STRUCTURE, MOLECULAR BIOLOGY, AND PATHOLOGY OF COLLAGEN

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Edited by Raul Fleischmajer, Bjorn R. Olsen, and Klaus Kühn

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STRUCTURE, MOLECULAR BIOLOGY, AND PATHOLOGY OF COLLAGEN [a]

Editors and Conference Organizers
RAUL FLEISCHMAJER, BJORN R. OLSEN, AND KLAUS KÜHN

CONTENTS

[a] The papers in this volume were presented at a conference entitled Structure, Molecular Biology, and Pathology of Collagen, which was held by the New York Academy of Sciences in Bethesda, Maryland on April 3-5, 1989.

Part II. Structure and Regulation of Collagen Genes

Part III. Collagen Biosynthesis and Supramolecular Assembly

Part IV. Developmental Biology of Collagen

Part V. Extracellular Matrix and Neurobiology

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Preface

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This volume contains the proceedings of the second New York Academy of Sciences Conference on Collagen, held on April 3-5, 1989 in Bethesda. We hope that this volume will be a useful reference source book for individuals, postdoctoral fellows, and others who are starting their scientific work in the area of extracellular matrix biology; for clinicians interested in diseases affecting connective tissues; and for established investigators in the need of updating their understanding of collagen molecular biology. Several chapters discuss the structure and molecular cloning of the nonfibrillar collagens. The discovery of these collagens coupled with data on other matrix molecules reveal an unexpected complexity of molecular interactions in the extracellular matrix. These complex interactions may play a fundamental role in the assembly of extracellular matrices during development. Therefore, this book will be of interest to developmental biologists.

Several chapters are devoted to a rapidly developing new field—namely, the regulation of collagen gene expression by specific factors (i.e., transforming growth factor-beta, interferon, and interleukin-1). Another emerging area is the role of certain components of the extracellular matrix such as laminin, merosin, and cytotactin in brain and nerve development. There are numerous contributions concerned with connective tissue pathology, particularly involving molecular defects of genetic disease and the use of transgenic mouse models for collagen defects. It is our hope that this volume will be instructive and will open new avenues of research to further improve our understanding of the structure and function of the extracellular matrix.

The Structure and Macromolecular Organization of Type IX Collagen in Cartilage[a]

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INTRODUCTION

Type IX collagen is a major connective tissue component of all cartilagenous tissues.[1-3] It is assembled from three genetically distinct chains to give a single molecule of chain composition α1(IX) α2(IX) α3(IX).[4,5] The structure of the molecule is now largely determined, and the complete amino acid sequence is available for the chicken α1(IX) and α2(IX) chains.[6] In addition, partial amino acid sequences are available for the human, bovine, and rat α1(IX) chains.[6] A model for the structure of type IX collagen is presented in FIGURE 1. This model was derived not only from the primary structure of the α1(IX) and α2(IX) chains, but also from electron microscopic observations of the intact molecule after rotary shadowing.[7] The latter studies show that the NC3 domain forms a hinge of variable angle and that the NC4 domain forms a compact knob. Further experiments using rotary shadowing of intact collagen fibrils, performed independently by two laboratories, show that type IX collagen is present on the surface of fibrils of type II collagen.[8] The COL3 and NC4 domains of each molecule were observed to project periodically from the surface of the fibril and were identified by the binding of monoclonal antibody 4D6 at the location of its epitope (see FIG. 1). This is illustrated in FIGURE 2A and 2B.

[a] This work was supported by Research Grant AM 30481 (to R.M.) from the National Institutes of Health, and by the Medical Research Council of Canada (MT-7796) and the Shriners of North America (to M.V.D.R.).

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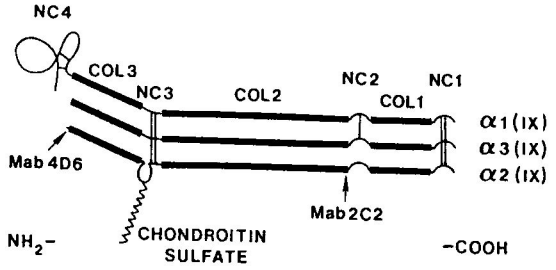

FIGURE 1. A model for the structure of type IX collagen. Note that the molecule contains three collagenous domains (COL1-COL3) and four noncollagenous domains (NC1-NC4). Interchain disulfide bridges are located at NC1, NC2, and NC3. The α1(IX) chain is longer than the other two chains, with a large NC4 domain. A single chondroitin sulfate chain is located at the NC3 domain of the α2(IX) chain. The locations of the epitopes for monoclonal antibodies 2C2 and 4D6, which recognize native but not denatured type IX collagen, are also shown.

