIA NH$_2$-propeptide (rhIIA-GST) or IIB NH$_2$-propeptide (rHIN-GST, exons 3-8 of the NH$_2$-propeptide) were mixed with BMP-2 as above and immunoprecipitated with BMP specific antisera. Immuno-precipitates were separated by electrophoresis on a 15% SDS polyacrylamide gel, transferred to PVDF membranes, and reacted with antisera to type IIA-GST.

To test whether BMP-2 binds to natural type IIA procollagen, the 3H- and 35S-labeled proteins collected from C5.18 cell medium were immunoprecipitated with BMP-2 antibody. In brief, 100 µl of labeled proteins diluted in NET-buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, pH 8.0, and 0.25% gelatin) to 1 ml was mixed with 10 µl of mouse-agarose (Sigma Chemical Co.) for 1 h at 0°C. Mouse serum-agarose was discharged after centrifugation. 200 ng of BMP-2 was added to the supernatant and incubated for 1 h at 4°C, then 5 µl of BMP-2 antibody was applied and incubated an additional 1 h at 4°C. After incubation, 20 µl of protein A–Sepharose beads (Pharmacia Biotech, Inc.) was added and incubated for 1 h at 4°C. Beads were pelleted for 1 min and the precipitated immunocomplexes were washed three times with 1 ml NET-buffer. The samples were resuspended in 30 µl Laemmli sample buffer and boiled 5 min. Normal mouse serum was used as negative control, instead of BMP-2 antibody. The type IIA procollagen and type IIB collagens were immunoprecipitated by rabbit antisera to type IIA-GST and rat antisera against the fibrillar domain of type II collagen. Labeled proteins were visualized by autoradiography after separation on 5% SDS polyacrylamide gel, transferred to PVDF membranes, and reacted with antiserum against the fibrillar domain of type II collagen. RNA probes were designed to detect the two splice forms of type II procollagen mRNA. A riboprobe (black bar) is specific for type IIA mR NA (+exon 2) and the oligonucleotide probe (gray bar) spans the splice junction and is therefore specific for type IIB procollagen mRNA. (B) Three antibodies were used to localize the type II procollagen domains. Triangles indicate potential epitopes for a rabbit antisera against human recombinant exon 2, squares indicate epitopes for a rat antisera against bovine type II collagen fibrillar domains, and circles indicate rabbit antisera against bovine type II collagen COOH-propeptide. The specific number of epitopes for each antisera is unknown.

**Results**

**Type IIA NH$_2$-propeptide Is Present in Precondensing Mesenchyme**

To determine whether type IIA procollagen is involved in early stages of chondrogenesis, we investigated the specific localization of the NH$_2$-propeptide before and during chondrogenesis. In the developing limb, distal skeletal structures differentiate later than proximal structures (Ham, 1974). Therefore, 50-d human embryonic limb tissue was used because many stages of chondrogenesis can be observed. Antibodies specific for different domains of the collagen molecule were used to localize the IIA NH$_2$-propeptide, COOH-propeptide, and triple-helical (fibrilar) domains of type II procollagen. RNA probes were used to confirm the distribution of mRNA. The approximate locations of epitopes and mRNA probes are shown in Fig. 1.

Double immunofluorescence was performed on tissue sections using the triple-helical antibody together with either the NH$_2$- or COOH-propeptide–specific primary antibodies and fluorescent secondary antibodies. Fluorescence was visualized by confocal laser-scanning microscopy (Fig. 2). In the condensing mesenchyme of the emerging digital rays, signal for type IIA NH$_2$-propeptide can be observed.

