Identification of Cross-linking Sites in Bovine Cartilage: Type IX Collagen Reveals an Antiparallel Type II-Type IX Molecular Relationship and Type IX to Type IX Bonding

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Jiann-Jiu Wu, Patricia E. Woods, and David R. Eyre
From the Orthopaedic Research Laboratories, University of Washington, Seattle, Washington 98195

Type IX collagen functions in covalent cross-linkage to type II collagen in cartilage (Eyre, D. R., Apone, S., Wu, J. J., Ericsson, L. H., and Walsh, K. A. (1987) FEBS Lett. 220, 337-341). To understand this molecular relationship better, an analysis of all cross-linking sites labeled by [3H]borohydride was undertaken using the protein prepared from fetal bovine cartilage. Sequence analysis of tryptic peptides containing the [3H]labeled cross-links showed that each of the chains of type IX collagen, α1(IX), α2(IX), and α3(IX), contained a site of cross-linking at the amino terminus of the COL2 triple-helix to which the α1(II)N-telopeptide could bond. The α3(IX)COL2 domain alone also had an attachment site for the α1(II)C-telopeptide. The distance between the α1(II)N-telopeptide and α1(II)C-telopeptide interaction sites, 137 residues, is equal to the length of the hole zone (0.6D) in a type II collagen fibril. This implies an antiparallel type II to type IX cross-linking relationship. Peptide analysis also revealed an unknown amino acid sequence linked to the COL2 cross-linking domains in both the α1(IX) and α3(IX) chains. Using antibodies to this novel peptide, its origin in the collagen α3(IX)NC1 domain was established. In summary, the results confirm extensive covalent cross-linking between type IX and type II collagen molecules and reveal the existence of type IX to type IX bonding. These data provide a molecular basis for the proposed function of type IX collagen as a critical contributor to the mechanical stability and resistance to swelling of the collagen type II fibril framework of cartilage.

Hyaline cartilage contains at least five genetically distinct types of collagen molecules, types II, VI, IX, X, and XI (1, 2). Type IX collagen accounts for about 1-2% of the collagenous protein of adult hyaline cartilage and at least 10% of that in fetal cartilage, the amount decreasing in ratio to type II collagen with increasing cartilage maturity (3). The type IX collagen molecule consists of three genetically distinct chains, α1(IX), α2(IX), and α3(IX). It has a native domain structure of three short triple-helical segments, COL1, COL2, and COL3 connected by nonhelical sequences, NC2 and NC3. In addition, there are noncollagenous domains, NC1 at the carboxyl terminus and a larger NC4 at the amino terminus (4). Pepsin extracts type IX collagen from cartilage by cleaving the molecule within its nonhelical regions to give three peptidolyase-resistant collagenous segments, COL1, COL2, and COL3. The COL1 and COL2 domains are recovered as interchain disulfide-bonded trimers. In earlier reports COL1 was variably termed LMW, CF2, C-PS2, and M2, and COL2 likewise was termed HMW, M, C-PS1, and M1 (5-11). Type IX collagen is also a proteoglycan (12-14). In chick cartilage a chondroitin sulfate chain is covalently linked to the α2(IX) chain at a specific serine residue in its NC3 domain (15-19). However, in bovine cartilage, most type IX collagen molecules do not appear to have an attached glycosaminoglycan chain, at least not of significant length to alter the chain's electrophoretic mobility on SDS-PAGE (20).

Type IX collagen is extensively cross-linked in cartilage and resists extraction by denaturants including 4 M guanidine HCl. Most of the cross-linking residues in pepsin-solubilized type IX collagen in mature articular cartilage are trivalent pyridinolines (21). Sequence analysis of peptides that contained the pyridinoline residues revealed that type IX molecules were covalently cross-linked to type II collagen telopeptides in cartilage (22-24). This finding was consistent with electron microscopic observations showing type IX collagen molecules decorating the surface of cartilage type II collagen fibrils (25, 26). In addition to the mature pyridinoline cross-links, divalent borohydride-reducible cross-links (dihydroxylysylonorleucine and dihydroxylysylisonorleucine) are also found in type IX collagen (27). The divalent residues were especially enriched in fetal cartilage type IX collagen but were also present in the protein extracted from the adult tissue (being the exclusive cross-links at certain molecular sites) and as ketoamines are believed to be the precursors of pyridinoline cross-links (28).