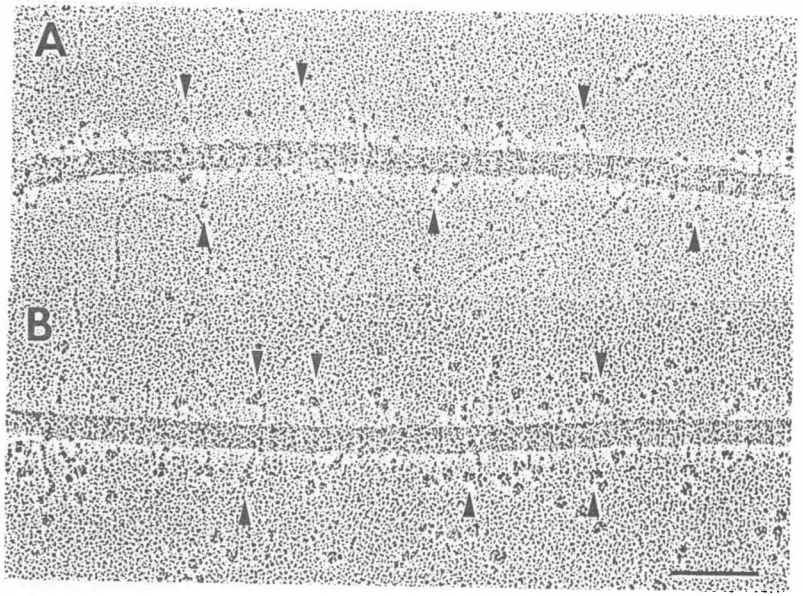

FIGURE 2. Rotary shadowing observations of isolated collagen fibrils from chicken hyaline cartilage. **A:** Note the projections and terminal knobs of the COL3 and NC4 domains that arise from the surface of the fibrils (*arrows*). **B:** The sample was incubated in the presence of monoclonal antibody 4D6 (50 μg/ml) before rotary shadowing. Note the binding of the antibody away from the surface of the fibril (*arrows*). This is the expected location of the epitope for 4D6, as previously determined for isolated type IX molecules. Isolation of the fibrils and rotary shadowing were performed as described previously.[8] *Bar* = 100 nm.

Isolation of Hydroxypyridinium-Containing Cross-Link Peptides between Type IX and Type II Collagen

To examine further the relationship between type II and type IX collagen, initial analyses were made of the pepsin-resistant fragments of the type IX molecule for the presence of the characteristic activation and emission spectra of the fluorescent hydroxypyridinium cross-link.[9,10] Further separation of the tryptic peptides by reverse phase HPLC resulted in the isolation of a peptide with a double sequence arising from the α2(IX) chain and the amino telopeptide of type II collagen. These previous results demonstrate that a lysine-derived cross-link occurs at the amino end of the COL2 domain of the α2(IX) chain and the amino telopeptide of type II collagen, and provides further evidence for type IX collagen being linked to the surface of fibrils of type II collagen. We now report the isolation of a second hydroxypyridinium-

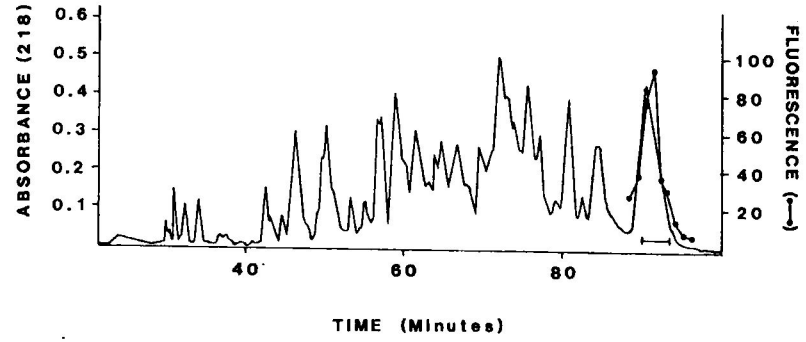

FIGURE 3. Fractionation of the tryptic peptides derived from the fragments C2 and C5 by reverse phase HPLC (C-18). Note that the last peak to elute contains all of the fluorescence. The ion pairing agent was 9 mM trifluoroacetic acid, and the column was eluted with a gradient of acetonitrile (0 → 45%) over a total time of 99 min. Earlier experiments showed that the fluorescent peak is derived only from the C5 fragment and is not contaminated by peptides from the C2 fragment (Shimokomaki and Mayne, unpublished result).

containing cross-link, which involves the α3(IX) chain and the amino telopeptide of type II collagen. It was found that the C5 fragment of the α3(IX) chain obtained after pepsin digestion showed the typical fluorescent activation and emission spectra of the hydroxypyridinium cross-link. Furthermore, separation of the tryptic peptides by reverse phase HPLC showed that all of the fluorescence was present in a single peak, which eluted very late from the column (see FIG. 3). Amino acid sequencing of the peptide showed a double sequence derived from the α3(IX) chain, with approximately 25% derived from the amino telopeptide of type II collagen. The sequence of the α3(IX) chain at this site is shown in FIGURE 4 and indicates that the cross-link must be in the COL2 domain, close to the same site as that for the α2(IX) chain. However, we have been unable to identify positively the lysine residue that is involved in the cross-linking reaction. The results provide for the first time the complete amino acid sequence for all three chains in the vicinity of the NC3 domain, and show that the α2(IX) chain has five additional amino acids, which contain the serine residue

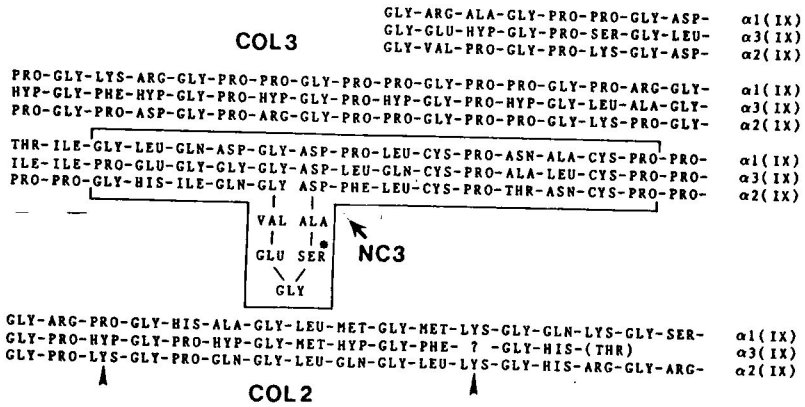

FIGURE 4. Amino acid sequences of the three chains of type IX collagen in the vicinity of the NC3 domain. The sequences of the α1(IX) and α2(IX) chains were obtained from Reference 10. Note that the α2(IX) chain contains five additional amino acids and that the serine residue (*) is the site of glycosylation giving rise to a chondroitin sulfate chain. Lysine residues that potentially may be involved in the formation of a hydroxypyridinium cross-link for the α2(IX) chain are shown by *arrows*. The location of the lysine residue that forms the cross-link for the α3(IX) chain has not been identified.