Figure 1. Diagram of alternative splicing of type IIA procollagen gene. (A) Probes were designed to detect the two splice forms of type II procollagen mRNA. A riboprobe (black bar) is specific for type IIA mRNA (+exon 2) and the oligonucleotide probe (gray bar) spans the splice junction and is therefore specific for type IIB procollagen mRNA. (B) Three antibodies were used to localize the type II procollagen domains. Triangles indicate potential epitopes for a rabbit antisera against human recombinant exon 2, squares indicate epitopes for a rat antisera against bovine type II collagen fibrillar domains, and circles indicate rabbit antisera against bovine type II collagen COOH-propeptide. The specific number of epitopes for each antisera is unknown.
colocalized with the triple-helical domain (Fig. 2, A–C). At this time, the cells are closely packed condensations and there is no evidence of chondrocyte-characteristic morphology. In serial sections, mRNA levels are below the level of detection with routine in situ hybridization. However, the more sensitive immunolocalization identifies these cells as the site of future cartilage differentiation. More proximal in the developing radius, different stages of chondrogenesis are present. D–F in Fig. 2 show the distribution of type IIA and IIB procollagen mRNA splice forms. Type IIA collagen mRNA is synthesized by chondroprogenitor (CP) cells and type IIB collagen by chondroblasts and chondrocytes (C). In chondroprogenitor tissue, where only type IIA procollagen mRNA is detected, both NH2-propeptide (red, Fig. 2 G) and triple-helical domains (green, Fig. 2 H) are colocalized (reddish/yellow, Fig. 2 I). There is a gradient of distribution of type IIA NH2-propeptide with the greatest immunoreactivity in the chondroprogenitor zone. The gradient distribution of fibrillar domain in H exceeds the range of sensitivity of the detector. Consequently, the green fluorescence in the CP region is underrepresented to reduce blurring due to the high signal in the C region. In the chondroblasts and chondrocytes, where type IIB mRNA is detected, the NH2-propeptide can still be visualized in the ECM (in Fig. 2, G and I). In contrast to the NH2-propeptide, double immunofluorescence using antibodies to the COOH-propeptide and triple-helical domains reveals a different pattern of fluorescence (Fig. 2, J and K). The COOH-propeptide is not colocalized with the triple-helical domains in the ECM, but appears to be localized inside the cells (red dots in Fig. 2 J and yellow dots in Fig. 2 L).

**Type IIA NH2-propeptide Is Deposited in the ECM**

To define more precisely the localization the type II procollagen domains during chondrogenesis, tissue sections were visualized using Delta Vision™ microscopy. The Delta Vision™ system utilizes broad field optics coupled with computerized deconvolution of the optical image using Fourier transformation. A Z-stack of optical sections through 3.2 μm can be viewed with a resolution of ~90 nm. Selected fields representing stages of chondrogenesis shown in Fig. 2 are presented in Fig. 3. In addition to the immunolabeling of collagen domains shown above in confocal micrographs, the fluorescent dye Hoechst 35258 can be used to identify nuclei. Immunoreactivity of the NH2-propeptide (red) and fibrillar domains (green) merged images are shown. Independent visualization of single fluorescence confirmed localization of both Cy3 (red) and
Figure 3. Double immunohistochemistry of growth cartilage in a 50-d gestation human fetal limb. A–D were reacted with antisera to type II collagen fibrillar domain (green) and type IIA procollagen NH₂-propeptide (red). In all panels, yellow or reddish-yellow color indicates colocalization. E and H were reacted with antibody to type II collagen fibrillar domain (green) and type II collagen COOH-propeptide (red). G was a negative control, and H showed the antibody to integrin β1 (red) and type II collagen fibrillar domain (green). Labels indicate nucleus (N), extracellular matrix (M), and secretory granules (SG). Arrow in B and C indicate ECM. Bars in A–D, 9.0 μm and in E–H, 5.4 μm.
confirm the extracellular localization of the NH2-matrix that contains primarily type IIB collagen. To further where streaks of type IIA procollagen remain in a immediately around the cell. Fig. 3 D shows the hypertrophic try granules surrounding the nucleus and deposited imm as synthesized type IIB procollagen can be seen in the secre try-to-mRNA demonstrated that these chondrocytes transcribe only type IIB procollagen mRNA and no longer synthesize the type IIA NH2-propeptide. The newly synthesized type IIB procollagen can be seen in the secretory granules surrounding the nucleus and deposited immediately around the cell. Fig. 3 D shows the hypertrophic zone where streaks of type IIA procollagen remain in a matrix that contains primarily type IIB collagen. To further confirm the extracellular localization of the NH2-propeptide, serial sections were stained with antibodies to type II procollagen COOH-propeptide, type II triple-helical domain, and integrin β1 (Fig. 3, E–H). In Fig. 3 (E and F), the double immunohistochemistry with anti–COOH-propeptide and anti-helical domain antibodies is shown. Note that only the triple-helical domain (green) is deposited into the ECM of chondroprogenitor cells while the COOH-propeptide (red) is colocalized with the triple-helical domain in the secretory granules (yellow in Fig. 3, E and F) or alone (red). The intercellular structures staining with the COOH-propeptide antisera (only red in Fig. 3, E and F) is currently under investigation. Most of these structures do not react with the Golgi apparatus or endoplasmic reticulum characteristic antibodies, such as anti-Golgi 58K protein and anti-Hsp47, respectively (data not shown). Preimmune serum used as the primary antiserum is shown as a negative control (Fig. 3 G) and the cell periphery was confirmed by localization of integrin β1 (Fig. 3 H). The yellow signal indicates that integrin β1 is colocalized with type II collagen triple-helical domains (Fig. 3 H).

