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), and various other species of interest.

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Abbreviations used in this paper: BMPs, bone morphogenetic proteins; C, chondrocytes; CP, chondroprogenitor cells; ECM, extracellular matrix; RT-PCR, reverse transcription-polymerase chain reaction; sog, short gastrulation gene.
lagen NH$_2$-propeptide has been proven. However, it has been suggested that propeptides play a role in the regulation of fibril diameter (Fleischmajer et al., 1990), and feedback regulation of collagen synthesis (Weisster et al., 1979; Horlein et al., 1981; Wu et al., 1986; Fouser et al., 1991); however, no definitive function has been proven.

Recently, two new proteins have been identified that contain multiple copies of a domain homologous to collagen NH$_2$-propeptides, sog (short gastrulation gene) in Drosophila (Francois and Bier, 1995), and chordin in Xenopus (Sasai et al., 1994). Elegant studies have shown that sog and chordin function to establish a dorsal–ventral pattern by binding to members of the TGF-$\beta$ superfamily (decoraptolegic and BMP-4, respectively) to establish a gradient of available morphogen (Francois et al., 1994; Sasai et al., 1994, 1995; Piccolo et al., 1996). The bone morphogenetic proteins (BMPs) are members of the TGF-$\beta$ superfamily and were originally identified because of their ability to induce cartilage and bone formation (Reddi, 1995; Hogan, 1996).

The present study was designed to explore the function of type IIA NH$_2$-propeptide in chondrogenesis. The hypothesis tested was that type IIA NH$_2$-propeptide is present in the ECM and can function to bind growth factors or cytokines. Of particular interest was whether type IIA NH$_2$-propeptide could bind to members of the TGF-$\beta$ 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 IIA 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 NH$_2$-propeptide binds to members of the TGF-$\beta$ superfamily, namely TGF-$\beta$1 and BMP-2. We propose that this interaction could potentially localize the factors capable of inducing chondrogenesis. These findings suggest a novel function for the collagen NH$_2$-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 X XI 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 IIA and IIB procollagen were used. A 207-bp cDNA, H-IIA, encoding exon 2 of human collagen type II (II) was used to detect type IIA procollagen mRNA. Primers (5'- primer, 5'-CCGT-GAAATTTCAAGAGGCGTGGACCTTGTTG-3'; 3' primer, 5'-GATT-GGATCCGGCGAAGTCAGTGGCAGAT-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. A sense 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 DNA template linearized with BamHI. The RNA transcripts were labeled with a 35S-UTP (New England Nuclear). 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'-CTCCGGTTGCGGACATCTTGC-3' (Ryan and Sandell, 1990). The probe was labeled with 5'-[a-thiol-35S]-UTP (New England Nuclear) using terminal deoxynucleotidyl transferase. In situ hybridization was performed as described previously (Sandell et al., 1993; Wicor, 1993).
Antibodies

Three antibodies were used for immunohistochemistry of type II procollagen, and another two were used to detect BMP-2 and IGF-1 by ELISA and Western blots. Rabbit anti sera against recombinant human type IIA-GST (IIA) only recognizes the exon 2 domain of type II procollagen (Oganessian et al., 1997). Rabbit anti sera against bovine COOH-propeptide of type II collagen (provided by D. A. Robin Poole) and rat anti sera against bovine type II collagen, IIF (provided by D. M. Cremer), recognize the triple-helical domain of type II collagen. Preimmune sera from the rabbit producing anti-type IIA procollagen antibodies and non-immune rat serum (Jackson Immunoresearch Laboratories, Inc.) were used as controls. A ntl-human integrin β1 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. IGF-1 antiserum was from Austral Biologicals. The BMP-2/4 mAb (A biH3b/17) was kindly provided by Dr. Elizabeth Morris (Genetics Institute, Cambridge, MA). This reagent, A biH3b/17, was made by standard mAb procedures using full length recombinant human BMP-2 as the immunogen. It reacts with both BMP-2 and BMP-4. Details of antibody specificity have been described in Yoshikawa et al. (1994) and Bostrom et al. (1995).

