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# Interaction Between Amino Propeptides of Type XI Procollagen 1 Chains

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## Interaction between Amino Propeptides of Type XI Procollagen $\alpha 1$ Chains\*

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Type XI collagen is a quantitatively minor yet essential constituent of the cartilage extracellular matrix. The amino propeptide of the  $\alpha 1$  chain remains attached to the rest of the molecule for a longer period of time after synthesis than the other amino propeptides of type XI collagen and has been localized to the surface of thin collagen fibrils. Yeast two-hybrid system was used to demonstrate that a homodimer of  $\alpha 1(\text{XI})$  amino propeptide ( $\alpha 1(\text{XI})\text{Npp}$ ) could form *in vivo*. Interaction was also confirmed using multi-angle laser light scattering, detecting an absolute weight average molar mass ranging from the size of a monomer to the size of a dimer (25,000–50,000 g/mol), respectively. Binding was shown to be saturable by ELISA. An interaction between recombinant  $\alpha 1(\text{XI})\text{Npp}$  and the endogenous  $\alpha 1(\text{XI})\text{Npp}$  was observed, and specificity for  $\alpha 1(\text{XI})\text{Npp}$  but not  $\alpha 2(\text{XI})\text{Npp}$  was demonstrated by co-precipitation. The interaction between the recombinant form of  $\alpha 1(\text{XI})\text{Npp}$  and the endogenous  $\alpha 1(\text{XI})\text{Npp}$  resulted in a stable association during the regeneration of cartilage extracellular matrix by fetal bovine chondrocytes maintained in pellet culture, generating a protein that migrated with an apparent molecular mass of 50–60 kDa on an SDS-polyacrylamide gel.

Type XI collagen is a constituent of the extracellular matrix of cartilage. It belongs to the family of fibrillar collagens, which includes types I, II, III, V, and XI (1). As with all of the fibrillar collagens, type XI contains a 300-nm uninterrupted triple helical domain (2). Type XI collagen forms the heterotypic collagen fibrils characteristic of cartilage along with types II and the nonfibrillar type IX collagen (3). Although minor in quantity, type XI collagen is thought to play a critical role in collagen fibril assembly and extracellular matrix organization in early development as demonstrated by the chondrodystrophic mouse (*cho/cho*) (4). Fibrils of developing cartilage are of uniform 20 nm in diameter and oriented more randomly than fibrils of adult articular cartilage.

Collagen fibril assembly in embryonic cartilage is known to rely upon the ratio of the collagenous constituents, types II and XI (5). Initially, type XI collagen may function to nucleate the formation of new collagen fibrils (6). The role of type XI colla-

gen in limitation of lateral growth of collagen fibrils is well established, although the molecular mechanism is not fully understood. Type XI collagen may also play a role in the maturation of thin fibrils into the larger diameter fibrils of the territorial zone of cartilage matrix, either by regulation of the accretion of more collagen molecules onto the surface of thin fibrils or by mediating the fusion of thin fibrils.

Type XI collagen is initially synthesized as a procollagen, which is subsequently proteolytically processed at both the amino and carboxyl termini (7). The amino-terminal processing site was identified 7 amino acids from the variable region (8, 9). Recently, type XI  $\alpha 1$  pro-collagen was identified as a substrate for the enzyme bone morphogenetic protein 1 (BMP-1)<sup>1</sup> (10). The carboxyl propeptide of the  $\alpha 1$  chain of type XI collagen is homologous to the other fibrillar collagens, whereas the amino propeptide of type XI  $\alpha 1$  pro-collagen is markedly different. In addition to the characteristic minor triple helix, the  $\alpha 1$  chain of XI contains a large non-triple helical domain at the amino terminus.

The  $\alpha 1$  chain of type XI collagen exists as a set of isoforms that arise by alternative splicing of the primary RNA transcript (11). The portion encoding the variable region gives rise to biochemically unique domains. However, common to all isoforms is the amino propeptide, the distal globular 233 amino acids, which is ultimately proteolytically removed during the process of extracellular matrix assembly and fibril formation. Proteolytic removal of the amino propeptide may rely on the action of BMP-1, as it does in the case of  $\alpha 1(\text{V})$  collagen chain (12).

The amino propeptide domain is structurally related to other amino propeptides including that of the  $\alpha 2(\text{XI})$  and  $\alpha 1(\text{V})$  collagen chains. The position of four cysteine residues are conserved, and sequence analysis predicts a conserved secondary structural motif rich in  $\beta$ -strand (13). This prediction has been supported by analysis of circular dichroism spectra, demonstrating a relatively high percentage  $\beta$ -strand and a markedly low percentage of  $\alpha$  helix (14). The dimensions of the globular amino propeptide domain of  $\alpha 1(\text{XI})$  collagen chain has been estimated by rotary shadowing to be at least 8 nm in diameter. This domain has been localized to the surface of collagen fibrils by immunoelectron microscopy (15).

Although the amino propeptides are ultimately removed from the collagen triple helical molecule by proteolytic processing, the rate and extent of removal vary. Although the processing of  $\alpha 1(\text{XI})$  collagen chain is relatively slow, thus achieving 50% completion by 18 h after synthesis in pulse-chase experi-

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<sup>1</sup> The abbreviations used are: BMP, bone morphogenetic protein; NTA, nitrilotriacetic acid;  $\alpha 1(\text{XI})\text{NTD}$ ,  $\alpha 1(\text{XI})$  amino-terminal domain containing the variable region; Npp, amino propeptide; ELISA, enzyme-linked immunosorbent assay.

ments, the processing of the  $\alpha 2(XI)$  collagen chain is much faster, 75% complete by 5 h after synthesis (16). This raises the possibility that the amino propeptides carry out different functions and that the  $\alpha 1(XI)$  amino propeptide may function while still retained on the surface of collagen fibrils for the extended period of time after synthesis. Globular domains poised on the surface of newly synthesized collagen fibrils may play a role in both the mechanism of growth of an individual fibril as well as lateral fusion of neighboring collagen fibrils or interaction between the collagen fibrils and other constituents of the extracellular matrix.