**Electron Microscopic Immunolocalization of Type IIA Procollagen Fibrils**

To determine the molecular organization of the NH2-propeptide, localization of type IIA procollagen in embryonic chondrogenic tissue was performed using electron microscopy. A antisera to the NH2-propeptide was used to localize the procollagen in tissue (Fig. 4 A). The results demonstrate localization of antibody-bound gold particles on the surface of collagen fibrils present in perichondrial tissue. The fibrils shown here also react with the type II collagen helical domain antibody. To further clarify the position of the NH2-propeptide within the fibrils, individual fibrils were released from tissue matrix by shearing in ammonium bicarbonate buffer using a tissue homogenizer (Fig. 4 B), incubated only with type IIA specific antibody (Fig. 4 C), then further incubated with 5-nm gold secondary antibody conjugate (Fig. 4 D). Before antibody treatment, the fibrils have an irregular surface (Fig. 4 B) and the periodic banding pattern of type II collagen characterized by Eikenberry et al. (1984). After incubation with type II antibody, protrusions from the fibril surface can be seen (arrow in Fig. 4 C). The identity of the protrusions as primary antibody is confirmed by secondary antibody-gold conjugate (black dots in Fig. 4 D). A determination of periodicity following gold conjugate is complicated by the additional length of the complex (primary antibody-secondary antibody-gold particles) and by some secondary antibodies carrying more than one gold particulate. Therefore, the estimate of antigen spacing was made from the primary antisera micrographs. Taken together, these results indicate that the NH2-propeptide is present at the surface of the type II collagen fibril and found at locations corresponding to the periodic repeat of the collagen molecule.
Type IIA Procollagen NH₂-propeptide Binds to TGF-β1 and BMP-2

The presence of type IIA NH₂-propeptide in ECM of chondroprogenitor cells suggests that it has a function before differentiation of the chondrocyte and could play a role in the induction of chondrogenesis. To assay for binding, immunoprecipitation of BMP-2 and IGF-1 with IIA NH₂-propeptide antibody was performed. rhIIA-GST protein isolated from the recombinant GST fusion protein was used. rhIIA (60 nM), human recombinant BMP-2 (15 nM), or IGF-1 (60 nM) was incubated for 1 h at room temperature in 1 ml of PBS binding buffer, immunoprecipitated with anti-IIA NH₂-propeptide antibody, and the amount of BMP-2 or IGF-1 bound to rhIIA protein was detected on Western blots with monoclonal anti-BMP-2 or IGF-1 antibody. As shown in Fig. 5 A (lane 1) BMP-2 can be immunoprecipitated by IIA NH₂-propeptide antiserum. Control reactions show no immunoprecipitation with BMP-2 alone (Fig. 5 A, lane 2) and no immunoprecipitation of the BMP-2-rhIIA protein complex with preimmune serum (Fig. 5 A, lane 3). No immunoreactivity for IGF-1 was detected when a mixture of IGF-1 and exon 2 protein was immunoprecipitated with NH₂-propeptide antiserum (Fig. 5 A, lane 5).

To determine whether BMP-2 binding was specific for the type IIA splice form of type II collagen, binding of BMP-2 to recombinant type IIA (rhIIA, exon 2) was compared with binding to recombinant type IIB NH₂-propeptide (rhIIN, exons 3–8; Fig. 5 B). Immunoprecipitation was performed by mixing 4.0 μg human recombinant type IIA fusion protein (rhIIA-GST), and 1.0 μg BMP-2 or human recombinant type IIB NH₂-propeptide (rhIIN-GST), and BMP-2 and precipitating with antibody to BMP-2. Western blot analysis was performed and recombinant type IIA fusion protein identified with specific antiserum. Type IIA (rhIIA-GST) was immunoprecipitated with antiserum to BMP (Fig. 5 B, lane 1), but recombinant type IIB NH₂-propeptide (rhIIN-GST; Fig. 5 B, lane 2) nor GST (Fig. 5 B, lane 3) could be immunoprecipitated. Fig. 5 B, lanes 4–6, shows that antiserum against rhIIA-GST can react with rhIIA (exon 2), rhIIN-GST (exons 3–8), and GST when they are run on the gel.