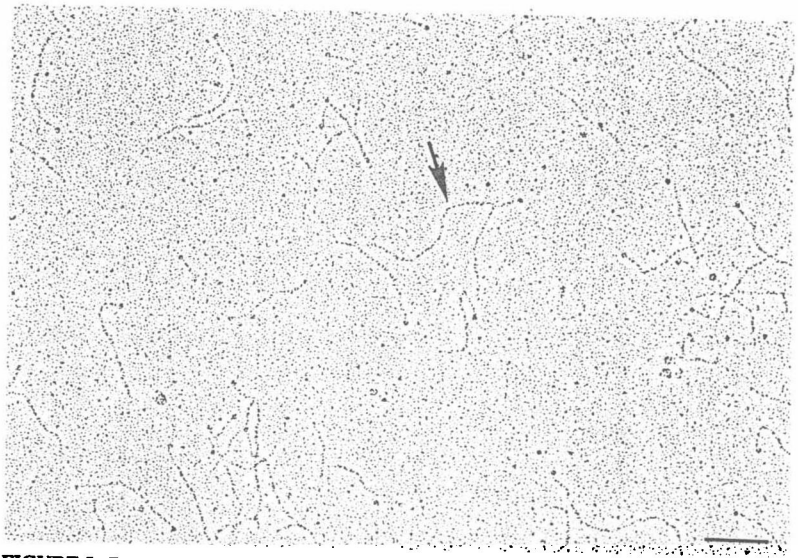

FIGURE 5. Rotary shadowing of a purified preparation of type IX collagen isolated after DEAE cellulose chromatography. Note the variable angle of the type IX molecules at the NC3 domain, and the consistent presence of the knob of the NC4 domain. The *arrow* shows a molecule in which an interstitial collagen molecule is apparently cross-linked to a type IX molecule at the NC3 domain. *Bar* = 100 nm.

to which the chondroitin sulfate chain is attached.[11,12] Whether this difference in length of the three chains at NC3 is also responsible for the formation of a flexible hinge in the molecule at this site is not known.

These results suggest that there is one major site of cross-linking between type IX and type II collagen that is located in COL2 close to NC3 and involves both the α2(IX) and α3(IX) chains. We have not found any evidence for hydroxypyridinium-containing cross-links in any of the other pepsin-resistant fragments of type IX collagen, nor have we found evidence for lysine-derived cross-links involving the carboxyl telopeptide of type II collagen and a pepsin-resistant fragment of type IX collagen.

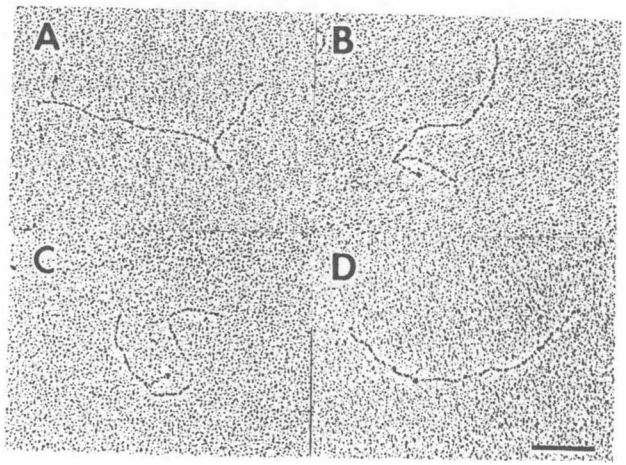

FIGURE 6. Electron micrographs of selected molecules showing an apparent cross-link between type IX collagen and an interstitial collagen molecule. In the molecule shown in panel D the COL3 and NC4 domains have probably folded back completely onto the COL2 domain as observed previously.[7] *Bar* = 100 nm.

Rotary Shadowing Observations of Type IX Collagen Preparations

Further evidence for the importance of crosslinking close to the NC3 domain was found by examination of highly purified preparations of type IX collagen obtained from the sternal cartilage of chickens made lathyritic by giving β-aminopropionitrile in the drinking water. Type IX collagen was isolated as described previously,[13] except that a fraction was prepared that eluted on DEAE cellulose between 0.2 M and 0.3 M NaCl, 0.05 M Tris HCl, pH 7.5. This fraction was subsequently dialyzed against 6.0 M urea, 0.01 M Tris HCl, pH 8.0 and eluted from DEAE cellulose between 0.12 M NaCl and 0.25 M NaCl. Examination of these preparations by SDS PAGE always showed a faint band at the location of the α1(II) chain in addition to type IX collagen. Examination of these preparations by rotary shadowing showed images as illustrated in FIGURE 5. FIGURE 6 shows a panel of such molecules, in which type IX molecules are apparently cross-linked to type II collagen at or close to NC3.