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. All primary antibodies were diluted in PBS containing normal donkey serum (1% vol/vol). A ntsese IIIA was used at a dilution of 1:400, IIC was 1:50, and integrin β1 was 1:50. For double immunostaining, primary antibodies (IIA and IIF, IIC and IIF, or integrin β1 and IIF) were mixed well and incubated with sections overnight at 4°C. A fter washing in PBS, sections were incubated sequentially with appropriate secondary antibodies [cyanine 3 conjugated donkey anti-rabbit IgG F(ab’)] with a dilution of 1:200, FITC conjugated donkey anti-rat IgG F(ab’) with a dilution of 1:100, or cyanine 3 conjugated donkey anti-mouse IgG F(ab’) with a dilution of 1:120, Jackson Immunoresearch 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 f ter washing, sections were mounted in fluorescent mounting medium (Vector Laboratories, Inc.) and viewed on a Nikon Microphot-FXA microscope. A f ter a substantial wash in PBS, the samples were immersed in goat anti-rabbit 5-nm secondary gold conjugate (Amersham Corp.) diluted 1:3 in PBS, pH 7.8, overnight at 4°C. The samples were washed, fixed in aldehydes containing 0.1% (wt/vol) tannic acid for 60 min followed by 1% OsO4 for 120 min, then dehydrated and embedded in Spurr’s epoxy.

Cell Cultures

RCJ 3.1 C518 cells were maintained in α-MEM supplemented with 10% heat-inactivated FCS (Gibco), and 1% penicillin-streptomycin. The cells were cultured at 37°C in a humidified 5% CO2 atmosphere.

Microscopy

Images were collected on a Biorad MRC 600 scanning laser confocal microscope using a Nikon Optiphot. Data were collected using either a Nikon 20X/0.50 or a 40X/0.70 NA objective. The Biorad A 1.2A 2 cubes were used with an Ar gon laser producing excitation at 514 nm and collecting emission at 520–560 nm (green) and 617/36 nm. Optical sections were collected at 200 nm with the 40X objective using an Olympus BX-60 microscope. Data were collected using either a Nikon 20X/0.50 or 100X/1.4 NA objective using oil with an i.r. = 1.515. Hoechst dye 33258 (blue) was excited at 360/20 nm and emission collected at 457/25 nm. Fluorescein (green) was excited at 490/20 nm and emission collected at 528/19 nm. Cyamine 3 (red) was excited at 555/14 nm and emission collected at 617/36 nm. Optical sections were collected in 200 nm per step and deconvolved with a measured optical transform function per Sedat and A gar (Hiraoka et al., 1990, 1991). Under these conditions we normally obtained 90 nm lateral and 400 nm axial resolution. Images were collected at 512 × 512 pixels at 12-bits/pixel. Final pixel depth is 16-bit. Images were exported as 24-bit TIFF images.

Immunoelectron Microscopy

The immunolocalization techniques used have been described previously (Reinhart et al., 1996). In brief, for en bloc localization of type IIA in fetal cartilage, samples were first exposed to chondroitinase ABC (Sigma Chemical Co.), 290 U/ml PBS for 2 h at 37°C, followed by rinsing with PBS. After a substantial wash in PBS, the samples were immersed in goat anti-rabbit 5-nm secondary gold conjugate (A mershan Corp.) diluted 1:3 in PBS, pH 7.8, overnight at 4°C. The samples were washed, fixed in aldehydes containing 0.1% (wt/vol) tannic acid for 60 min followed by 1% OsO4 for 120 min, then dehydrated and embedded in Spurr’s epoxy.