To understand the precise orientation, alignment, and chemical mechanism of interaction of type IX collagen molecules with the surface of type II fibrils, we set out to define all the sites of intermolecular cross-linking in the type IX collagen molecule. The source of tissue was fetal bovine type IX collagen. The strategy was to label acid-stable, divalent cross-links with tritiated borohydride and use the tritium label to isolate the cross-linked peptide fragments from the mixture of protease- and chemical-cleavage products.

The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; D-period, the 67-nm repeating periodicity of types I, II, and III collagen fibrils; PVDF, polyvinylidene difluoride; RP-HPLC, reversed-phase high-performance liquid chromatography; N- and C-telopeptides, short sequences that form the amino and carboxyl ends of types I, II, and III collagen chains.

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3To whom correspondence should be addressed: Dept. of Orthopaedics, RK-10, University of Washington, Seattle, WA 98195.
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Fig. 1. Molecular sieve chromatography of pepsin-extracted fetal bovine type IX collagen. The \[^{3}H\] NaBH₄-reduced type IX collagen (10 mg) was chromatographed on an agarose A5m (Bio-Rad) molecular sieve column (170 x 1.5 cm) to resolve COL1 and COL2 domains. The eluent was 2 M guanidine HCl, 0.05 M Tris-HCl, pH 7.5, at a flow rate of 7.2 ml/h, collecting 2.4-ml fractions. Aliquots of collected fractions (100 \(\mu l\)) were assayed for tritium activity. All the tritium-labeled borohydride reducible cross-linking residues were recovered in the COL2 fraction.

Fig. 2. Reversed-phase HPLC of individual COL2 chains. Cysteine residues were reduced and carboxymethylated prior to chromatography on a C8 reversed-phase HPLC. The C8 column (Brownlee Aquapore RP-300; 4.6 mm x 25 cm) was eluted with a linear gradient (20-35%) of solvent B in 30 min at a flow rate of 1 ml/min. Solvent A was 0.1% trifluoroacetic acid (v/v) in water, and solvent B was 0.085% trifluoroacetic acid (v/v) in acetonitrile:n-propanol (3:1, v/v). Fractions were pooled and analyzed by SDS-7.5% PAGE (Inset). Bands after transblotting to PVDF membrane were identified by amino-terminal protein sequence analysis.

Materials and Methods

Preparation of Type IX Collagen—Fetal (5-6 month) bovine epiphyseal cartilage was dissected as the source of collagen. Type IX collagen fragments were extracted by limited pepsin digestion of homogenized tissue, previously extracted with 4 M guanidine HCl, 0.05 M Tris-HCl, pH 7.4, at 4 °C then washed thoroughly with water, and purified by salt precipitation as described previously (21). To label the borohydride-reducible cross-linking residues, the preparation was reacted with \[^{3}H\]NaBH₄ (10 mCi/mM) in 0.1 M sodium phosphate, pH 7.4, dialyzed against 0.1 M acetic acid, and freeze-dried (29). Intact type IX collagen molecules were prepared by extracting slices of fetal bovine epiphyseal cartilage from 1 M NaCl, 0.05 M Tris-HCl, pH 7.4, and purified by salt fractionation (30).

Column Chromatography—The COL1 and COL2 disulfide-bonded chain fragments of the type IX molecule were resolved by molecular sieve chromatography on an agarose A5m column (170 x 1.5 cm, 200-400 mesh, Bio-Rad Labs), eluted by 2 M guanidine-HCl, 0.05 M Tris-HCl, pH 7.5. After reduction and carboxymethylation of cysteine residues in the isolated fragments (31), the individual chains of the COL2 fragment were partially resolved by reversed-phase HPLC on a C8 column (Brownlee Aquapore RP-300; 4.6 mm x 25 cm) with a linear gradient of acetonitrile:n-propanol (3:1, v/v) in aqueous 0.1% (v/v) trifluoroacetic acid from 20 to 35% in 30 min. Ion-exchange HPLC was performed on a DEAE-5-PW column (75 x 7.5 mm, Bio-Rad), eluting with a linear gradient of 0-0.15 M NaCl in 40 ml of 0.02 M Tris-HCl, pH 7.5, containing 10% (v/v) acetonitrile. Eluent was monitored for 220-nm absorbance, and aliquots (100 \(\mu l\)) of collected fractions were assayed for \(^3\)H activity. Peptides containing pyridinealine cross-links were detected by monitoring for fluorescence (excitation, 297 nm; emission, 340 nm) using a Kratos Model 970 HPLC fluorescence detector (32).

