Covalent Cross-linking of the NC1 Domain of Collagen Type IX to Collagen Type II in Cartilage*

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David R. Eyre‡, Terri Pietka, Mary Ann Weis, and Jiann-Jiu Wu

From the Orthopaedic Research Laboratories, University of Washington, Seattle, Washington 98195

From a study to understand the mechanism of covalent interaction between collagen types II and IX, we present experimental evidence for a previously unrecognized molecular site of cross-linking. The location relative to previously defined cross-linking sites predicts a specific manner of interaction and folding of collagen IX on the surface of nascent collagen II fibrils. The initial evidence came from Western blot analysis of type IX collagen extracted by pepsin from fetal human cartilage, which showed a molecular species that had properties indicating an adduct between the α1(II) chain and the C-terminal domain (COL1) of type IX collagen. A similar component was isolated from bovine cartilage in sufficient quantity to confirm this identity by N-terminal sequence analysis. Using an antibody that recognized the putative cross-linking sequence at the C terminus of the α1(IX) chain, cross-linked peptides were isolated by immunaffinity chromatography from proteolytic digests of human cartilage collagen. They were characterized by immunochemistry, N-terminal sequence analysis, and mass spectrometry. The results establish a link between a lysine near the C terminus (in the NC1 domain) of α1(IX) and the known cross-linking lysine at residue 930 of the α1(II) triple helix. This cross-link is speculated to form early in the process of interaction between collagen IX molecules and collagen II polymers. A model of molecular folding and further cross-linking is predicted that can spatially accommodate the formation of all six known cross-linking interactions to the collagen IX molecule on a fibril surface. Of particular biological significance, this model can accommodate potential interfibrillar links as well as intrafibrillar links between the collagen IX molecules themselves, so providing a mechanism whereby collagen IX could stabilize a collagen fibril network.

Collagen type IX is a member of the fibril-associated collagen with interrupted triple helix (FACIT) family of collagen molecules, which share homologous domains and are all believed to function in the extracellular matrix in association with collagen fibril surfaces (1, 2). Collagen IX is unique among the FACIT molecules in binding covalently to fibril surfaces. The cross-links are formed through the lysyl oxida-

dase mechanism (3–7). Collagen IX is found mostly in cartil-
gages, but it also occurs in the eye (vitreum, 8, 9) and avian cornea (8), ear (tectorial membrane (10)), and intervertebral disc (11, 12), always in co-existence with type II collagen. A special role for collagen IX in the organization of type II collagen fibril networks has apparently evolved, which for articular cartilage seems to be essential for the long term normal functioning of joints. The evidence for this comes from the study of genetic defects. Mutations in all three collagen IX genes have been linked to a chondrodysplasia syndrome (multiple epiphyseal dysplasia), which features early onset osteoarthritis (13).

All three chains of the vertebrate collagen IX molecule, α1(IX), α2(IX), and α3(IX), contain intermolecular cross-linking sites, each with chain-specific properties (3–7). All the cross-links are of the lysyl oxidase-mediated type, formed from modified lysine residues between type IX and type II collagen molecules or between type IX collagen molecules. The first evidence for this was an observation that type I collagen triple helical domains purified from pepsin digests of bovine articular cartilage contained pyridinoline cross-linking residues (3). The concentration was higher on a mole/mole basis than in type II collagen isolated from the same tissue. Further analysis showed that the most abundant cross-links in fetal cartilage were divalent keto-amines, the same intermediates found on the pathway to pyridinoline formation within collagen fibrils (6). Analysis of the structures of cross-linked peptides isolated from type IX collagen showed one class that had derived from the reaction of a hydroxylysine aldehyde in the N-telopeptide of type II collagen to a hydroxylysine at the N terminus of the triple helical COL2 domain (4–6). All three chains, α1(IX), α2(IX), and α3(IX), were able to cross-link to the α1(II) N-telopeptide. In addition, the α1(II) C-telopeptide was similarly cross-linked to another site in the middle of the COL2 domain but only to α3(IX) (4, 6, 7). Studies on fetal human cartilage showed this same pattern of collagen IX cross-linking as in bovine cartilage (7). From both human and bovine tissues, cross-linked peptides were also identified that had originated from an interaction between two type IX collagen molecules and in yields that indicated about an equal prominence as for IX-to-II linkages (6, 7). The IX-to-IX cross-links were from the NC1 domain (C terminus) of α3(IX) to the same COL2 site (in α1(IX) or α3(IX)) to which the α1(II) N-telopeptide could link. Of all the cross-linked peptides isolated from collagen IX, only two had properties that showed an origin from a lysyl oxidase-generated aldehyde in the type IX collagen molecule itself. These peptides had linked α3(IX)NC1 to a site in α1(IX) or α3(IX)COL2. All others were apparently based on precursor hydroxylysine aldehydes in α1(II)N- or C-telopeptides.