DISCUSSION

The present results provide additional information describing the interaction between type II and type IX collagen to form the fibrils of hyaline cartilage. It appears that each fibril has a coating of type IX collagen with the COL3 and NC4 domains projecting from the surface. Potential functions for type IX collagen may therefore be (1) to limit the lateral growth of fibrils and perhaps to influence fibril diameter; (2) to prevent fibril-to-fibril interactions and assist in maintaining a network of fibrils within which the proteoglycan aggregates are constrained; (3) to interact directly with the proteoglycans, potentially via the NC4 domain. At present, there is no evidence in support any of these possibilities. However, a potential insight into the function of type IX collagen may come from the recent observations that the molecule is missing the NC4 domain of the α1(IX) chain in the primary chick cornea[14,15] and adult chicken vitreous.[16] Tissue-specific forms of type IX collagen therefore exist and in the cornea arise by use of an alternative promoter during mRNA synthesis.[17]

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REFERENCES

1. VAN DER REST, M. & R. MAYNE. 1987. Type IX collagen. *In* Structure and Function of Collagen Types. R. Mayne & R. E. Burgeson, Eds.: 195-221. Academic Press. Orlando, FL.
2. OLSEN, B. R., D. GERECKE, M. GORDON, G. GREEN, T. KIMURA, H. KONOMI, Y. MURAGAKI, Y. NINOMIYA, I. NISHIMURA & S. SUGRUE. 1989. A new dimension in the extracellular matrix. *In* Collagen: Biochemistry, Biotechnology and Molecular Biology, Vol. 4. B. R. Olsen & M. Nimni, Eds. CRC Press. Boca Raton, FL. In press.
3. BRUCKNER, P., M. MENDLER, B. STEINMANN, S. HUBER & K. H. WINTERHALTER. 1988. The structure of human collagen type IX and its organization into fetal and infant cartilage fibrils. J. Biol. Chem. **263:** 16911-16917.
4. VAN DER REST, M., R. MAYNE, Y. NINOMIYA, N. G. SEIDAH, M. CHRETIEN & B. R. OLSEN. 1985. The structure of type IX collagen. J. Biol. Chem. **260:** 220-225.
5. MAYNE, R., M. VAN DER REST, Y. NINOMIYA & B. R. OLSEN. 1985. The structure of type IX collagen. Ann. N. Y. Acad. Sci. **460:** 38-46.
6. NINOMIYA, Y., P. CASTAGNOLA, D. GERECKE, M. GORDON, O. JACENKO, P. LUVALLE, M. MCCARTHY, Y. MURAGAKI, I. NISHIMURA, S. OH, N. ROSENBLUM, N. SATO, S. SUGRUE, R. TAYLOR, G. VASIOS, N. YAMAGUCHI & B. R. OLSEN. 1989. Molecular biology of collagens with short triple helical domains. *In* Collagen Genes: Structure, Regulation and Abnormalities. C. D. Boyd, P. Byers & L. Sandell, Eds. Academic Press. Orlando, FL. In press.
7. IRWIN, M. H., S. H. SILVERS & R. MAYNE. 1985. Monoclonal antibody against chicken type IX collagen: Preparation, characterization and recognition of the intact form of type IX collagen secreted by chondrocytes. J. Cell Biol. **101:** 814-823.

8. VAUGHAN, L., M. MENDLER, S. HUBER, P. BRUCKNER, K. WINTERHALTER, M. H. IRWIN & R. MAYNE. 1988. D-periodic distribution of collagen type IX along cartilage fibrils. J. Cell Biol. **106:** 991-997.
9. EYRE, D. R., S. APON, J-J. WU, L. H. ERICSSON & K. A. WALSH. 1987. Collagen type IX: Evidence for covalent linkages to type II collagen in cartilage. FEBS Lett. *220:* 337-341.
10. VAN DER REST, M. & R. MAYNE. 1988. Type IX collagen proteoglycan from cartilage is covalently cross-linked to type II collagen. J. Biol. Chem. **263:** 1615-1618.
11. MCCORMICK, D., M. VAN DER REST, J. GOODSHIP, G. LOZANO, Y. NINOMIYA & B. R. OLSEN. 1987. Structure of the glycosaminoglycan domain in the type IX collagen-proteoglycan. Proc. Natl. Acad. Sci. USA **84:** 4044-4048.
12. HUBER, S., K. H. WINTERHALTER & L. VAUGHAN. 1988. Isolation and sequence analysis of the glycosaminoglycan attachment site of type IX collagen. J. Biol. Chem. **263:** 752-756.
13. VAUGHAN, L., S. HUBER, M. CHIQUET & K. H. WINTERHALTER. 1987. A major, six-armed glycoprotein from embryonic cartilage. EMBO J. **6:** 349-353.
14. SVOBODA, K. K., I. NISHIMURA, S. P. SUGRUE, Y. NINOMIYA & B. R. OLSEN. 1988. Embryonic chicken cornea and cartilage synthesize type IX collagen molecules with different amino-terminal domains. Proc. Natl. Acad. Sci. USA **85:** 7496-7500.
15. NISHIMURA, I. & B. R. OLSEN. 1988. Different forms of type IX collagen in cartilage and cornea due to the use of alternative promoters in the α1(IX) gene. J. Cell Biol. **107:** 232a.
16. WRIGHT, D. W. & R. MAYNE. 1988. Vitreous humor of chicken contains two fibrillar systems: An analysis of their structure. J. Ultrastruct. Mol. Struct. Res. **100:** 224-234.
17. NISHIMURA, I., Y. MURAGAKI, M. HAYASHI, Y. NINOMIYA & B. R. OLSEN. 1990. Tissue specific expression of type IV collagen. Ann. N.Y. Acad. Sci. This volume.