We report the interaction between amino propeptide domains of  $\alpha 1(XI)$  collagen chains. The interaction had a dissociation constant on the order of 130 nM. This interaction was stabilized in cell culture by an association that was not disrupted by reducing agents, EDTA, SDS, or heat. Such an interaction may explain, in part, how collagen XI functions to regulate growth of collagen fibrils in developing tissues.

#### EXPERIMENTAL PROCEDURES

**Synthesis and Purification of Recombinant Rat  $\alpha 1(XI)Npp$  Domain**—A cDNA fragment encoding the amino propeptide of  $\alpha 1$  chain of type XI collagen was inserted into expression vector pET11a (Stratagene). BL21 DE3 transformants were screened for expression of recombinant protein on SDS-polyacrylamide gels and subsequent transfer to polyvinylidene difluoride membrane for detection of recombinant proteins by nickel affinity blot using nickel-NTA conjugated to alkaline phosphatase (Qiagen). Cells from transformant cultures were collected by centrifugation, and proteins were solubilized using bacterial protein extraction reagent (Pierce) with 1 unit of DNase I per 10 ml of cell suspension. This extraction solubilized the majority of cellular proteins, leaving recombinant  $\alpha 1(XI)$  amino propeptide ( $\alpha 1(XI)Npp$ ) insoluble in the inclusion bodies. Protein from inclusion bodies was solubilized and unfolded in 20 mM Tris-HCl, pH 7.5, with freshly prepared 8 M urea, and 14 mM  $\beta$ -mercaptoethanol at a protein concentration of  $\sim 1$  mg/ml. Refolding was initiated by diluting solubilized unfolded protein dropwise and with stirring into 100 volumes of 20 mM Tris-HCl, pH 7.5, containing 100 mM KCl, 20% glycerol, and 2 M urea at 4 °C. Incubation at 4 °C was continued for 16 h. Refolded protein was concentrated by ultrafiltration using a Centrprep filtration unit (Amicon). Insoluble material, if present, was removed by centrifugation. Refolded protein was further purified by chelation chromatography on a Hi-Trap nickel column (Amersham Biosciences) (Fig. 1). Disulfide-bonded cysteine pairs were verified by tryptic digest followed by protein sequencing of those bands present in the absence of 10 mM DTT but absent when the sample was treated with reducing agent as described previously (17). Refolded protein was characterized by circular dichroism and compared with native protein isolated from tissue (17). Npp without a His<sub>6</sub> tag was generated by removing the carboxyl portion of a recombinant  $\alpha 1(XI)$  amino-terminal domain containing the variable region ( $\alpha 1(XI)NTD$ ), which contains a functional BMP-1 proteolytic processing site (10). Recombinant protein was incubated in the presence of purified recombinant human BMP-1 (FibroGen, Inc.) as described previously (10).

**Yeast Two-hybrid System**—A protein-protein interaction was tested *in vivo* using Matchmaker GAL4 (Clontech, Inc.) yeast two-hybrid system. cDNA encoding  $\alpha 1(XI)Npp$  was obtained by reverse transcription-PCR using the following primers: 5'-cca tgg ttg ata tac tga aag ctt tag at-3' and 5'-att agg atc etc atc tat gtg cgg ctc ctg-3'. This fragment was used to make two different gene fusions: one with the DNA binding domain of GAL4 and the other with the transcriptional activation domain of GAL4 in the plasmids pGBKT7 and pGADT7, respectively.

Yeast host strain AH109 was transformed using the lithium acetate method (17). Selection of positive transformants was carried out by plating on SD minimal medium agar plates lacking the nutrient leucine and tryptophan to select for the plasmids derived from pGBKT7 and pGADT7, respectively. Plates lacking both nutrients were used to select for double transformants.

To select for colonies in which the two-hybrid proteins interact, double transformants were plated on SD minimal medium lacking tryptophan, leucine, and histidine. Protein-protein interaction was verified by determining the expression of  $\beta$ -galactosidase activity by colony-lift filter assay and by determining the level of expression in liquid culture using chlorophenol red- $\beta$ -D-galactopyranoside as substrate (Clontech).

**Weight Average Molecular Weight Determination by Light Scattering**—Recombinant  $\alpha 1(XI)Npp$  was applied to a TSK 3000SW size exclusion column at 10 °C in the following buffer: 30 mM Na<sub>2</sub>HPO<sub>4</sub>, 45 mM KH<sub>2</sub>PO<sub>4</sub>, pH 6.6, containing 100 mM KCl. The eluting peak was analyzed using a refractive index detector, a UV monitor, and by multi-angle laser light scatter (DAWN, Wyatt Technology, Santa Barbara, CA) to determine the absolute molecular weight of  $\alpha 1(XI)Npp$  in solution. A vertically polarized laser beam was passed through the flow cell containing the sample. The scattered light was simultaneously detected by 18 separate detectors at different angles. The intensity of the scattered light was digitized and transmitted to a computer for analysis by software provided by the manufacturer. The weight-average molar mass was determined from the Rayleigh ratio of scattered light, molecular concentration in grams/milliliters, and the specific refractive index increment of the dissolved molecules (dn/dc). The concentration was determined from the refractive index at each slice of the chromatogram and also from the absorbance at 280 nm and the 0.1% extinction coefficient, calculated for recombinant rat  $\alpha 1(XI)Npp$  to be 0.548 assuming all cysteine residues exist as half-cystines. The dn/dc value used was 0.185. Recombinant  $\alpha 1(XI)Npp$  was analyzed under three salt conditions: 75, 175, and 500 mM with respect to additive concentration of KCl and buffer components. Molar mass was determined for individual slices of data across the eluting size exclusion chromatographic peak and plotted as a function of elution volume. Molecular weight at the leading and trailing edges of the chromatographic peak was compared with to determine whether recombinant  $\alpha 1(XI)Npp$  multimerized under the buffer conditions of the column.