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‡ To whom correspondence should be addressed: Dept. of Orthopaedics & Sports Medicine, Box 356500, University of Washington, Seattle, WA 98195. Tel.: 206-543-4700, Fax: 206-685-4700; E-mail: deyre@u.washington.edu.

The abbreviations used are: FACIT, fibril-associated collagen with interrupted triple helix; COL1, COL2, etc., triple helical domains; NC1, NC2, etc., non-triple helical domains; CB9,7, cyanogen bromide peptide; HPLC, high performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; mAb, monoclonal antibody.
From transmission electron microscopy we know that type IX collagen molecules physically decorate the surface of type II collagen fibrils in developing cartilage (14) and are most concentrated on the thinnest fibrils (15) that form the network or basket around chondrocytes (16). From the yield of cross-linked peptides we know that most of the collagen IX molecules in cartilage, even in fetal tissue, occur in covalent linkage to type II collagen fibrils (5, 7). The lack of extractability of collagen IX in protein denaturants confirms this. The relative yields of the different cross-linked peptides also predict that most collagen IX molecules are also linked to each other. These various observations make it challenging to present a molecular model that can explain how, in a network of fine collagen fibrils coated with collagen IX molecules, IX-to-IX molecular links can be so abundant when the opportunity for fibril-to-fibril surface interactions would seem to be relatively limited. Any viable molecular model of heterofibril assembly needs, however, to accommodate the cross-linking stoichiometry. Here we provide evidence for an additional, major site of interaction between type II and IX collagens that further constrains how the molecules can be organized but from its position suggests how the collagen IX molecules are folded on the surface of the collagen II polymer to accommodate all the cross-links.

EXPERIMENTAL PROCEDURES

Preparation of Human Collagen Samples—Ribs, knees, and hip were harvested and minced from 94- to 98-day-old human fetuses (Central Laboratory for Human Embryology, University of Washington). The minced tissue samples were extracted in 4 M guanidine HCl, 0.05 M Tris, pH 7.4, containing protease inhibitors (2 mM EDTA, 5 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, and 5 mM phenanthroline) at 4°C for 48 h to remove proteoglycans and non-cross-linked proteins. The tissue residue was washed with water, suspended in 3% acetic acid, and digested with pepsin using a ratio of 1:50 (w/w) (pepsin:wet tissue) for 24 h at 4°C. Pepsatin was added to stop the reaction, and the digest was centrifuged to remove insoluble tissue. The solubilized collagen was then precipitated with 2.2 M NaCl to remove most of the pepsin. After precipitation, the collagen was resuspended in 3% acetic acid, dialyzed, freeze-dried, and then digested with CNBr in 70% formic acid under N₂ for 24 h at room temperature. The CNBr digest was freeze-dried.

Column Chromatography—Monoclonal antibody 2B4 was used to prepare an affinity column. This mouse antibody was originally prepared to recognize a proteolytic cleavage product in the C-telopeptide domain of the α1(IX) chain, a, replicate aliquots of a 4 M guanidine HCl cartilage extract were analyzed by SDS-6% PAGE with and without disulfide cleavage by dithiothreitol (DTT). The left panel shows 2B4 Western results, and the right panel shows Coomassie Blue staining. The dimer (β2(IX)) persists because of a resistant cystine disulfide. b, replicate aliquots of pepsin-solubilized fetal cartilage collagen were run on SDS-7.5% PAGE for Western analysis with and without dithiothreitol and for Coomassie Blue staining. Strong immunostaining of a minor protein band that shifts down on reduction, running between the prominent a and β chains of type II collagen, which also show immunoreactivity.

by SDS-PAGE using the method of Laemmli (18). For N-terminal microsequencing, peptide bands were transferred to polyvinylidene difluoride membrane (SequimBlot, Bio-Rad) using a MilliBlot-SED electroblotting apparatus. For Western blot analysis, peptide bands were transferred to another polyvinylidene difluoride membrane (Westran, Schleicher & Schuell).