Trypsin Digestion—Isolated type IX collagen chain fragments were heat-denatured and digested with trypsin (Boehringer sequencing grade) at an enzyme-to-substrate ratio of 1:50 (w/w) in 0.02 M NH₄HCO₃ at 37 °C for 4 h. Tryptic peptides containing \(^3\)H-labeled reducible cross-links were purified by reversed-phase and ion-exchange HPLC.

Bacterial Collagenase Digestion of Cartilage Matrix—500 mg of insoluble residue after 4 M guanidine-hydrochloride extraction (24 h, 4 °C) of bovine fetal cartilage was suspended in 0.05 M Tris-HCl, pH 7.4, containing 10 mM CaCl₂, 0.2 M NaCl, and the proteinase inhibitors 10 mM N-ethylmaleimide and 2 mM phenylmethylsulfonyl fluoride, heat-denatured at 70 °C for 15 min, equilibrated to 37 °C, and digested with 2,000 units of bacterial collagenase type III (Sigma) at 37 °C for 24 h. The digest was dialyzed against 0.1 M acetic acid, the precipitate was removed by centrifugation, and the supernatant was lyophilized. Resulting peptides were suspended in 4 M guanidine HCl, 0.05 M Tris-HCl, pH 8.0, and cysteine residues were reduced with diithiothreitol and then alkylated with \[^{14}C\]iodoacetate (5.3 pmol, 31). The reaction mixture was desalted on a P2 column (30 x 1.5 cm, 200-400 mesh, Bio-Rad) eluted by 10% (v/v) acetic acid. Peptides containing \(^{14}C\) activity were purified by ion-exchange and reversed-phase HPLC and identified by protein microsequencing.

Periodate Cleavage of Borohydride-reduced Cross-links—Purified \(^3\)H-labeled type IX chain fragments were dissolved in 0.02 M acetic acid and reacted with an equal volume of 0.02 M NaIO₄ for 3 h in the dark at room temperature. The reaction was stopped by adding a drop of ethylene glycol, followed by excess cold sodium borohydride (29). The collagen telopeptides released by this cleavage were resolved by reversed-phase HPLC and identified by protein microsequencing.

Gel Electrophoresis and Electroblotting—Proteins were resolved by SDS-PAGE (33). For amino-terminal microsequencing or Western blot analysis, the proteins were transferred after electrophoresis to PVDF membrane (Immobilon-P, Millipore) (34) using a MilliBlot SDE electroblotting apparatus (Millipore). The membrane was washed thoroughly in ultrapure water (Millipore Milli-Q) to remove salts, then stained with Coomassie Brilliant Blue to identify protein bands. The bands were excised and subjected to protein sequence analysis.
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FIG. 3. Reversed-phase HPLC fractionation of tryptic peptides prepared from pepsin fragment COL2A. The sample (1.2 mg) was eluted from a C8 column (Brownlee Aquapore RP-300; 25 cm x 4.6 mm) with a linear gradient (0–30%) of solvent B in A over 60 min at a flow rate of 1 ml/min. Solvent A was 0.1% trifluoroacetic acid (v/v) in water, and solvent B was 0.085% trifluoroacetic acid (v/v) in acetonitrile:propanol (3:1, v/v). The amino-terminal sequences of the 3H-labeled cross-linked peptides A1 (fraction 36) and A2 (fraction 38) are shown as deduced from the automated Edman degradation results. The two-chained structures are derived from the d(II)N-telopeptide linked to the α(IX)COL2 domain. P, 4-hydroxyproline; R, hydroxylysine; and X, cross-linking hydroxylysine residue.

Protein Elution from PVDF Blots—For elution of protein bands, the above procedure was followed to the stage before staining with Coomassie Blue. Protein bands were visualized by transillumination as the membrane began to dry after washing and were excised. Protein was eluted from the membrane by incubating in aqueous 40% (v/v) acetonitrile at 37 °C for 3 h, followed by incubating in 40% (v/v) acetonitrile containing 0.05% (v/v) trifluoroacetic acid for 20 min at 50 °C after removing the supernatant. Extraction steps were then repeated. Supernatants were combined and lyophilized.