Immunoprecipitation

RT-PCR was carried out to amplify a 315-bp fragment encoding the entire common domain of the type II collagen NH2-propeptide from 54-d human fetal embryonic tissue total RNA. The forward 35-mer primer was 5′-AATGGATCCCAACCAGGACCAAAGGGACAGA-3′, and the reverse 35-mer primer was 5′-AATATGCGGCCGCCAT-3′. PCR products were digested with BamHI and NotI, and cloned into a pGEX-4T-2 vector (Pharmacia Biotech, Inc.). cDNA sequencing was used to confirm the correctness of the PCR fragment. The expression and purification of the recombinant human type II collagen NH2-propeptide was analyzed by rabbit anti–IIA + GST antibody or goat anti-GST antibody (Pharmacia Biotech, Inc.) on Western blotting. RT-PCR was carried out to amplify a 315-bp fragment encoding the entire common domain of the type II collagen NH2-propeptide from 54-d human fetal embryonic tissue total RNA. The forward 35-mer primer was 5′-AATGGATCCCAACCAGGACCAAAGGGACAGA-3′, and the reverse 35-mer primer was 5′-AATATGCGGCCGCCAT-3′. PCR products were digested with BamHI and NotI, and cloned into a pGEX-4T-2 vector (Pharmacia Biotech, Inc.), cDNA sequencing was used to confirm the correctness of the PCR fragment. The expression and purification of the recombinant human type II collagen NH2-propeptide was analyzed by rabbit anti–IIA + GST antibody or goat anti-GST antibody (Pharmacia Biotech, Inc.) on Western blotting.

60 nM recombinant human type IIA procollagen NH2-propeptide (rhIIA-GST, exons 2-5) was added to the samples and proteins 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 1:1,000, were used. A ntl-rabbit– and rat IgG conjugated with HRP (Jackson Immunoresearch Laboratories, Inc.) were applied and detected with Supersignal® 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.

Expression of Recombinant Human Type IIB 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′, and the reverse 35-mer primer was 5′-AATATGCGGCCGCCAT-3′. The reverse 29-mer primer was 5′-AATGCGGCCGCAT-3′. PCR products were digested with BamHI and NotI, and cloned into a pGEX-4T-2 vector (Pharmacia Biotech, Inc.), cDNA sequencing was used to confirm the correctness of the PCR fragment. The expression and purification of the recombinant human type II collagen NH2-propeptide was analyzed by rabbit anti–IIA + GST antibody or goat anti-GST antibody (Pharmacia Biotech, Inc.) on Western blotting.

Zhu et al. Type IIA Procollagen and BMP 1071
IGF-1 (R & D Systems), or 15 mM human BMP-2 (Genetics Institute) was incubated for 1 h at room temperature in 1 ml of PBS containing 1 mM CaCl\textsubscript{2}, 3 mM MgCl\textsubscript{2}, and 1 mg/ml BSA. 10 \mu l rabbit antiserum against NH\textsubscript{2}-propeptide or preimmune serum was added to the samples and incubated for 2 h at 4\textdegree C. 20 \mu l 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 \mu l Laeemmli 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 anti-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\textsubscript{2}-propeptide (rhIIA-GST) or II NH\textsubscript{2}-propeptide (rhIIN-GST, exons 3-8 of the NH\textsubscript{2}-propeptide) were mixed with BMP-2 as above and immunoprecipitated with B1 anti B1 specific antiserum. Immunoprecipitates were separated by electrophoresis on a 15% SDS polyacrylamide gel, transferred to PVDF membranes, and reacted with antiserum to type IIA-GST.

To test whether BMP-2 binds to natural type IIA procollagen, the \textsuperscript{3}H- and \textsuperscript{15}N-labeled proteins collected from C5.18 cell medium were immunoprecipitated with BMP-2 antibody. In brief, 100 \mu l 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 \mu l of mouse serum–agarose (Sigma Chemical Co.) for 1 h at 0\textdegree 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\textdegree C, then 5 \mu l of BMP-2 antibody was applied and incubated an additional 1 h at 4\textdegree C. A fter incubation, 20 \mu l of protein A-Sepharose beads (Pharmacia Biotech, Inc.) was added and incubated for 1 h at 4\textdegree 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 \mu l Laeemmli sample buffer and boiled 5 min. Normal mouse serum was used as negative control, in- stead of BMP-2 antibody. The type IIA procollagen and type II collagens were immunoprecipitated by rabbit antiserum to type IIA-GST and rat antiserum against the fibrillar domain of type II collagen. Labeled proteins were visualized by autoradiography after separation on 5% SDS polyacrylamide gel using A amplify (Nycomed A mersham Inc.).