Western Blot Analysis—Transblotted membranes were incubated with monoclonal antibodies 2B4 and 1C10. 1C10 recognizes a sequence-specific epitope (GFTGLQGLP, residues 933–941 of the α1(III) helical domain) in denatured α1(IIICB9,7) (19). Following incubation with the primary antibodies, Biotin-SP-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories) was applied followed by incubation with ExtrAvidin®-alkaline phosphatase conjugate (Sigma). The Western blots were developed using bromochloroindolyl phosphate and nitro blue tetrazolium.

Bacterial Collagenase Digestion—Freeze-dried, pepsin-solubilized, 2.2 M NaCl-precipitated human fetal cartilage collagen was reconstituted in 50 mM Hepes, pH 7.2, and passed through the mAb 2B4 affinity column under gravity. The column was sequentially washed with 25 mM Hepes, 125 mM NaCl, pH 7.2, and 25 mM Hepes, 125 mM NaCl, 0.1% Tween 20, pH 7.2. Three pools, 1) the pass-through, 2) the combined washings, and 3) the bound fraction, were assayed by SDS-PAGE, immunoblotting, and reverse-phase HPLC column fractions were analyzed by LC/MS.

Enzyme-linked Immunosorant Assay—Reverse-phase HPLC fractions were diluted in phosphate-buffered saline (1:10, v/v) and coated on polystyrene microtiter plates (Nunc MaxiSorp). After blocking with 1% bovine serum albumin (w/v) in Tris-buffered saline (0.15 M NaCl, 0.02 M Tris, 0.1% Tween 20, pH 7.5), plates were incubated with either 2B4 or 1C10 followed by horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories) to detect bound primary antibodies. Plates were washed with 0.15 M NaCl containing 0.05% Tween 20 (v/v) between each step. Color was developed using 3,3’,5,5’-tetramethylbenzidine dihydrochloride, and absorbance was measured at 450 nm using either a Titertek Multiskan-plus plate reader or a Bio-Tek ELx800 plate reader.

N-terminal Sequence Analysis—Peptides were subjected to N-terminal sequencing on a Porton 2090E gas phase sequencer equipped with online HPLC analysis of phenylthiodyantoin derivatives.
Cross-linking between Collagen Types IX and II

Mass Spectrometry—Stained (Coomassie Blue G-250) protein bands excised from SDS-polyacrylamide gels were subjected to tandem mass spectrometric analysis after in-gel digestion with endoproteinase Asp-N or trypsin using an established procedure for in-gel protein digestion (20). Recovered peptides were analyzed by nanobore LQ (Vydac C8 MS 0.3 mm × 15 cm) with on-line electrospray introduction into an ion-trap mass spectrometer (Thermo Finnigan LCQ Deca XP). Tandem spectra were analyzed by Sequest software to identify peptides searching against a National Center for Biotechnology Information-derived data base of collagen genes (21) to identify tryptic and endoproteinase Asp-N linear sequences. The cross-linked peptide recovered by 2B4 data base of collagen genes (21) to identify tryptic and endoproteinase Asp-N linear sequences. The cross-linked peptide recovered by 2B4 affinity from a bacterial collagenase digest of pepsin-solubilized fetal collagen. The column (agarose A5m column, 1.5 × 170 cm, Bio-Rad) was eluted with 2 M guanidine HCl, 0.65 M Tris-HCl, pH 7.5, and fractions were analyzed by SDS-5% PAGE. The fraction indicated was sufficiently enriched in the components equivalent to those of human collagen (Fig. 1) for Edman N-terminal sequence analysis. The two running sequences shown were derived from the running band after reduction. The upper sequence is from the N terminus of the bovine α1(II) helical domain generated by pepsin (23). The lower sequence is from bovine α1(IX) beginning in the NC2 domain and running into COL1 (23). P* indicates 4-hydroxyproline. DTT, dithiothreitol.