CNBr Cleavage—Protein eluted from the PVDF blot was treated with CNBr in 70% formic acid under N2 at room temperature for 24 h on a shaker. The digest was diluted 15-fold with water and freeze-dried (32).

Protein Sequencing—Amino-terminal sequence analyses were performed on a Porton 2090E gas-phase microsequencer equipped with on-line high-performance liquid chromatography analysis of phenylthiodyanantoin derivatives.

Antibody Preparation and Immunoblotting—A 10-residue peptide (AAVGEKSGPR) corresponding to a novel sequence identified in cross-linked peptides from type IX collagen (vide infra) was synthesized using an automated peptide synthesizer. A cysteine residue was added to the carboxy terminus for use in coupling to the carrier protein. Peptide was coupled to the carrier protein, keyhole limpet hemocyanin (Pierce) using the reagent, sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane 1-carboxylate (Pierce). Rabbits were immunized by subcutaneous injection with 1 mg of hemocyanin-peptide conjugates in 0.5 ml of phosphate-buffered saline solution emulsified with an equal volume of Freund’s complete adjuvant. Three subcutaneous booster injections of 1 mg of antigen emulsified with Freund’s incomplete adjuvant were given after 3, 6, and 10 weeks of initial injection. For Western blot analysis, polyclonal antisera were raised against the synthetic peptide conjugate was diluted 1:100 in phosphate-buffered saline containing 0.1% bovine serum albumin and examined for its reactivity against the intact type IX collagen chains previously transblotted to PVDF membrane.

RESULTS

Pepsin-extracted type IX collagen was reacted with [3H]borohydride to label the acid-stable, reducible cross-linking...
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Fig. 5. Reversed-phase HPLC fractionation of tritium-labeled telopeptides released from fetal bovine type IX collagen COL2 domains by periodate oxidation. a, α1(IX)COL2 plus α3(IX)COL2; b, α2(IX)COL2. Column conditions are as described in Fig. 3, except that the elution was with 0.1% trifluoroacetic acid for 5 min, followed by a linear gradient (0–35%) of solvent B in A over 35 min. In the sequences shown, X was the tritium-labeled 5-hydroxynorvaline residue derived from the cross-link.

amino acid residues before fractionating the two main protein domains by molecular sieve chromatography. The results showed that the 3H-labeled divalent cross-linking residues were associated exclusively with the COL2 domain of the molecule (Fig. 1). This distribution resembles the distribution of pyridinoline cross-linking residues previously reported for type IX collagen extracted from mature bovine articular cartilage (22).

After the isolated COL2 fraction had been treated to reduce and carboxymethylate cysteine residues, two components were resolved by chromatography on a C8 reversed-phase HPLC, both containing 3H activity (COL2A and COL2B, Fig. 2). The earlier eluting component (COL2A) gave a single 33-kDa band on SDS-PAGE (Fig. 2, inset). The amino-terminal protein sequence of this band from a PVDF blot identified it as the COL2 domain of the α2(IX) chain by similarity to the chicken α2(IX) cDNA sequence (35). The second peak (COL2B) gave two bands at 33 and 41 kDa on SDS-PAGE, which are the COL2 domains of the α1(IX) and α3(IX) chains, respectively (sequences are shown in Fig. 6). Both had incorporated 3H label, indicating that all three chains of the COL2 domain of type IX collagen from fetal cartilage included reducible divalent cross-linking residues.

Structural Identification of Cross-linking Site—To identify the cross-linking sites, COL2A and COL2B chain fragments were digested with trypsin, and the peptides were resolved by reversed-phase HPLC. Fig. 3 shows the elution profile by UV absorbance and 3H activity of tryptic peptides prepared from α2(IX)COL2 (COL2A in Fig. 2). Two main 3H-labeled peaks were found (A1 and A2). Sequence analysis of A1 gave two running sequences in each cycle (in 1:1 molar ratio). These could be interpreted as the α1(II)N-telopeptide generated by pepsin and the amino terminus of the α2(IX)COL2 domain. Tritium activity measured in aliquots from each cycle of Edman degradation off the sequencer located the cross-linking site at cycle 12 indicating that the peptide was derived from the linkage site between a hydroxylysine at residue 3 of the α2(IX)COL2 triple-helical domain and a cross-linking hydroxylysine residue in the α1(II)N-telopeptide sequence. Amino-terminal sequence results on the other cross-linked peptide (A2) also gave a two-chained structure. Here the sequences were identical to those derived from the A1 peptide, except that A2 gave an additional methionine residue at the carboxy terminus of the α1(II)N-telopeptide. Peptides A1 and A2 therefore most probably result from variable cleavage by pepsin of the α1(II)N-telopeptide.