**Antibodies**—A peptide coding for the human  $\alpha 1(XI)Npp$  was used to make the  $\alpha 1(XI)Npp$  antibody that showed specificity for the endogenous  $\alpha 1(XI)Npp$  but not the recombinant  $\alpha 1(XI)Npp$ . Preparation and characterization of this antibody was reported previously (18). The ability of the  $\alpha 1(XI)Npp$  antibody to discriminate between endogenous and recombinant  $\alpha 1(XI)Npp$  was demonstrated by immunoblot and nickel affinity blot (Fig. 6). Samples of increasing amounts of recombinant  $\alpha 1(XI)Npp$  were loaded onto an SDS gel and subsequently transferred to polyvinylidene difluoride membrane. Nickel-NTA-alkaline phosphatase conjugate was used to detect recombinant protein from a sample that was three orders of magnitude more dilute than the concentration needed for detection by the polyclonal antibody to  $\alpha 1(XI)Npp$ . Generation of the  $\alpha 2(XI)Npp$  antibody was carried out using the sequence RERPQRQPSHRTQ from bovine sequence (18) as the immunizing peptide. Antibody to the variable region of the recombinant  $\alpha 1(XI)$  amino-terminal domain was described previously (19).

**ELISA Assay**—Recombinant  $\alpha 1(XI)Npp$  was adsorbed to a microtiter plate in 96-well format at room temperature. To block unoccupied sites on plastic surface, wells were incubated with buffered skim milk powder solution for 1 h at room temperature. Recombinant  $\alpha 1(XI)NTD$  was allowed to interact with the  $\alpha 1(XI)Npp$  bound to the plate for 2 h at room temperature. Antibody to the unique variable region was used to detect interaction. Absorbance at 405 nm was plotted as a function of the concentration of bound  $\alpha 1(XI)NTD$  with variable region detected. This antibody did not recognize  $\alpha 1(XI)Npp$ . Nonspecific binding of the primary antibody was tested in the absence of antigen. Nonspecific binding of the secondary antibody was tested in the absence of primary antibody. Bovine serum albumin was bound to the 96-well plate as a control to detect nonspecific binding of  $\alpha 1(XI)NTD$ . Data was subjected to Scatchard analysis to determine  $K_D$  and  $B_{max}$ .

**Co-precipitation of Recombinant and Endogenous  $\alpha 1(XI)Npp$  Domains**—Recombinant  $\alpha 1(XI)Npp$ , synthesized with six histidine residues in tandem at the carboxyl terminus of the protein, was bound to nickel-NTA-agarose (Qiagen) to investigate the specificity of the interaction between  $\alpha 1(XI)Npp$  domains. Protein was extracted from bovine chondrocyte pellet cultures using 50 mM Tris-HCl, pH 7.5, 1 M NaCl, and 5 mM EDTA. No denaturants such as guanidine hydrochloride or urea were present during the extraction. Salt concentration was reduced to 150 mM by dialysis prior to incubation with  $\alpha 1(XI)Npp$ -bound nickel-agarose. Material that bound to the recombinant  $\alpha 1(XI)Npp$  was separated from that which did not bind by centrifugation. Samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblot using an antibody that recognized  $\alpha 1(XI)Npp$  and one that recognized a very similar domain,  $\alpha 2(XI)Npp$ .

**Pellet Culture of Bovine Chondrocytes**—Fetal bovine chondrocytes were maintained in pellet culture (20). Where indicated in the text,  $\beta$ -aminopropionitrile fumarate was included in pellet culture medium at a final concentration of 64  $\mu$ g/ml, putrescine was included at a final concentration of 2 mM, AEBSF (4-(2-aminoethyl)-benzene sulfonyl-

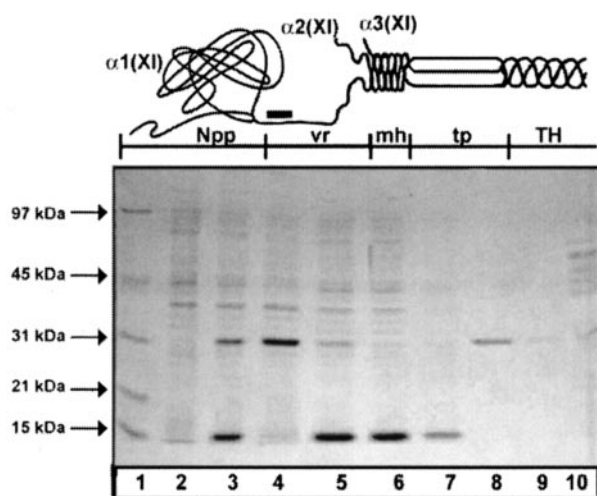


FIG. 1. **Expression of recombinant rat  $\alpha 1(XI)Npp$ .** A, schematic representation of the amino-terminal domain of a type XI collagen molecule. The Npp of the  $\alpha 1(XI)$  chain is shown as a terminal globular domain attached to the minor helix (*mh*) via an extended variable region (*vr*). The  $\alpha 2(XI)Npp$  is absent from this schematic to represent the rapid proteolytic processing of this chain. Between the minor helix and the major triple helix (*TH*) lies the amino telopeptide (*tp*). Black bar represents location of antibody recognition site. B, extracts of *E. coli* cells grown in the presence of IPTG were resolved by SDS-PAGE on a 12% polyacrylamide gel stained with Coomassie Blue. Lane 1, molecular weight markers; lane 2, mid-log culture grown in the absence of IPTG; lane 3, mid-log culture grown in the presence of IPTG; lane 4, insoluble protein after extraction of *E. coli* cell pellet; lane 5, soluble protein after protein extraction; lane 6, flow-through from nickel-affinity column; lane 7, column wash; lanes 8 and 9, elution of purified, refolded recombinant protein; lane 10, recombinant protein markers.

fluoride-HCl) was included at a final concentration of 0.1 mM, and recombinant  $\alpha 1(XI)Npp$  was included in tissue culture medium at a concentration of 10  $\mu\text{g/ml}$  for 24 h. Proteins from medium and cell pellets were analyzed by SDS-PAGE in the presence of  $\beta$ -mercaptoethanol and subsequent immunoblot and nickel affinity blot.