Immunohistochemistry—10-μm-thick frozen sections from 16.7-week human fetal femoral head and adult (25-year-old) human articular cartilage (from femoral head) (Northwest Tissue Center) were fixed in ice-cold acetone for 5 min and treated with 2 mg/ml porcine testicular hyaluronidase (Sigma) in phosphate-buffered saline, pH 7.5, at 37 °C for 30 min. The sections were probed with mAb 2B4 (1:1000 dilution of 1.1 mg/ml) using biotin-labeled goat anti-mouse secondary antibody and developed using the Vectastain Elite ABC and VIP substrate kits (Vector Laboratories). The staining pattern of the tissue was documented with a Nikon Micro photomicroscope equipped with a Photometrics Senysa CCD digital camera.

RESULTS

mAb 2B4, which was prepared against the type II collagen C-telopeptide domain, recognizes the cleaved C terminus of the sequence EKGPDP (17). It also binds with a high affinity to a similar sequence encoded by COL9A1 (National Center for Biotechnology Information accession number gi 3510535, Ref. 22) that occurs at the C terminus of the chain (NKGPDP). Thus, using mAb 2B4 for Western blot analyses of collagen preparations from human cartilage enriched in type IX collagen, the anticipated selective binding to the intact α1(IX) chain and to cross-linked peptide fragments of it was confirmed. Fig. 1 shows 2B4 binding to intact α1(IX) chains in a 4 M guanidine HCl extract of human fetal cartilage (Fig. 1α) and to components of the pepsin-solubilized collagen from the same tissue (Fig. 1b). In Fig. 1b the mobilities of the two strongly immunoreactive bands running above the α1(II) chain, one before and the other shifted lower after disulfide cleavage, had properties suggesting an interaction product between the disulfide-bonded COL1 trimer from type IX collagen and the α1(II) chain. On a duplicate gel/Western using another antibody, 1C10, which is specific for an epitope in the α1(II) chain (see “Experimental Procedures”), the presence of α1(II) in the 2B4 immunoreactive bands was confirmed (data not shown). Fig. 2

a) Fetal

b) Adult

FIG. 2. Immunohistochemical comparison of fetal and adult human cartilage using the 2B4 antibody. Frozen sections of fetal cartilage (a) showed a much stronger, uniform staining of extracellular matrix compared with adult articular cartilage (b), consistent with the known 10-fold higher content of type IX collagen in fetal cartilage. Collagen IX in adult tissue is also seen to be most concentrated pericellularly.

FIG. 3. Molecular sieve chromatography of pepsin-solubilized type II collagen from fetal bovine cartilage. The column (agarose A5m column, 1.5 × 170 cm, Bio-Rad) was eluted with 2 M guanidine HCl, 0.65 M Tris-HCl, pH 7.5, and fractions were analyzed by SDS-5% PAGE. The fraction indicated was sufficiently enriched in the components equivalent to those of human collagen (Fig. 1) for Edman N-terminal sequence analysis. The two running sequences shown were derived from the running band after reduction. The upper sequence is from the N terminus of the bovine α1(II) helical domain generated by pepsin (23). The lower sequence is from bovine α1(IX) beginning in the NC2 domain and running into COL1 (23). P* indicates 4-hydroxyproline. DTT, dithiothreitol.

FIG. 4. SDS-PAGE/Western blot analysis of CNBr-digested fetal human cartilage collagen. a, replicate aliquots of CNBr-digested insoluble collagen matrix were analyzed by SDS-12.5% PAGE and compared by 1C10 Western blot, 2B4 Western blot, and Coomassie Blue staining. mAb 1C10 recognizes an epitope in α1(II) CB9,7 and detects all forms of this peptide, including cross-linked peptides and CNBr partial cleavage products. mAb 2B4 detects the cleaved α1(IX)NC1 sequence cross-linked to CB9,7 as well as incomplete cleavage products running higher in the gel. b, duplicate aliquots of 2B4-affinity purified material from CNBr-digested, pepsin-solubilized fetal cartilage collagen. Lanes 1 and 4, 2B4-bound peptides; lanes 2 and 5, column pass-through; lanes 3 and 6, column wash. Lanes 1–3 were developed with mAb 1C10, and lanes 4–6 were developed with mAb 2B4. In lanes 1 and 4, the lower band (a doublet) is α1(IX) NC1 cross-linked to α1(II)CB9,7. Pepsin cleavage at more than one site in the NC1 domain can explain the doublet (see Fig. 5 legend). The other, much slower band in lanes 1 and 4 is the partial cleavage product α1(II)CB10,9.7 linked to α1(IX)NC1. Peptides were resolved by SDS-12.5% PAGE after disulfide cleavage with dithiothreitol.
shows that fetal cartilage matrix reacts strongly with mAb 2B4, whereas adult cartilage does not.