Fig. 4 shows the elution profile of the trypsin digest of the combined α1(IX)COL2 and α3(IX)COL2 components (i.e. fraction COL2B described above and shown in Fig. 2). Tryptic peptides containing 3H-labeled reducible cross-links from this chromatogram were sequenced after purification by ion-exchange HPLC and reversed-phase HPLC (chromatographic steps not shown). All such peptides revealed two-chained structures. The results are summarized in Fig. 4. They show that the cross-linked tryptic peptides under peaks B1 and B3 (Fig. 4) were both derived from a site of covalent interaction between a hydroxylysine at a helical site in the α1(IX)COL2 domain with a hydroxylysine aldehyde in an α1(II)N-telopeptide. The two sequences in purified B4 peptide were matched to an α1(II)C-telopeptide linked to a helical sequence previously identified in the major pyridinoline-containing peptide isolated from type IX collagen of adult bovine articular cartilage (22). The cross-linked peptide purified from peak B5 gave sequences matching what we believe is the amino terminus of the α3(IX)COL2 triple-helical domain and the α1(II)N-telopeptide. The latter appears to be linked to a hydroxylysine at residue 12 of the α3(IX)COL2 triple helix.

In addition to forming cross-links with α1(IX)N-telopeptides, the same α1(IX) and α3(IX) COL2 cross-linking sites were also recovered in additional peptides cross-linked to a novel amino acid sequence, AAVGEXSGPR (where X is the cross-linking residue), which did not appear to be derived from type II collagen or any known protein (Fig. 4, peaks B2 and B5).

Periodate Cleavage—Borohydride-reduced cross-links were cleaved by periodate to release the individual peptide chains. Using this method, the above novel peptide sequence (AAVGEXSGPR) was also recovered from the pooled fractions containing cross-linked α1(IX) and α3(IX)COL2 domains (COL2B, Fig. 2). Periodate treatment of this fraction released three 3H-labeled peptides that were resolved by RP-HPLC (Fig. 5a). In elution order, the first had a sequence identical to a fragment of the N-telopeptide of type II collagen; the second was identical to the novel peptide sequence identified...
Fig. 6. A summary of the triple-helical cross-linking amino acid sequences in bovine type IX collagen. Sequences were compared with cDNA deduced sequences for α1(IX) and α2(IX) and with protein sequence for α3(IX) to confirm the identity of each sequence (24, 35, 36). Solid and open arrows indicate sites of attachment for type II and type IX telopeptides. All three chains, α1(IX), α2(IX), and α3(IX), have sites for linkage to the α2(II)N-telopeptide at specific hydroxylysine residues close to the amino terminus of COL2. The α3(IX) chain alone contains a second triple helical site that can cross-link specifically to the α1(II)C-telopeptide. The same two α1(II)N-telopeptide sites in the α1(IX) and α3(IX) COL2 domains can also link to a novel peptide sequence which is derived from the NC1 domain of α3(IX).

Fig. 7. Reversed-phase HPLC fractionation of CNBr peptides derived from the α3(IX)COL2 pepsin fragment. The α3(IX)COL2 band was extracted from a PVDF blot and digested with CNBr (see “Materials and Methods” for details). The digest was eluted from a C8 column (Brownlee Aquapore RP300; 4.6 mm × 25 cm) with a linear gradient (10-37%) of acetonitrile:propanol (31, 49-50 min in addition to the 13-, 16-, and 27-kDa CNBr peptides that the 27-kDa peptide was a partial cleavage product of the 11-kDa peptide, indicating that the 27-kDa peptide had the identical amino-terminal sequence to the 11-kDa peptide, indicating that the 27-kDa peptide was a partial cleavage product of CNBr digestion, in which a methionine residue between the adjacent 11- and 16-kDa peptides had failed to cleave. The relative positions of the 13-, 11-, and 16-kDa CNBr peptides within the 41-kDa domain were thus established and are shown in Fig. 8. By sequence homology, these results also indicate that the two attachment sites for α1(II) telopeptides (N and C) in the α3(IX) chain are separated by about 140 residues, or 0.6D (where D = 67 nm or 234 amino acid residues), the exact length of the hole region in the packing arrangement of a collagen fibril (37).