## RESULTS

**Recombinant Rat  $\alpha 1(XI)Npp$  Domain**—A 682-bp fragment encoding  $\alpha 1(XI)Npp$  was obtained by reverse transcription-PCR and inserted into the pET11a expression vector. Recombinant  $\alpha 1(XI)Npp$  was expressed as a fusion protein with gene-10 gene product of bacteriophage T7, resulting in the addition of amino acids MAS at the amino terminus of the protein. Protein was expressed by BL21(DE3) *Escherichia coli* cells upon isopropyl-1-thio- $\beta$ -D-galactopyranoside induction. The DNA sequence of the insert was determined by automated fluorescence DNA sequencing. The terminal methionine was removed in the bacterial expression system as verified by LCQ<sup>TM</sup>MS/MS. The sequence of interest proceeded from the first proline after the signal peptide processing site of  $\alpha 1(XI)$  and continued to the glutamine one amino acid before the amino propeptide processing site. Codons for six histidine residues were included in the downstream PCR primer immediately preceding a stop codon. The penultimate amino acid, alanine, was changed conservatively to a valine to allow the antibody to discriminate between endogenous bovine  $\alpha 1(XI)Npp$  and recombinant rat  $\alpha 1(XI)Npp$ . In addition, this change modified the sequence at the proteolytic processing site of  $\alpha 1(XI)$  collagen, reducing the chance that the histidine tag would be removed from the recombinant protein. Recombinant  $\alpha 1(XI)Npp$  with a His<sub>6</sub> tag had a molecular weight of 24,826 g/mol and a theoretical pI of 6.91. Recombinant  $\alpha 1(XI)Npp$  was recovered from inclusion bodies. After unfolding and refolding, recombinant  $\alpha 1(XI)Npp$  was purified by nickel-chelation chromatography (Fig. 1). Puri-

fied bacterial recombinant  $\alpha 1(XI)Npp$  co-migrated with human embryonic kidney 293 recombinant  $\alpha 1(XI)Npp$  by SDS-polyacrylamide gel electrophoresis (data not shown). Unfolded and refolded  $\alpha 1(XI)Npp$  was previously characterized by spectropolarimetry, electron microscopy, and LCQ-MS/MS (17).

**Detection of Interaction Using Yeast Two-hybrid System**—Expression vectors encoding fusion proteins between the DNA binding domain of GAL4 and  $\alpha 1(XI)Npp$  and between the transcriptional activation domain of GAL4 and  $\alpha 1(XI)Npp$  were constructed. Cells cotransfected with both constructs were able to grow in the absence of histidine (Fig. 2), indicating an interaction between the  $\alpha 1(XI)Npp$  domain portion of each of the fusion proteins. Interaction was also demonstrated by detection of  $\beta$ -galactosidase activity (Fig. 2C). The level of  $\beta$ -galactosidase activity in double transformants was  $\sim 10$ -fold higher than background.

**Size Exclusion Chromatography and Multi-angle Laser Light Scatter**—At physiological ionic strength (175 mM), the weight average molar mass varied from the molecular mass of a monomer (25,000 g/mol) at the trailing edge of the size exclusion chromatography peak to 40,000 g/mol, an average molar mass lying between that of a dimer (50,000 g/mol) and a monomer at the leading edge of the peak (Fig. 3). Interaction was favored by low ionic strength (75 mM) and disrupted by high ionic strength (500 mM). Maximum molecular weight observed corresponded to a dimer with no indication of higher multimerization. Multimerization was inferred by an increase in molar mass of purified recombinant  $\alpha 1(XI)Npp$ .

**ELISA**—Binding was shown to be saturable by ELISA (Fig. 4) in which recombinant  $\alpha 1(XI)Npp$  was bound to the plate and a tagged recombinant isoform of  $\alpha 1(XI)$  amino-terminal domain was allowed to interact with the  $\alpha 1(XI)Npp$  bound to the plate. Antibody to the unique region of the alternate isoform was used to detect interaction. This antibody did not recognize  $\alpha 1(XI)Npp$ . The dissociation constant was determined to be on the order of 0.13  $\mu\text{M}$ .

**Specificity of  $\alpha 1(XI)Npp$  Interaction: Co-precipitation of Endogenous and Recombinant  $\alpha 1(XI)Npp$** —Bovine chondrocytes maintained in pellet culture were used as a source of cartilage extracellular matrix proteins. Extracts were prepared from 10-day pellets using 1 M NaCl in Tris buffer in the absence of denaturants. These extracts were incubated with recombinant  $\alpha 1(XI)Npp$  bound to agarose beads. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and subsequent immunoblot (Fig. 5). As a control, endogenous  $\alpha 2(XI)Npp$  was analyzed for its ability to bind to recombinant  $\alpha 1(XI)Npp$  under the same conditions. Using an  $\alpha 2(XI)$ -specific antibody,  $\alpha 2(XI)Npp$  was found primarily in the free fraction, whereas bovine  $\alpha 1(XI)Npp$  bound to the recombinant  $\alpha 1(XI)Npp$ -agarose beads (Fig. 5).