The Structure of Type XII Collagen[a]

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INTRODUCTION

Type XII collagen was initially discovered by isolation and sequencing of cDNAs.[1] The first type XII cDNA, pMG377, was isolated from a 17-day chick embryo tendon library, and defined two regions of the α1(XII) collagen chain: a non-triple-helical carboxy-terminal domain, called NC1, and an adjacent triple-helical domain, COL1 (FIG. 1). The deduced amino acid sequence of COL1 was strikingly similar to the COL1 domain of type IX collagen chains, which are found in cartilage. The presence of cysteinyl residues at the end of COL1 and five residues into NC1 of α1(XII) were also found in α1(IX) and α2(IX) polypeptide chains.[2,3] Alignment of the chains using these cysteinyl residues demonstrated that two imperfections in the triple helix of α1(XII) fell in the same relative positions in α1(IX). The overall amino acid sequence similarity between the α1(IX) and the α1(XII) polypeptides was about 50%.[1]

Multidomain Structure of Type XII Collagen Chains

Subsequently, two additional overlapping α1(XII) cDNAs were isolated from a λgt10 chick tendon cDNA library, and their sequences have defined three more domains in the α1(XII) polypeptide chain: a second triple-helical domain and two additional non-triple-helical domains.[4] When compared to α1(IX) and α2(IX) chains, the deduced amino acid sequence of α1(XII) is similar to α1(IX) in restricted regions,

[a]This work was supported by National Institutes of Health Grants AR36819 and AR36820, the Canadian MRC Grant MT-7796, and the Shriners of North America.

as shown by the shaded areas in FIGURE 1. These regions will be discussed starting at the carboxyl end. NC1 and NC2 contain conserved cysteinyl residues in both α1(XII) and α1(IX), but for the most part the similarity between the two chains that is seen at the carboxyl end is in their COL1 domains. Working toward the amino ends of the molecules, one finds, perhaps surprisingly, that the sequence of the COL2 domain of α1(XII) has no similarity to any region of COL2 or COL3 of α1(IX) or α2(IX). This domain could not be compared to the α3(IX) COL2 and COL3, since complete amino acid sequences for the α3(IX) chain are not yet available. Because the cDNAs are not complete copies of the α1(XII) mRNA, the sequence of most of the amino-terminal domain, called NC3, has not yet been determined. However, the cDNAs do encode about 300 amino acid residues at the carboxyl end of NC3. Within this domain, the carboxyl most 26 residues contain 4 cysteines, and show no similarity to any portion of type IX collagen chains. However, the adjacent 213 residues in α1(XII) contain an additional 4 cysteines and are quite similar to 209 of the 243 residues in the amino-terminal globular domain NC4 of α1(IX) [α2(IX), and α3(IX) chains lack this domain].[5] On the amino acid level, these regions share about 34% identity; on the nucleotide level, 46%. The four conserved cysteinyl residues within the 213 residue region of α1(XII) are found in analogous positions within the 209 residues of the α1(IX) NC4.[5]

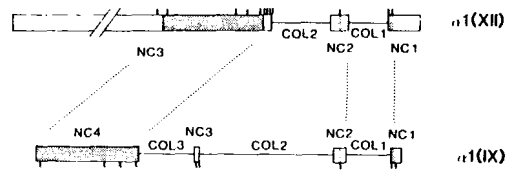

FIGURE 1. Schematic diagram comparing α1(XII) (*top*) and α1(IX) (*bottom*) collagen polypeptide chains. *Shading* of non-triple-helical regions indicates areas of similarity between the chains. Sticklike *projections* indicate the positions of cysteinyl residues.

Partial Homology between Type XII and IX Collagen Genes

The similarities between portions of α1(XII) and type IX collagen chains suggest partial homology between types XII and IX collagen genes. This homology is clear from the similarities in exon sizes that exist between the α1(IX) and α1(XII) genes. These similarities further show that these genes are distinctly different from fibrillar collagen genes. The gene encoding the α2(IX) chain has been completely characterized, and is therefore used for comparison.[6] At the 3′ end of this gene are four exons that are similar to exons in the α1(XII) gene (FIG. 2). The 3′-most exon of the α2(IX) gene is a 400-bp exon that encodes all of the NC1 domain and 40 ⅔ amino acid residues of the COL1 domain.[7] In the α1(XII) gene, the 3′-most exon has not yet been isolated. However, an exon encoding part of NC1 and 40 ⅔ amino acid residues of COL1 has been sequenced, and this sequence demonstrates that the exon/intron junctions at the 5′ end of this exon in the type XII gene and exon 1 in the α2(IX) gene are in the same positions relative to the cDNA sequences.

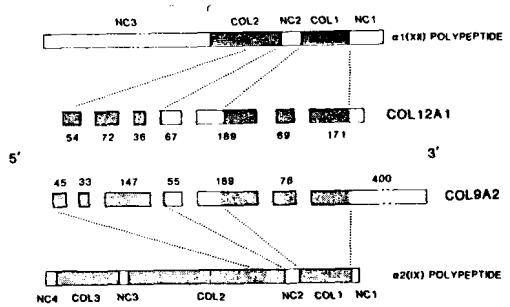

FIGURE 2. The *upper panel* shows the α1(XII) collagen polypeptide and partial exon structure of the α1(XII) collagen gene, COL12A1. The *lower panel* shows the α2(IX) collagen polypeptide and partial exon structure of the α2(IX) collagen gene, COL9A2. *Dotted lines* indicate correspondence between exons and polypeptide regions. *Shaded areas* indicate triple-helical sequence domains.