Imunochemical Analysis—To identify the source of the novel peptide sequence found cross-linked to α1(IX) and α3(IX) chains, polyclonal antibodies were raised against a peptide synthesized to reproduce this novel sequence. The antibody preparation was used to seek out the parent protein from which the peptide epitope had originated. Western blot analysis showed that it reacted specifically with the α3 chain of a neutral salt extract of cartilage that contained intact type IX collagen molecules (Fig. 9), indicating that the novel peptide sequence was located within the α3(IX) chain.

Site of the Novel Cross-linking Sequence in the α3(IX)NC1 Domain—To determine where this peptide sequence was lo-
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Fig. 8. Sites of cross-linking in the bovine a3(IX)COL2 domain. The sequences of the interaction sites for the type II collagen N-telopeptide and C-telopeptide are shown. The distance between the two sites is exactly 0.6D (137 residues based on the chicken a3(IX) cDNA (39)), the length of the hole zone in a collagen fibril, which implies an antiparallel cross-linking relationship between type IX and type II collagen molecules.

Fig. 9. SDS-polyacrylamide electrophoresis and Western blot of whole type IX collagen chains using a polyclonal antibody raised against the synthetic peptide, AAVGEKSGPR. Individual chains of intact type IX collagen were resolved on SDS-6% PAGE after disulfide cleavage (dithiothreitol) and transblotted to PVDF membrane. Lane 1, stained with Coomassie Brilliant Blue; lane 2, reacted with antibody.

Fig. 10. Anion-exchange HPLC fractionation of a bacterial-collagenase digest of fetal bovine cartilage collagen. Peptides containing 14C-labeled carboxymethylcysteine were resolved on a DEAE-5-PW column (75 x 7.5 mm, Bio-Rad), by elution with a linear gradient of 0–0.15 M NaCl in 60 ml of 0.02 M Tris-HCl, pH 7.5, containing 10% (v/v) acetonitrile followed by a 0.5 M NaCl strip. Fractions indicated by the bar were selected for further purification.

DISCUSSION

Type IX collagen functions in cartilage matrix as a cross-linked copolymer with type II collagen. The cross-linking residues in type IX collagen consist of trivalent pyridinolines and divalent borohydride-reducible ketoamines (21, 22). The ratio between these two forms of cross-link varies with the maturity of the tissue source. Type IX collagen purified from steer articular cartilage after pepsin extraction contains 1.2–1.5 mol of pyridinoline and 0.5–0.8 mol of dihydroxylysino-norleucine per mol of type IX collagen. Fetal cartilage type IX collagen contains about 0.5 mol of pyridinoline and 1.5 mol of dihydroxylysino-norleucine per mol (27).

Since an analysis of pyridinoline-containing peptides may not define all cross-linking sites, the present study was designed to define both borohydride-reducible (ketoamine) and pyridinoline cross-linking sites in the bovine type IX collagen molecule. If any acid-labile borohydride-reducible cross-links existed in type IX collagen in the tissue, they would probably not be detected by the methods adopted, since the protein was extracted with pepsin at low pH before borohydride treatment. The results on fetal cartilage confirm our previous finding that cartilage type IX collagen is extensively cross-linked to type II collagen by pyridinoline and borohydride-reducible divalent cross-linking residues (21, 22, 27). The results also reveal new cross-linking sites for borohydride-
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FIG. 11. Reversed-phase HPLC of $^{14}$C-carboxymethylated peptides pooled from DEAE-HPLC as shown in Fig. 10. The sample was eluted with a linear gradient (10-40%) of solvent B in A (see Fig. 3) in 60 min. The aminoterminus sequence that was derived from the peptide recovered in fractions 37-39 is shown. The underlined sequence matches exactly the novel peptide sequence identified in the cross-linked peptides.

FIG. 12. Molecular models of potential type IX-type II collagen interactions based on the cross-linking sites found in bovine type IX collagen. The predicted antiparallel relationship between type II and type IX collagen molecules and the $\alpha_3$(IX)NC1 to $\alpha_1$(IX) and $\alpha_3$(IX)COL2 interaction sites are accommodated in these three speculative arrangements. A predicted feature of such head-to-tail type IX-type IX linkages, given the likely flexible-hinge character of the NC2 and NC3 molecular domains, is an inherent capacity for tethering neighboring type II fibrils to each other at virtually any angular relationship.

dehyde is presumably formed as the precursor of cross-link formation.