**Specificity of Antibody for the Endogenous  $\alpha 1(XI)Npp$** —The ability of the  $\alpha 1(XI)Npp$  antibody to discriminate between endogenous and recombinant  $\alpha 1(XI)Npp$  was demonstrated by immunoblot and nickel affinity blot (Fig. 6). Samples of increasing amounts of recombinant  $\alpha 1(XI)Npp$  were loaded onto an SDS gel and subsequently transferred to polyvinylidene difluoride membrane. Nickel-NTA-alkaline phosphatase conjugate was used to detect recombinant protein from a sample that was three orders of magnitude more dilute than the concentration needed for detection by the polyclonal antibody to  $\alpha 1(XI)Npp$ . Location of the epitope site within the amino propeptide and differences in amino acid sequence among the peptide antigen, endogenous bovine sequence, and the sequence within the recombinant protein are demonstrated in Fig. 6C.

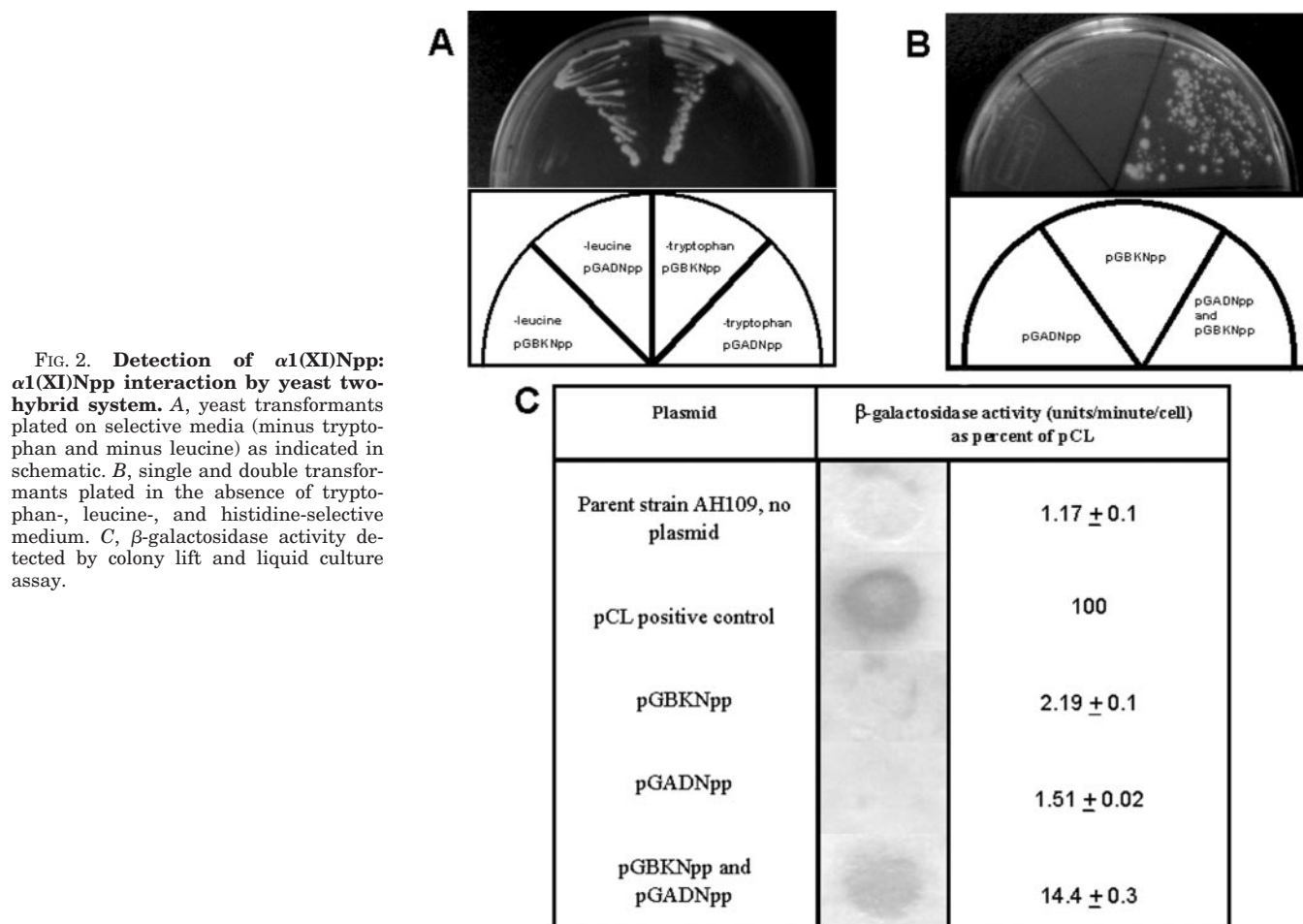


FIG. 2. Detection of  $\alpha 1(XI)Npp$ :  $\alpha 1(XI)Npp$  interaction by yeast two-hybrid system. *A*, yeast transformants plated on selective media (minus tryptophan and minus leucine) as indicated in schematic. *B*, single and double transformants plated in the absence of tryptophan-, leucine-, and histidine-selective medium. *C*,  $\beta$ -galactosidase activity detected by colony lift and liquid culture assay.