Proceeding in an upstream direction, the next exon in each gene corresponds to an interior region of COL1, encoding only triple-helical sequences. Following this exon is a 189 bp exon in both genes that encodes the remainder of the COL1 domain and part of the NC2 domain. The next upstream exon in both genes encodes the rest of NC2 and 3 amino acid residues of the COL2 domains. The similarities of the cDNA sequences, the primary structure of the polypeptides, and the gene structures demonstrate that portions of types IX and XII collagens are indeed homologous and are likely to have a common ancestor.

Is Type XII Collagen a Fibril-Associated Molecule?

The homology between the structures of types IX and XII collagen suggest that type XII, like type IX, may be associated with collagen fibrils. For example, type IX collagen is cross-linked to type II collagen molecules in cartilage fibrils,[8,9] with the amino-terminal globular domain of the α1(IX) projecting from the fibril surface.[10] Since the cDNAs offer no information on the distribution and role of intact type XII collagen molecules in tissues, the protein was isolated and characterized.

To examine the tissue distribution of type XII collagen, a monoclonal antibody was made to a synthetic peptide, whose sequence was deduced from the cDNA sequence of NC1.[11] It was demonstrated that the monoclonal antibody specifically recognized the polypeptide encoded by the cDNA in the following way: Chick embryo tendon extracts were digested with cyanogen bromide. The resulting peptides were separated by antibody affinity chromatography. Amino acid sequence analysis of the immuno-affinity purified peptide agreed perfectly with the cDNA-deduced amino acid sequence.[12] The monoclonal antibody reacts with native and denatured type XII collagen, and has been used for immunohistochemistry of various chick tissues.[11]

The immunohistochemical results indicate that the type XII collagen epitope is present in many, but not all, tissues containing type I collagen.[11] The most notable

exception is bone matrix. Antibodies to type I collagen react with the bone matrix and the periosteum, but the type XII collagen antibody reacts mostly with the tissue surrounding the bone and with the bone matrix only where it is close to the periosteum.[11] While the colocalization of type XII collagen with type I collagen is suggestive of an interaction similar to that of types IX and II collagens, localization of type XII collagen on the surface of type I-containing fibrils has not yet been demonstrated.

The Molecular Structure of Type XII Collagen

On Western blots of 1-M NaCl chick tendon extracts, the antibody reacts with a 220 kDa polypeptide, and with a band of much greater molecular weight, compared to globular molecular weight markers (FIG. 3).[11] To separate the type XII collagen from other components of the tendon extract, the extract was subjected to chromatography through Sephacryl S-500.[12] Each fraction eluted from the column was tested for immunoreactivity to the antibody. The type XII collagen-containing fractions were analyzed by SDS PAGE, as shown in FIGURE 4.[12] Lane 1 shows that the major polypeptide species in the immunoreactive fraction is 220 kDa in size; the minor species is larger than 250 kDa. When the fraction containing the two polypeptides is digested by collagenase, a single band at 190 kDa is observed (lane 2). This large collagenase-resistant fragment corresponds to the NC3 domain, which must contain about 1600 amino acid residues of non-triple-helical character. Chondroitinase ABC digestion does not alter the mobility of the SDS PAGE bands, indicating that, unlike type IX collagen, type XII is not a proteoglycan as well as a collagen.[12]

The Sephacryl S-500 column has also been run under nondenaturing conditions.[12] The immunoreactive fractions containing the intact type XII collagen molecules were examined by rotary shadowing and electron microscopy. The images obtained are quite different from the rotary shadowing image of type IX collagen (FIG. 5).[13] Type IX molecules have a globule attached to the shorter portion of a kinked collagenous tail (FIG. 5A). The structures observed in the type XII fraction have a 75-nm tail

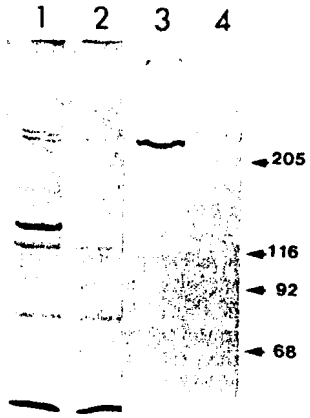

FIGURE 3. SDS PAGE (under reducing conditions) of a 1-M NaCl extract from 17-day chick embryo tendons. *Lane* 1: Coomassie blue staining of the tendon extract. *Lane* 2: Coomassie blue staining of the tendon extract treated with bacterial collagenase before electrophoresis. *Lanes* 3 and 4: Identical samples as in lanes 1 and 2, respectively, transferred to a polyvinylidene difluoride membrane and immunostained with the monoclonal antibody to α1(XII). The major immunoreactive band migrates at 220 kDa; the minor, close to the top of the gel (modified from Reference 11).

attached through a central globule to three thicker 60-nm "fingers."[12] The 75-nm tail frequently shows a kink (FIG. 5B). When the type XII collagen fraction is denatured and allowed to renature, the images seen in FIGURE 5C are observed. These molecules are structures that have a lollipop appearance: a 75-nm tail attached to a large globule.