The results of the present and earlier studies (21, 22, 27) indicate that all the borohydride-reducible and mature pyridinoline cross-linking residues in type IX collagen have one arm located in the COL2 domain. Thus all three COL2 domains of $\alpha_1$(IX), $\alpha_2$(IX), and $\alpha_3$(IX) form covalent cross-links to the amino-telopeptide of type II collagen. Only the $\alpha_3$(IX)COL2 domain, however, contained a second helical cross-linking site, to which the $\alpha_1$(II)C-telopeptide was found to be linked. In addition to cross-linking to the $\alpha_1$(II)N-telopeptide, the same helical sites at the amino terminus of $\alpha_1$(IX) and $\alpha_3$(IX)COL2 domains were recovered in peptides covalently cross-linked to the $\alpha_3$(IX)C-telopeptide (i.e. the $\alpha_3$(IX)NC1 domain). These results prove that intermolecular bonds must be formed between type IX collagen molecules themselves as well as between type II and type IX collagen molecules. It is not yet clear whether covalent interactions between type IX collagen molecules are restricted to dimer formation or can, for example, mediate a more complex polymeric type IX structure.

Periodate cleavage proved to be an informative method for isolating and identifying the source of the aldehyde-containing partner that participates at each of the cross-linking sites. Thus, sodium metaperiodate (Na$I_3O_4$) selectively cleaves the borohydride-reduced ketoamine cross-links of collagen. After the reaction, the $^3$H label stays with the amino acid cross-linking partner that originally was a hydroxylysine aldehyde formed by lysyl oxidase from a hydroxylysine side chain (29). In types I, II, and III collagens this appears always to be a telopeptide sequence (28). Released telopeptides can be readily purified from the reaction products and identified by protein microsequencing. This method has been successfully applied to the isolation and identification of telopeptides linked to type II collagen (38), and in the present study to type IX collagen. The method has potential for exploring other heterotypic interactions believed to occur between different types of collagen in the extracellular matrix of cartilage and other tissues.

Type IX collagen molecules decorate the surface of type II collagen fibrils (25, 26) where they are presumably covalently linked (22, 23). However, prior to the present study, the orientation of type IX molecules relative to type II collagen molecules on the fibril surface (whether, for example, they are packed parallel or antiparallel to type II fibrils) was unknown, although a model was proposed that assumed a parallel orientation (23). Identification of the precise molec-
ular cross-linking sites had the potential to resolve this issue and perhaps also to reveal details of the mechanism of docking and spatial interaction between type IX and type II collagen molecules. The present results show that the distance between the amino- and carboxyl-telopeptide attachment sites along the COL2 triple-helix was about 140 residues, or 0.6D, the length of the hole region in the near quarter-stagger molecular packing arrangement that is the basis of a banded collagen fibril (37). This strongly suggests that type IX collagen molecules decorate the surface of:type II collagen molecules in an antiparallel relationship. Thus, an antiparallel but not a parallel orientation would allow both α1(II)-N-telopeptide and α1(II)-C-telopeptide sites to be occupied in a single type IX collagen molecule on a type II fibril surface.

Recently, cDNA clones coding for the entire chicken α3(IX) chain have been sequenced (39). From the deduced amino acid sequence, the precise distance of the α1(II)-C-telopeptide attachment site from the α1(II)-N-telopeptide attachment site in the α3(IX)/COL2 domain is 137 residues. The results of the present study allow the spatial interactions between type IX collagen molecules and type II collagen fibrils to be precisely modeled (Fig. 12). Three possible examples are shown of type IX collagen molecules linked both to the surface of type II collagen fibrils and to other type IX molecules, thus effectively providing links between different fibrils. Our favored mode of interaction between type IX collagen molecules themselves is the overlapping head-to-tail parallel arrangement shown, but the findings do not rule out, for example, antiparallel dimers.

These observations and conclusions are relevant in understanding the underlying molecular mechanisms, whereby articular cartilage is caused to increase in hydration and swell (40, 41) and the collagen fibrils to spread apart (41) as part of the earliest known events to occur in traumatic injury and perhaps also to reveal details of the mechanism of docking and break the restraints that this protein may impose on the type II collagen fibril network in cartilage.

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