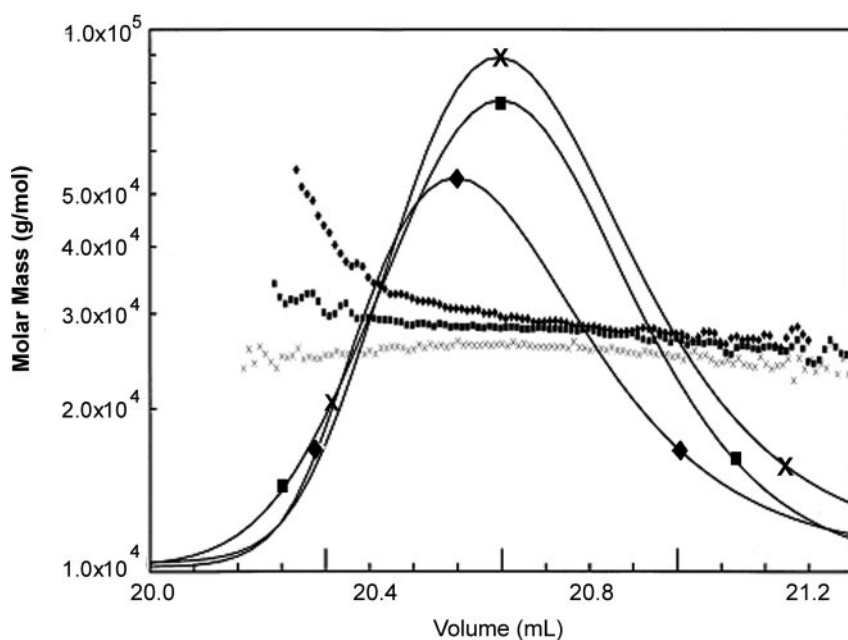


FIG. 3. Npp:Npp interaction detected by size exclusion chromatography and light scattering. Weight average molar mass plotted as a function of elution volume from TSK 3000SW column. Buffer conditions varied from low ionic strength, 75 mM (diamonds), physiological ionic strength, 175 mM (squares), and high ionic strength, 500 mM ( $\times$ ). Average molecular weight values are shown for the trailing and leading edges of chromatographic peaks.

*Interaction in Chondrocyte Pellet Culture System Resulted in a Stable Association*—Recombinant  $\alpha 1(XI)Npp$  was converted from a protein that migrated as an  $\sim 30$ -kDa protein to one that had an apparent molecular mass of  $\sim 60$  kDa under denaturing and reducing conditions when incubated for 24 h with bovine chondrocytes maintained in pellet culture. Both the 30- and 60-kDa bands were detectable via the His<sub>6</sub> tag by Nickel affinity blot (Fig. 7). Using the  $\alpha 1(XI)Npp$  antibody that can dis-

criminate between recombinant and endogenous bovine  $\alpha 1(XI)Npp$ , the 60-kDa band was also recognized by this antibody, suggesting the formation of a dimer of  $\alpha 1(XI)Npp$  monomers, one contributed by the endogenous bovine  $\alpha 1(XI)$  and the other by the added recombinant  $\alpha 1(XI)Npp$ . In addition, a band with an apparent molecular mass of 35 kDa was identified as immunologically related to endogenous  $\alpha 1(XI)Npp$ . The nature of this band is currently unknown. Stability of the 60-kDa band

FIG. 4. **ELISA assay.** Binding was shown to be saturable by ELISA assay in which recombinant  $\alpha 1(XI)Npp$  was bound to the plate and tagged recombinant  $\alpha 1(XI)$  amino-terminal domain was allowed to interact with the bound  $\alpha 1(XI)Npp$ . Antibody to the unique tag region was used to detect interaction. Absorbance at 405 nm was plotted as a function of tagged  $\alpha 1(XI)$  concentration (■). This antibody did not recognize  $\alpha 1(XI)Npp$ . *Inset* depicts the results of Scatchard analysis of binding. Bovine serum albumin was used as a control for nonspecific interaction (×). Error bars indicate  $\pm$  S.E.

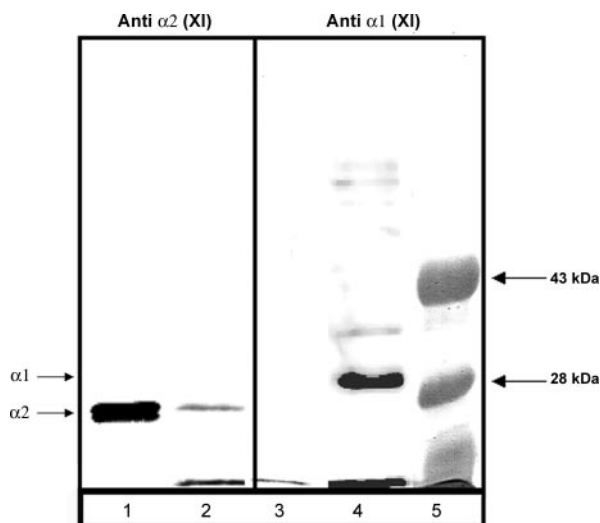
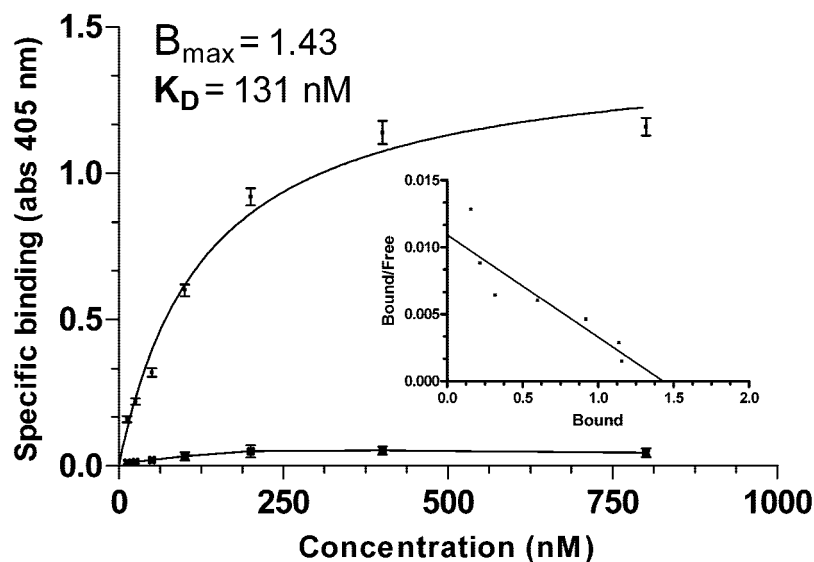