**Solid Phase Binding Assay**

96-well flat bottomed plates (Costar, High Binding, E.I.A./R.I.A. #3590) were coated overnight at 4\textdegree C with 5 or 10 ng/well TGF-β1, BMP-2, bFGF, IGF-1, and GST in 0.1 M Tris-HCl, 50 mM NaCl, pH 7.4 (Tris-NaCl), respectively. Plates were washed three times with PBS, pH 7.2, containing 0.1% (vol/vol) Tween 20 (PBS/Tween). To block nonspecific binding, plates were incubated for 1 h at 20\textdegree C with PBS/Tween containing 3% (wt/vol) BSA and washed four times in PBS/Tween. 20 \mu l of rhIIA-GST fusion protein and GST (Oganesian et al., 1997), from 1 to 5,000 ng/well, in Tris-NaCl were added to the coated wells and incubated at 37\textdegree C for 2 h. Plates were washed five times with PBS/Tween. Plates were incubated for 4 h with PBS/Tween/BSA buffer, then incubated for 2 h at 20\textdegree C with a 1:1,000 dilution of anti-IIA-GST antibodies in PBS/Tween. Plates were washed five times with PBS/Tween and incubated for 2 h at 20\textdegree C with a 1:5,000 dilution of goat anti-rabbit IgG–alkaline phosphatase conjugate in PBS/Tween and washed five times with PBS/Tween. Plates were incubated for 30–60 min with 3 mM p-nitro-phenylphosphate substrate in 0.05 M Na\textsubscript{2}CO\textsubscript{3} and 0.005 mM MgCl\textsubscript{2} buffer, and absorbance was measured at 405 nm using a Hewlett Packard ELISA microplate reader. In addition, the substrates and ligands were reversed. rhIIA-GST fusion protein or IIA protein (only exon 2) alone was plated at 10 ng/well. BMP-2 and mAb against rhBMP-2 were incubated sequentially as above. Then, secondary antibody and color reactive substrate were used to detect the binding. Each data point was in duplicate from three independent experiments.

**Results**

**Type IIA NH\textsubscript{2}-propeptide Is Present in Prechondrogenic Mesenchyme**

To determine whether type IIA procollagen is involved in early stages of chondrogenesis, we investigated the specific localization of the NH\textsubscript{2}-propeptide before and during chondrogenesis. In the developing limb, distal skeletal structures differentiate later than proximal structures (H am, 1974). Therefore, 50-d human embryonic limb tissue was used because many stages of chondrogenesis can be observed. A antibodies specific for different domains of the collagen molecule were used to localize the IIA NH\textsubscript{2}-propeptide, COOH-propeptide, and triple-helical (fibrillar) 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\textsubscript{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\textsubscript{2}-propeptide can be observed.
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 NH$_2$-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 NH$_2$-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 NH$_2$-propeptide can still be visualized in the ECM (C in Fig. 2, G and I). In contrast to the NH$_2$-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 NH$_2$-propeptide Is Deposited in the ECM**