BMP-2 Binds Only to the Type IIA Procollagen Isoform

Media from C5.18 cultured chondroblasts was used to demonstrate binding of natural type IIA procollagen to BMP-2. Fig. 6 A shows that cells express mRNA for both type IIA and IIB procollagens. Protein products were separated on a 5% SDS-polyacrylamide gel and transferred to PVDF membrane for Western blot analysis of type II collagens (Fig. 6 B). Lanes 1 and 3 show immunoreactivity with the type IIA NH₂-propeptide antiserum and type II COOH-propeptide antiserum, identifying this band as pNC type IIA procollagen, shown previously for human cells (Oganesian et al., 1997). A antisera to the fibrillar domain of type II collagen indicates the presence of multiple forms of type II collagen in the medium (Fig. 6 B, lane 2). These forms include type IIA pNC procollagen, type IIB pNC procollagen, type II pC procollagen, and mature α chains (Sandell et al., 1991). Pepsin solubilized type II collagen α chain is shown in Fig. 6 B, lane 4. Specific antisera were used to precipitate procollagens from the medium, type IIA procollagen (Fig. 6 C, lane 1), and all type II collagens (Fig. 6 C, lane 2). When recombinant BMP-2 was added to the medium and proteins immunoprecipitated with BMP-2 antibody, type IIA procollagen alone was observed (Fig. 6 C, lane 3).

To estimate the strength of interaction between NH₂-propeptide and BMP-2, the binding of various growth factors to alternatively spliced type IIA procollagen NH₂-propeptide domain (rhIIA) expressed as a GST-fusion protein was tested. The growth factors bFGF, IGF-1, IGF-2, BMP-2, and TGF-β1 were compared for their ability to bind to the GST-rhIIA protein (Fig. 7). BMP-2 bound to the GST-rhIIA protein (lane 1) and was competed for with excess of IGF-1 (lane 2) and IGF-2 (lane 3). TGF-β1 (lane 4) and bFGF (lane 5) did not bind to the GST-rhIIA protein. These results indicate that type IIA NH₂-propeptide binds specifically to BMP-2 and not to other growth factors.
BMP-2, and TGF-β1, all known to be involved in chondrogenesis, were tested in a solid phase binding assay. Fig. 7 A shows the results of binding of rhIIA-GST to immobilized BMP-2, bFGF, and IGF-1. rhIIA-GST was added in increasing concentrations and the amount bound was measured with antiserum to NH2-propeptide. No binding of rhIIA-GST was observed with bFGF and IGF-1 to 10 μg/well (Fig. 7 A). Similar results were observed with TGF-β1 (Fig. 7 B). Similar results were also obtained when substrates and ligands were reversed, i.e., rhIIA-GST was coated on plates and exposed to BMP-2. An antibody to BMP-2 was used to detect binding (data not shown). Scatchard plot analysis of the interaction indicated a $K_D$ of 5.23 nM for TGF-β1 and 5.23 nM for BMP-2.

**Discussion**

The mechanism of induction and differentiation of the skeleton represents a basic developmental question and thus has attracted a great deal of attention. Substantial progress has been made in clarifying the roles of patterning genes such as pax, hox, hedgehogs, FGFs, genes that induce musculoskeletal cell phenotypes such as the Myo D family of transcription factors, and the extracellular signaling factors, BMPs. The findings presented here indicate that type IIA procollagen could potentially play a role in induction and differentiation of the skeleton. Type IIA procollagen is synthesized by chondroprogenitor cells and deposited into the ECM. It retains the NH2-propeptide, but the COOH-propeptide is removed. The NH2-propeptide of type IIA procollagen binds to BMP-2 and TGF-β1, factors present in the tissue and known to induce chondrogenesis in vivo (Wang et al., 1990) and in vitro, respec-
Phenotype of noggin−/− embryos (Piccolo et al., 1996). Recently, noggin has been shown to be involved in chondrogenesis. That is, in noggin-deficient mice, among other central nervous system and somite patterning defects, cartilage condensations initiate normally but develop hyperplasia, and development of joints in the limb fails (Brunet et al., 1998). The involvement of noggin in chondrogenesis is intriguing, and the relationship between noggin and type IIA collagen NH2-propeptide binding to BMP is unknown. However, the expression pattern of noggin is quite different from type IIA procollagen and more closely resembles the type IIB procollagen splice form (primarily expressed in chondroblasts and chondrocytes; Ng et al., 1993; Sandell et al., 1994). Consequently, its role in chondrogenesis is likely to be different from type IIA procollagen. The primary sequence of noggin is not homologous to type IIA NH2-propeptide.

Reddi et al. have investigated the binding of ECM proteins to TGF-β and bone morphogenetic proteins. They have shown that TGF-β, BMP-3 (Paralkar et al., 1991), and BMP-7 (Vukicevic et al., 1994) bind avidly to type IV collagen, and to a lesser extent, types I, V, and IX collagens and heparin. They do not bind to types II, III, IV, or X collagens, laminin, fibronec-tin, or proteoglycans (Paralkar et al., 1990, 1991, 1992; Vukicevic et al., 1994). Consistent with these results, we show the fibrillar domain of type II collagen does not bind to BMP-2. In general, only relative binding affinities were reported. However, the Kd of BMP-7 and type IV collagen was estimated to be 5 × 10−11 M (Vukicevic et al., 1994).