FIG. 5. **Specificity of  $\alpha 1(XI)Npp$  interaction by co-precipitation of endogenous  $\alpha 1(XI)Npp$  with recombinant  $\alpha 1(XI)Npp$ -nickel-agarose.** Proteins were extracted from chondrocyte pellet cultures in 1 M NaCl-containing buffer. Buffer conditions were changed by dialysis to reduce the salt concentration to 150 mM NaCl, and extract was incubated with  $\alpha 1(XI)Npp$ -bound nickel-agarose beads. Proteins were resolved by SDS-PAGE 12% polyacrylamide gel and analyzed by immunoblot using an antibody to  $\alpha 1(XI)Npp$  and an antibody to  $\alpha 2(XI)Npp$ . *Lanes 1 and 3*, material not bound to  $\alpha 1(XI)Npp$ -bound nickel-agarose beads. *Lanes 2 and 4*, material bound to  $\alpha 1(XI)Npp$ -bound nickel-agarose beads. *Lanes 1 and 2*, immunoblot with  $\alpha 2(XI)Npp$  antibody. *Lanes 3 and 4*, immunoblot with  $\alpha 1(XI)Npp$  antibody. *Lane 5*, molecular weight markers. *Arrows on left-hand side* indicate position of the  $\alpha 1(XI)Npp$  and the  $\alpha 2(XI)Npp$ .

was investigated by subjecting samples to prolonged incubation in the presence of 250 mM imidazole, EDTA, or  $\beta$ -mercaptoethanol at 90 °C with no change observed in migration on SDS gels. The generation of the 60-kDa band during 24 h in bovine pellet culture was not inhibited by inclusion of  $\beta$ -aminopropionitrile fumarate to inhibit lysine-derived covalent cross-linking or by inclusion of putrescine to inhibit transglutaminase in the culture (data not shown). To rule out a possible interaction mediated by the His<sub>6</sub> tag present on the recombinant protein, recombinant  $\alpha 1(XI)Npp$  was generated without a His<sub>6</sub> tag, by removing the carboxyl domain from the recombinant  $\alpha 1(XI)$  amino-terminal domain isoform by enzymatic proteolysis with BMP-1 (Fig. 7, lanes 6 and 7). The resulting recombinant

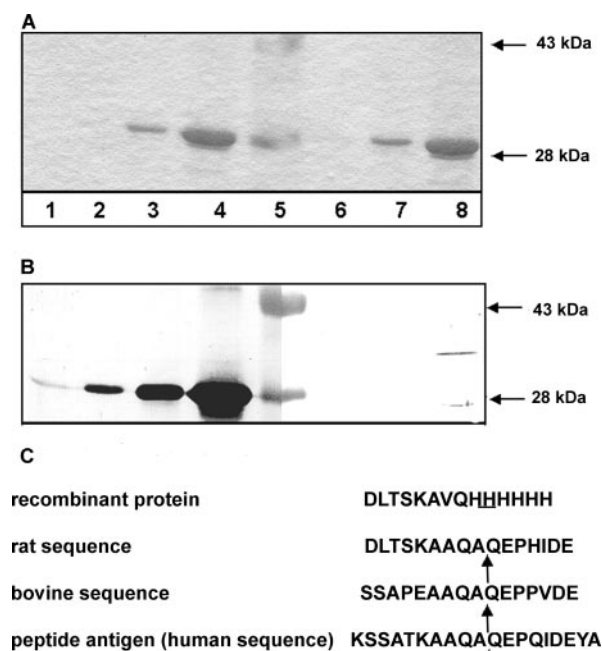
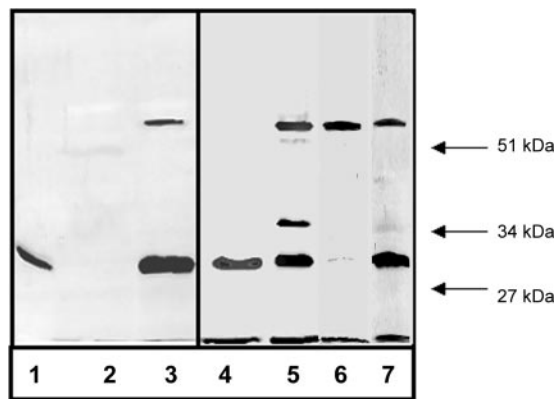


FIG. 6. **Characterization of specificity of antibody and recombinant  $\alpha 1(XI)Npp$ .** Specific changes made to the recombinant  $\alpha 1(XI)Npp$  allowed the antibody to distinguish endogenous bovine  $\alpha 1(XI)Npp$  from recombinant rat  $\alpha 1(XI)Npp$ . *A*, recombinant  $\alpha 1(XI)Npp$  in 10 $\times$  increasing incremental concentration curve. *Lane 1*, 0.01- $\mu$ g protein; *lane 2*, 0.1- $\mu$ g protein; *lane 3*, 1- $\mu$ g protein; *lane 4*, 10- $\mu$ g protein; *lane 5*, molecular weight markers, 43 and 28 kDa; *lane 6*, 0.1- $\mu$ g protein; *lane 7*, 1.0- $\mu$ g protein; *lane 8*, 10- $\mu$ g protein. *B*, nickel affinity and immunoblot of recombinant protein. *Lanes 1-4*, nickel affinity blot; *lanes 6-8*, immunoblot with  $\alpha 1(XI)Npp$  antibody. *C*, sequence of antigenic peptide compared with the analogous site in bovine and rat  $\alpha 1(XI)Npp$  and with the sequence of the recombinant protein. Although the antibody recognized endogenous rat  $\alpha 1(XI)$  collagen chains, it did not recognize the recombinant protein. The proteolytic processing site is indicated by the arrow.

$\alpha 1(XI)Npp$  was included in chondrocyte cell culture with similar results (Fig. 7).