To confirm this molecular identification by sequence analysis, a large batch of pepsin-solubilized collagen was prepared from fetal bovine cartilage. A similar minor band, which shifted to lower molecular weight on reduction, was detected in a Coomassie Blue-stained gel, and a fraction containing this material was enriched by molecular sieve chromatography (Fig. 3). Sequence analysis of the N termini of the excised, reduced bands gave two running sequences, one matching the N terminus of the \( \alpha_1(II) \) chain generated by pepsin (23) and the other matching a sequence at the N terminus of the COL1 domain of \( \alpha_1(IX) \).

Having established cross-linking between the COL1/NC1 domain of type IX collagen and an \( \alpha_1(II) \) chain, we then sought the exact sites of interaction. We used human cartilage for these experiments; 2B4 does not recognize the bovine \( \alpha_1(IX) \) chain, which presumably differs in sequence at the C terminus from human \( \alpha_1(IX) \) NC1.4 Fig. 4 shows Western blots of CNBr digests of cartilage matrix (Fig. 4a) and of pepsin-extracted collagen after immunopurification on a 2B4 affinity column (Fig. 4b). The main product from the immunopurified pool ran slightly slower than uncross-linked CB9,7. This mobility is consistent with a cross-link between the \( \alpha_1(IX) \) NC1 domain (bearing the 2B4 epitope) and \( \alpha_1(II)/CB9,7 \). The COL9A1 sequence encodes a methionine that would be cleaved by CNBr to release a short C-terminal peptide, QAQQKFGPDP, containing the putative cross-linking lysine and the 2B4 epitope (22).

A mAb 2B4 immunoaffinity column was used to enrich and purify the cross-linked NC1 to the CB9,7 fragment for sequence analysis. Fig. 5a shows the elution profile of the resulting affinity-purified material on reverse-phase HPLC monitored by 220 nm absorbance and pyridinoline fluorescence. Fig. 5, b and c, show Western blot analyses across the fractions using mAbs 1C10 (CB9,7 helical sequence specificity) and 2B4. The results are consistent with a cross-link from \( \alpha_1(IX)/NC1 \) to a site in CB9,7. To confirm this, the remainder of fractions 47–52 was pooled, run on SDS-PAGE, and transblotted to polyvinylidene difluoride membrane, and the Coomassie Blue-positive band corresponding to the 2B4 immunoreactive band was subjected to N-terminal sequence analysis. Essentially two running sequences were observed as shown. One is from the N terminus of \( \alpha_1(II)/CB9,7 \) (GPSGPAGARGIQGPQGPR) and the other from the pepsin-generated NC1 fragment with evidence of two cleavage products, the main version, AGQRAFN, and a lesser form beginning FN. Immunopurification from a CNBr digest of total fetal cartilage collagen (guanidine HCl-insoluble tissue) gave similar results except that the NC1 domain had the N-terminal sequence QAGQRAFN, the product of CNBr cleavage, rather than the two products (minus Q and minus QAGQRA) from pepsin-solubilized collagen. The 2B4 affinity-purified CB fragments were excised from SDS-PAGE and analyzed by in-line HPLC/ion-trap mass spectrometry (LC/MS) after endoproteinase Asp-N or trypsin digestion (data not shown). The results of this peptide analysis confirmed the identity of CB9,7, but the cross-linked peptide itself was not identified in the MS profile of the LC eluent.