The localization of type IIA procollagen shown here is consistent with a role for propeptide in regulating the distribution of BMPs. This localization could potentially apply in four primary, but distinct processes. The first is the localization of type IIA procollagen at epithelial–mesenchymal boundaries. Wood et al. (1991) immunolocalized type II collagen and we and others (Sandell et al., 1994; Lui et al., 1995) have shown that these cells synthesize predominantly type IIA mRNA. Lui et al. (1995) showed type II collagen mRNA is initially synthesized by neuroepithelial cells, then by both epithelial and mesenchymal cells, then only mesenchymal cells. The mesenchymal cells proceed to chondrogenesis because they express the receptors necessary to respond to the inducing agent. Secondly, type IIA procollagen is localized in prechondrogenic condensations before differentiation into chondrocytes, as shown above. Thirdly, type IIA procollagen is transiently expressed in other areas where BMPs are involved in induction of differentiation and could be involved as nonchondrogenic processes. For example, type IIA procollagen mRNA has been found in early kidney development, skin before terminal differentiation of keratinocytes, developing aorta, lung buds, salivary gland, adrenal cortex, notochord, somites, and apical ectodermal ridge in mice (Ng et al., 1993; Sandell et al., 1994) and in humans (Sandell, 1994; Lui et al., 1995). Fourthly, type IIA is present in periosteum and perichondrium, predominant sites of ectopic bone formation.

The mechanism of BMP induction of mesenchymal cells after binding and localization by type IIA procollagen remains to be clarified. It is possible that IIA-bound BMPs could induce chondrogenesis. On the other hand, the NH2-
propeptide–BMP complex could be liberated by an amino propeptidase or stromelysin, both known to be able to cleave the propeptide (Wu et al., 1991) when these enzymes become available in the ECM. Lastly, the NH2-propeptide–BMP complex could be disengaged, releasing BMP to bind to the cellular receptor. Piccolo et al. (1996) have hypothesized that chordin inactivates potential binding of BMP-2 to the cellular receptors, based on inhibition of BMP-2 stimulation of osteogenesis in C3H 10T1/2 cells. While the binding mechanism between the chordin–BMP-4 and NH2-propeptide–BMP-2 complexes may be similar, the functional outcome may be quite distinct. Chordin is synthesized and secreted as a soluble protein, while type IIA procollagen is deposited into the ECM. The NH2-propeptide can remain attached to the triple-helical domain or be liberated by cleavage. Chordin is thought to function by removing BMP-4 from the site of potential inductive activity, in this case inducing ventralization in Xenopus. A similar interaction occurs in the dorsal ventral patterning in Drosophila. That is, the sog (Francesco et al., 1994), a homologue of type IIA NH2-propeptide and chordin (Francesco and Bier, 1995), functions as an antagonist of decapentaplegic, a member of the TGF-β superfamily (Padgett et al., 1993). The similar functional outcome of interactions of chordin–BMP-4 and sog–decapentaplegic establishes a conserved mechanism for dorsal–ventral patterning that is shared by vertebrates and arthropods (Piccolo et al., 1996).

The binding of type IIA NH2-propeptide to BMPs suggests a novel function for this protein domain. We show that type IIA procollagen is synthesized and deposited into the ECM. This fibrillar domain of the collagen could then provide a substrate for mesenchymal cells while the triple-helical domain is used to control the amount of morphogenetic agent the cells are exposed to. Subsequently, type IIA procollagen is synthesized in the perichondrium and periosteum (Oganesian et al., 1997). The NH2-propeptide localizes the protein capable of inducing chondrogenesis. As the cells differentiate into chondrocytes, exon 2 encoding the NH2-propeptide is removed by alternative splicing of the mRNA. Consequently, by controlling the availability of NH2-propeptide, a mechanism is built in to control the amount of morphogenetic agent the cells are exposed to. Subsequently, type IIA procollagen is synthesized in the perichondrium and periosteum (Oganesian et al., 1997) where it can help establish a reservoir of BMP. Pattern induction, whether in early body axis or elements of the skeletal system, is thus guided by the result of a gradient of morphogen bound to a specific protein domain. We propose that the interactions of sog, chordin, and type IIA procollagen NH2-propeptide with members of the TGF-β superfamily represent a biological paradigm whereby the presentation of morphogenetic proteins can be regulated.
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