#### DISCUSSION

The  $\alpha 1$  chain of type XI collagen exists as a set of isoforms that arise by alternative splicing of the primary RNA transcript. The portion encoding the variable region gives rise to biochemically unique domains. However, common to all isoforms is the amino propeptide, the distal globular 233 amino



**FIG. 7. Incubation of recombinant  $\alpha 1$ (XI)Npp with bovine chondrocytes in pellet culture.** A, conversion of monomer to dimer by covalent interaction between endogenous bovine  $\alpha 1$ (XI)Npp and recombinant  $\alpha 1$ (XI)Npp. Lanes 1–3, nickel affinity blot; lanes 4–7, immunoblot with antibody to endogenous  $\alpha 1$ (XI)Npp. Lane 1, purified recombinant  $\alpha 1$ (XI)Npp containing a His<sub>6</sub> tag migrated with an approximate size of 30 kDa on an SDS (11.5%) polyacrylamide gel after 24-h incubation at 37 °C in tissue culture medium in the absence of cells; lanes 2 and 4, no proteins were detected by nickel-NTA-alkaline phosphatase from control cell pellet cultures incubated in the absence of recombinant  $\alpha 1$ (XI)Npp for 24 h; however, endogenous  $\alpha 1$ (XI)Npp was detected; lanes 3 and 5, material extracted (in 1 M NaCl-containing buffer) from cell pellet cultures, incubated in the presence of 10  $\mu$ g/ml recombinant  $\alpha 1$ (XI)Npp, contained the 30-kDa recombinant protein and some of the endogenous  $\alpha 1$ (XI)Npp (apparent molecular mass, 30 kDa) and in addition contained larger proteins including a 35-kDa protein and a protein with an apparent molecular mass of 60 kDa, which was recognized by nickel-NTA-alkaline phosphatase (lane 3) and the endogenous-specific  $\alpha 1$ (XI)Npp antibody (lane 5). Lane 6, material extracted (in 1 M NaCl-containing buffer) from cell pellet cultures incubated in the presence of 1  $\mu$ g/ml recombinant  $\alpha 1$ (XI)Npp without a His<sub>6</sub> tag. The 60-kDa band is present as well as a minor band present at 30 kDa. Lane 7, material extracted (in 1 M NaCl-containing buffer) from cell pellet cultures incubated in the presence of 10  $\mu$ g/ml recombinant  $\alpha 1$ (XI)Npp without a His<sub>6</sub> tag. The 60-kDa band is present as well as the 30-kDa band and a minor band, which migrates with an apparent molecular mass of 35 kDa.

acids, which is ultimately proteolytically removed during the process of extracellular matrix assembly and fibril formation. Although the amino propeptide domain is thought to play a role in the regulation of fibril diameter, the molecular mechanism of this event is not fully understood.

A homotypic interaction was detected among the  $\alpha 1$ (XI)Npp domains. The possibility of such an interaction was supported by the use of multi-angle laser light scattering by which purified recombinant Npp was found to exist in equilibrium between monomer and dimer. Yeast-two hybrid system also supported the possibility that an interaction between two  $\alpha 1$ (XI)Npp domains could exist.

Freshly isolated chondrocytes were maintained as pellet cultures under conditions that allow the maintenance of the chondrocyte phenotype and reestablishment of a characteristic cartilage extracellular matrix. The newly synthesized matrix constituents were used as a source of cartilage matrix candidates for this study. An interaction between recombinant rat  $\alpha 1$ (XI)Npp and endogenous bovine  $\alpha 1$ (XI)Npp was detected by co-precipitation. The interaction resulted in a stable and apparently covalent cross-link. The interaction was neither a result of lysine-derived cross-linking nor transglutaminase-mediated cross-links as  $\beta$ -aminopropionitrile fumarate or putrescine did not inhibit the cross-link. The interaction was also not mediated by the His<sub>6</sub> tag present on the recombinant protein. Noncollagenous domains of other collagens have been reported to form stable interactions including type X collagen (21–23), type IV collagen (24, 25), type IX collagen (26), and type VIII collagen (27). However, no such interaction has been described

for any of the other fibrillar collagens and no such interactions have been described between propeptide domains. In the case of types IV and X, the interactions were hydrophobic in nature rather than due to covalent bond formation. In the case of the type XI  $\alpha 1$  Npp dimer, the stable interaction takes place only in the presence of living cells because incubation of purified Npp does not result in the appearance of higher molecular weight bands on subsequent SDS gels, neither does the incubation of mixtures of recombinant isoforms of  $\alpha 1$ (XI) amino-terminal domain that contains any of the possible variable domains adjacent to the Npp domain (data not shown). This interaction may be because of hydrophobic interaction or may be the result of covalent bond formation between the two Npp subunits. Data from the light scattering experiments do not support a hydrophobic interaction, because a decrease in average molecular weight was observed with increasing ionic strength of the buffer. If the stable interaction is the result of the formation of a covalent bond, it is unknown what enzyme is responsible at this time.

Type XI collagen serves primarily a regulatory function in collagen fibrillogenesis. The interaction between  $\alpha 1$ (XI)Npp domains demonstrated in this study may be coupled to enzymatic events including covalent cross-linking of amino propeptide domains and proteolytic cleavage to remove these domains from collagen fibrils. Such an interaction could contribute to our understanding of 1) initial nucleation of collagen fibrils, 2) lateral fusion of preexisting thin fibrils, and 3) increase in diameter because of accretion of individual collagen molecules to the surface of a growing collagen fibril.

The role of type XI collagen in limitation of lateral growth of collagen fibrils is well established, although the molecular mechanism is not fully understood. The  $\alpha 1$ (XI)Npp has been localized to the surface of thin collagen fibrils, it is relatively long-lived at the surface, and it is clear from the molecular dimensions that the  $\alpha 1$ (XI)Npp, the variable region, and the minor helix would not be accommodated within the gap region and would therefore sterically hinder further accretion of type II collagen molecules to the surface of a growing fibril. An interaction between Npp domains that favor proteolytic removal of globular domains from the surface may facilitate lateral growth of collagen fibrils.

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