We believe the rotary shadowing images observed with type XII collagen-containing fractions can be explained based on a model of the polypeptide as shown in FIGURE 6. The 75-nm tail corresponds to the triple-helical domains COL1 and COL2. Consistent with this are the results of analysis of the pepsin-resistant fragments obtained from pepsin extracts of chick embryo tendons. Unreduced pepsin-resistant fragments of type XII collagen migrate on SDS PAGE as 46-kDa and 32-kDa bands.[14] When reduced, the fragments migrate as bands with molecular masses of 16 kDa and 10 kDa.[14] Partial amino acid sequence analysis of these fragments agrees perfectly with the sequences predicted by the cDNA; the 10-kDa fragment

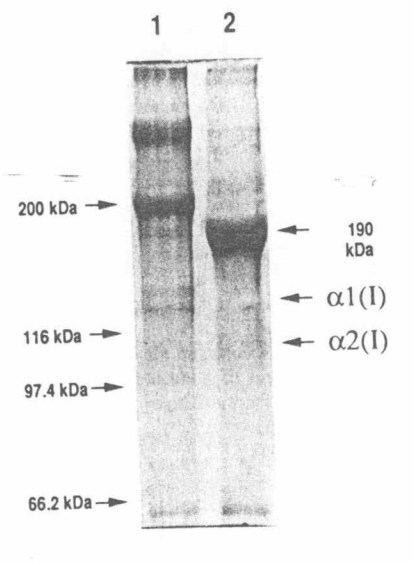

FIGURE 4. SDS PAGE (under reducing conditions) of immunoreactive fractions from a Sephacryl S-500 chromatography column (run under denaturing conditions for fractionation of 1-M NaCl chick tendon extracts) stained with Coomassie blue. *Lane* 1 shows the two major bands in this immunoreactive fraction: one band is 220 kDa; the other is halfway between the 220-kDa band and the top of the gel. *Lane* 2 shows the products of this fraction after digestion with bacterial collagenase (modified from Reference 12).

represents the COL1 domain, and the 16-kDa fragment corresponds to COL2. The sequence analysis also indicates that type XII collagen is a homotrimer of three α1(XII) chains.[12] Since the total number of amino acid residues in COL1 and COL2 combined is 255, the 0.289-nm rise per residue typical of a triple-helical conformation would result in a calculated 73.7-nm triple-helical tail for type XII collagen. This is in very good agreement with the observed 75-nm tail. In the rotary shadowing image, the kink in the tail divides it into pieces with relative sizes of about 1.3 to 1. This is consistent with the COL2 domain being 152 residues and the COL1 domain being 103. The three "fingers" therefore must result from the NC domains of the three chains in the homotrimer. As mentioned, the NC3 domain is approximately six times larger than the combined collagenous regions, yet in the intact molecule the NC3 domains fold into a conformation that produces three thick 60-nm projections, while the relatively small COL1 and COL2 domains generate a thin 75-nm rod.

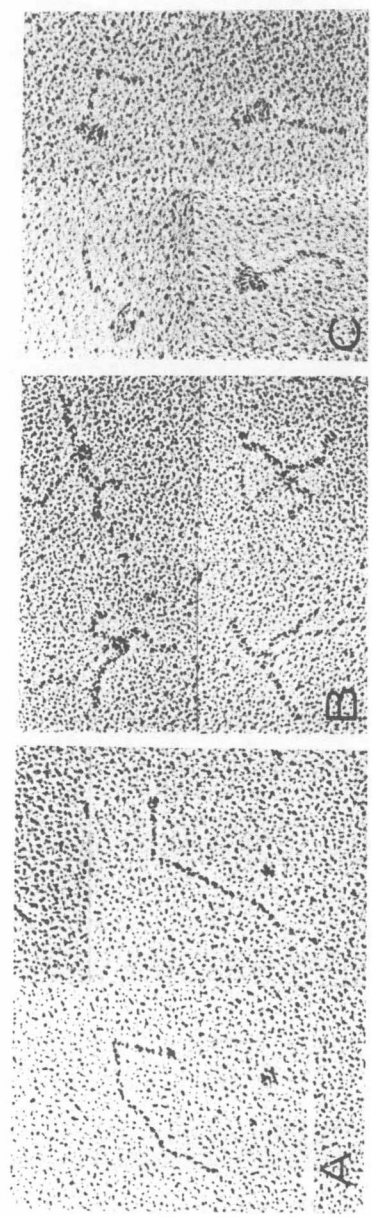

FIGURE 5. Rotary shadowing images of types IX and XII collagen. Panel A: type IX images (courtesy of Dr. R. Mayne). Panel B: image of native material isolated from the Sephacryl S-500 column run under nondenaturing conditions (modified from Reference 12). Panel C: image of denatured and partially renatured material from the immunoreactive fractions of the Sephacryl S-500 column, run under denaturing conditions (modified from Reference 12).

CONCLUSIONS

In summary, types IX and XII collagen are members of a subclass of collagen proteins, but there are some intriguing differences between the molecules. Type IX collagen is a heterotrimer of three different chains; type XII is a homotrimer. On the amino acid level, the COL1 domain of type XII is similar to, but slightly smaller than, that of type IX chains. The α1(XII) COL2 domain has no apparent amino acid or nucleotide sequence similarity to either the COL2 or COL3 domains of type IX. This domain in type XII is about half the size of the type IX COL2, but about 10% larger than the type IX COL3. The non-triple-helical regions of type XII are larger than the analogous regions in type IX collagen. Other than conserved cysteinyl residues, NC1 and NC2 domains are unique between the two species. There is a partial similarity, however, between the non-triple-helical domains NC3 and NC4, found in α1(XII) and α1(IX), respectively. The α1(XII) NC3 domain is only partially characterized from cDNAs, but from protein data it is known to be about six times larger than the α1(IX) NC4. Because type XII collagen is a homotrimer, the large NC3 domains of α1(XII) chains appear in rotary shadowing images as three "fingers" attached to the collagenous tail by a small globule. The NC2 domain separating COL1 and COL2 corresponds to a kink in the collagenous tail.