To define more precisely the localization the type I 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 NH$_2$-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.
FITC (green) in regions that appear orange or orange-red. As shown above, chondroprogenitor cells (Fig. 3 A) synthesize type IIA procollagen mRNA while more mature chondrocytes (Fig. 3, B–D) synthesize type IIB procollagen. In the chondroprogenitor tissue, the cells are tightly packed with large nuclei, little cytoplasm, and very little ECM is observed. However, the small amount of staining around the cells can clearly be seen in this merged image to be reddish yellow (Fig. 3 A) indicating colocalization of NH$_2$-propeptide and the triple-helical domains. In chondroblasts (Fig. 3 B), an accumulation of type IIA NH$_2$-propeptide and fibrillar collagen can be observed. Less mature cells are in the upper left half of the photograph while the more mature chondrocytes are in the lower right half of the photograph. In the zone of mature chondrocytes (Fig. 3 C), the cells are even larger and contain distinct secretory granules lying close to the nucleus. More cellular detail in these rounded cells can now be resolved. In the ECM, reddish orange-staining areas of propeptide are localized in the interterritorial matrix where it has been displaced by newly synthesized type IIB procollagen (green). In previous studies and shown above, in situ hybridization to mRNA demonstrated that these chondrocytes transcribe only type IIB procollagen mRNA and no longer synthesize the type IIA NH$_2$-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 NH$_2$-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 antisemur (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 antisemur 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 NH$_2$-propeptide, localization of type IIA procollagen in embryonic chondrogenic tissue was performed and visualized using electron microscopy. A titserum to the NH$_2$-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 NH$_2$-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 IIA 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 antisemur photomicrographs. Taken together, these results indicate that the NH$_2$-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 antisera 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 procollagen proteins. 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 I COOH-propeptide antiserum, identifying this band as pNC type IIA procollagen, shown previously for human cells (Oganesian et al., 1997). An antiserum 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 procollagen 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,
BMP-2, and TGF-β1, all known to be involved in chondrogenesis, were tested in a solid phase binding assay. Fig. 7A 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 antisera to NH2-propeptide. No binding of rhIIA-GST was observed with bFGF and IGF-1 up to 10 μg/well (Fig. 7A). Similar results were observed with TGF-β1 (Fig. 7B). 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 KD of 7.65 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-
tively (Denker et al., 1995). These results show for the first time that type IIA pN-procollagen is deposited into the ECM and suggest a novel function for the collagen NH$_2$-propeptide. Type IIA procollagen is the predominant form of type II collagen in chondroprogenitor tissue and remains in the tissue after cells switch synthesis to type IIB collagen. Over time however, the predominant collagen becomes type IIB collagen, and the type IIA procollagen is removed. We do not know what enzymes are involved in type IIA procollagen turnover or whether the NH$_2$-propeptide alone is cleaved from the collagen fibril, although the NH$_2$-propeptide can be cleaved by stromelysin, which cleaves between the N-protease cleavage site and the beginning of the major triple helix (Wu et al., 1991), an enzyme known to be increased in hypertrophic cartilage (Zhu, Y., and L.J. Sandell, unpublished observations) and the collagen N-protease which cleaves 8 amino acids downstream of the major triple helix of the propeptide (Procop et al., 1998). Piccolo et al. (1997) have shown recently that the chordin–BMP-4 complex is proteolytically processed in chordin by the matrix metalloprotease xolloid, thereby releasing active BMP-4. Cleavage of chordin alone inhibits its ability to bind BMP-4. A similar cleavage mechanism by a related enzyme, tolloid, occurs in the sog-dpp complex (Marques et al., 1997). For type IIA procollagen, an analogous cleavage mechanism may exist, as both N-protease and stromelysin are members of the same class of astacin proteases as tolloid and xolloid.

The data presented here suggest that BMPs may be localized to sites of chondrogenesis by direct interaction with the NH$_2$-propeptide of type IIA procollagen. Support for this hypothesis is derived from a similar interaction of chordin and sog, homologues of the NH$_2$-propeptide, with BMP-4 (Piccolo et al., 1996) and decapentaplegic (Sasai et al., 1995). The interactions regulate presentation of the BMP-4 (Piccolo et al., 1996) and decapentaplegic (Sasai, 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 NH$_2$-propeptide.

Reddi and colleagues have investigated the binding of ECM proteins to TGF-$eta$ and bone morphogenetic proteins. They have shown that TGF-$eta$, 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, V, or X collagens, laminin, fibronectin, 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 $K_a$ of BMP-7 and type IV collagen was estimated to be $5 \times 10^{-11}$ 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 type II A bound BMPs could induce chondrogenesis. On the other hand, the NH$_2$-
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 (Francois et al., 1994), a homologue of type II NH₂-propeptide and chordin (Francois 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 IIAprocollagen is synthesized and deposited into the ECM. This fibrillar domain of the collagen could indicate a functional outcome of interactions of chordin–BMP-4 and sog–decapentaplegic that are conserved mechanisms for dorsal–ventral patterning that is shared by vertebrates and arthropods (Piccolo et al., 1996).

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