Final identification of the two lysine residues involved in the divalent cross-link was confirmed by digesting a pool of pepsin-solubilized type II collagen from fetal human cartilage with bacterial collagenase and using the 2B4 affinity column to isolate the \( \alpha_1(IX)/NC1 \)-containing peptide. The peptide fraction was analyzed by nanobore HPLC/electrospray mass spectrometry.
Fig. 6. Mass spectrometry of the immunopurified cross-linked peptide from bacterial collagenase-digested fetal human cartilage collagen. Pepsin-solubilized collagen was digested with bacterial collagenase and applied to a 2B4 affinity column, and the eluted bound fraction was analyzed by reverse-phase HPLC. The immunoreactive fractions were taken for LC/mass spectrometry. a, upper panel, full MS scan of the main form of the peptide detected in the LC eluent. The lower two panels show fragmentation spectra (MS/MS) of the 4 and 3 charge states of this peptide, which are consistent with the two peptide sequences cross-linked as shown. b, upper panel, full MS scan of a minor form of essentially the same peptide. The lower panel shows the MS/MS fragmentation spectrum of the 3 charge state. The determined molecular masses match structures based on a keto-amine cross-link derived from the two lysine residues which in c has lost two molecules of water and a proton compared with d. The keto-amine is the known cross-link from a hydroxylysine aldehyde (presumably in α1(IX)NC1) and a hydroxylysine residue (at Lys-930 in α1(II)CB9,7). The pyrrole dehydration product (c) is speculative but fits the mass. P* indicates 4-hydroxyproline.

etry. Fig. 6 summarizes the results of this analysis, which identifies the basic composition of the two peptides cross-linked through their lysine residues. The MS results showed the presence of two forms of essentially the same cross-linked peptide that differed in mass of the cross-linking residue by two water molecules. One form matches the mass of the open chain keto-
amino cross-link, hydroxylysino-ketonorleucine. The other, minus 37 mass units, equal to two water molecules and an additional proton, would fit the pyrrole structure shown (Fig. 6d), but other condensed structures are possible to explain this mass difference.

**DISCUSSION**

The Western blot results in Fig. 1a establish the specificity of mAb 2B4 for the human α1(IX) chain and lack of recognition of intact α1(II) chains from the same cartilage extract. These and other observations indicate binding of 2B4 to the expressed natural C terminus of the α1(IX) chain but not to the same pentapeptide cross-linking sequence (KGDPD) in the α1(II) C-telopeptide domain. The latter is recognized only after it becomes C-terminal through proteolysis, for example through degradation by matrix metalloproteinases as has been reported (17). This specificity was important to establish since 2B4 was raised to recognize the proteolytic neoeptope in degradation products of type II collagen (17), potentially explaining the novel immunoreactive bands in Fig. 1b. It seemed more likely, therefore, that α1(IX)/NC1 was responsible for 2B4 binding to these components from fetal cartilage. Adult human cartilage did not reveal such bands or significant immunoreactivity of α1(II) chains when similarly analyzed (not shown). The results of Fig. 2 further support this specificity with a lack of significant staining of adult cartilage when 2B4 was used for immunohistochemistry on frozen sections, whereas fetal cartilage stained strongly throughout the matrix. These findings indicate that 2B4 binds to a native epitope in type IX collagen but not in undegraded type II collagen and are consistent with the much higher content and uniform distribution of type IX collagen in fetal cartilage matrix (10% of total collagen) compared with adult cartilages (1% or less of total collagen (6, 24)).

The recovery of a similar pool of II-to-IX cross-linked chains from fetal bovine cartilage (Fig. 3) shows that this property is not restricted to human fetal tissue, and the sequencing results confirmed the component identities suspected from SDS-PAGE mobilities and Western blotting. The predicted molecular identity was of an α1(IX)/COL1 domain with its NC1 sequence still attached to which an α1(II) chain was cross-linked. Incomplete cleavage of the NC1 domain by pepsin would explain the 2B4 immunoreactivity of both the minor molecular species (α1(II) chains cross-linked to NC1 with COL1 still attached) and α1(II) chains (those having a cleaved NC1 domain cross-linked to them). The latter short sequence would not significantly alter the mobility of α1(II) on SDS-PAGE, being of similar length to the pepsin-cleaved α1(II) telopeptides. This conclusion was confirmed by the results of analysis of CNBr peptides before and after immunoaffinity purification (Figs. 4 and 5). The lack of significant fluorescence in the cross-linked peptide (Fig. 5), the N-terminal sequencing results, and the mass spectral data (Fig. 6) taken together identify the site of cross-linking between two lysines and the cross-link as a divalent keto-amine with no evidence of pyridinoline (trivalent) cross-link formation at this locus from α1(IX)/NC1. The pyridinoline cross-links found in type IX collagen were all formed between collagen II telopeptides and the COL2 domain (4).

These results establish a major, previously unrecognized site of intermolecular cross-linking between types IX and II collagen molecules in cartilage. Compared with known cross-linking sites in type IX collagen (4–7), this one is unique in linking the molecule to the triple helix of type II collagen. The earlier studies (4–7) defined sites of linkage from type II collagen telopeptides to the triple helical COL2 domain of type IX collagen. Based on evidence that COL1, the triple helical domain most conserved in sequence across all FACIT collagen family members (25, 26), together with the NC1 domain and its conserved cysteine pair is physically responsible for the interaction of collagen IX molecules with fibril surfaces (26), we speculate that the identified covalent bond from α1(IX)/NC1 to type II collagen is the first cross-link to form (both in the interaction pathway and in evolutionary terms).

This is shown in a molecular model of the proposed interaction and packing of collagen IX molecules on the collagen II fibril surface (Fig. 7) that best fits the spatial distribution of all the sites of covalent cross-linking. It is similar to one of a series of models proposed by Miles et al. (27) on theoretical grounds and before the current NC1 link to type II collagen was identified. In it, the COL1 domain folds back through NC2 and before the current NC1 link to type II collagen was identified. This arrangement gives the correct linear distances and the required antiparallel relationship between COL2 and the type II helix to accommodate both the new cross-link and those reported earlier (4, 6, 7). Particularly notable is the correct linear juxtaposition of collagen IX molecules on the fibril relative to each other (staggered axially by D (67 nm) the distance by which collagen molecules are staggered in banded fibrils) to allow IX-to-IX intermolecular cross-links to form between α3(IX)/NC1 and the COL2 N terminus. The previously reported interaction sites for α3(IX)/NC1 on
COL2 are in α1(IX) and α3(IX) (6, 7, 28). The abundance of IX-to-IX cross-links can be explained, therefore, by an interaction of adjacent type IX collagen molecules along the fibril surface. The periodicity of collagen II molecules acts as a template for this interaction. As shown (Fig. 7) the model also has the potential to allow interfibrillar links between collagen IX molecules when the opportunity arises between intersecting nascent fibrils coated with collagen IX molecules. Proving the existence of such interfibrillar bonds will be a challenge, but immunoelectron microscopic observations have previously been interpreted as showing evidence for focal concentrations of collagen IX at sites of fibril intersection in the matrix of developing cartilage (29) as well as a periodic distribution along thin fibrils (14).

Each chain of the vertebrate collagen IX molecule has evolved its own distinctive pattern of cross-linking interactions in the collagen heteropolymer. The α1(IX) chain (COL9A1) is probably the ancestral form. Deletion of this gene in mice produced a functional knock-out of collagen IX protein despite normal expression of COL9A2 and COL9A3 mRNA (30). There is also evidence for a homolog of α1(IX) in a basal chordate (31). It seems likely that α2(IX) (COL9A2) and α3(IX) (COL9A3) in higher vertebrates are the result of gene duplications and subsequent mutations that have added and altered functional cross-linking properties. With regard to the cross-link to type II collagen based on α1(IX)NC1 identified here, it is notable that the α3(IX)NC1 sequence was shown to be cross-linked exclusively to the COL2 domain of a second type IX molecule (6). Is this an evolved function from that of ancestral α1(IX)NC1? We do not know, but we do have preliminary evidence that a lysine residue in α2(IX)NC1 can also link to type II collagen at the Lys-930 locus. If so, the NC1 domains of all three gene products have retained a cross-linking function. Using synthetic peptides, it was previously shown that the NC1 domains of type II collagen at the Lys-930 locus.5 If so, the NC1 domains of type IX collagen (25) and type II collagen (6). Is this an evolved function from that of ancestral α1(IX)NC1? We do not know, but we do have preliminary evidence that a lysine residue in α2(IX)NC1 can also link to type II collagen at the Lys-930 locus. If so, the NC1 domains of all three gene products have retained a cross-linking function. Using synthetic peptides, it was previously shown that the NC1 domains of type II collagen at the Lys-930 locus.5 If so, the NC1 domains of type IX collagen (25) and type II collagen (6). Is this an evolved function from that of ancestral α1(IX)NC1?

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