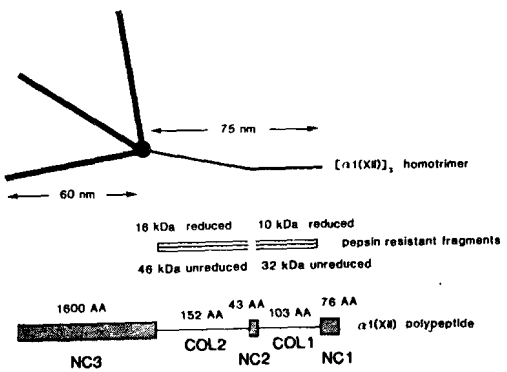

FIGURE 6. Schematic diagram of the homotrimeric type XII collagen molecule, with dimensions determined from the rotary shadowing images (*top*). A schematic diagram of two unreduced pepsin-resistant fragments of type XII collagen is shown in the *middle*. Disulfide bonds are shown as *vertical lines*. Molecular masses of the reduced and unreduced fragments, as determined by SDS PAGE, are indicated above and below the fragments. A schematic diagram of the α1(XII) polypeptide chain is shown at the *bottom*. Triple-helical domains are shown as *thin lines;* non-triple-helical domains as *shaded boxes*. The size of each domain in amino acid residues is indicated.

If type XII collagen interacts with type I-containing fibrils, analogous to the interaction of types IX and II collagens, the type XII COL1 domain would most likely be on the surface of the fibrils, allowing the COL2 domain and the NC3 "fingers" to project outward from the surface. If one supposes that the various types of heterotypic fibrillar collagen matrices present in tissues would be associated with collagens

such as types IX and XII, then one would expect that the subclass of collagens containing these molecules would have additional members. Limited peptide sequencing of a pepsin-resistant triple-helical fragment from bovine and chick skin demonstrates that the fragment is derived from a collagen that is a close relative of type XII collagen.[15] A skin cDNA library is presently being screened to elucidate the structure of this molecule.

The precise functions of different members of such a subclass of fibril-associated collagens could be quite different because of the large differences that may be present in the non-triple-helical domains projected into the perifibrillar space. However, we speculate that a major characteristic of this collagen subclass would be the conservation of a triple-helical domain that allows lateral association with the surface of collagen fibrils.

REFERENCES

1. GORDON, M. K., D. R. GERECKE & B. R. OLSEN. 1987. Type XII Collagen: Distinct extracellular matrix component discovered by cDNA cloning. Proc. Natl. Acad. Sci. USA **84:** 6040-6044.
2. NINOMIYA, Y. & B. R. OLSEN. 1984. Synthesis and characterization of cDNA encoding a cartilage-specific short collagen. Proc. Natl. Acad. Sci. USA **81:** 3014-3018.
3. NINOMIYA, Y., M. VAN DER REST, R. MAYNE, G. LOZANO & B. R. OLSEN. 1985. Construction and characterization of cDNA encoding the α2 chain of chicken type IX collagen. Biochemistry **24:** 4223-4229.
4. GORDON, M. K., D. R. GERECKE, B. DUBLET, M. VAN DER REST & B. R. OLSEN. 1989. J. Biol. Chem. In press.
5. VASIOS, G., I. NISHIMURA, H. KONOMI, M. VAN DER REST, Y. NINOMIYA & B. R. OLSEN. 1988. Cartilage type IX collagen-proteoglycan contains a large amino-terminal globular domain encoded by multiple exons. J. Biol. Chem. **263:** 2324-2429.
6. NINOMIYA, Y., P. CASTAGNOLA, D. R. GERECKE, M. K. GORDON, O. JACENKO, P. LUVALLE, M. MCCARTHY, Y. MURAGAKI, I. NISHIMURA, S. OH, N. ROSENBLUM, N. SATO, S. SUGRUE, R. TAYLOR, G. VASIOS, N. YAMAGUCHI & B. R. OLSEN. 1989. The molecular biology of collagens with short triple-helical domains. *In* Collagen Genes: Structure, Regulation and Abnormalities. C. Boyd, P. Byers & L. Sandell, Eds. Academic Press. New York, NY. In press.
7. LOZANO, G., Y. NINOMIYA, H. THOMPSON & B. R. OLSEN. 1985. A distinct class of vertebrate collagen genes encode chicken type IX collagen polypeptides. Proc. Natl. Acad. Sci. USA **82:** 4050-4054.
8. VAN DER REST, M. & R. MAYNE. 1988. Type IX collagen proteoglycan from cartilage is covalently cross-linked to type II collagen. J. Biol. Chem. **263:** 1615-1618.
9. EYRE, D. R., S. APON, J.-J. WU, L. H. ERICSSON & K. A. WALSH. 1987. Collagen type IX: Evidence for covalent linkage to type II collagen in cartilage. FEBS Lett. **220:** 337-341.
10. VAUGHAN, L., M. MENDLER, S. HUBER, P. BRUCKNER, K. H. WINTERHALTER, M. I. IRWIN & R. MAYNE. 1988. D-periodic distribution of collagen type IX along cartilage fibrils. J. Cell Biol. **106:** 991-997.
11. SUGRUE, S. P., M. K. GORDON, J. SEYER, B. DUBLET, M. VAN DER REST & B. R. OLSEN. 1989. Immunoidentification of type XII collagen in embryonic tissues. J. Cell Biol. **109:** 939-945.
12. DUBLET, B., S. OH, S. P. SUGRUE, M. K. GORDON, D. R. GERECKE, B. R. OLSEN & M. VAN DER REST. 1989. The structure of avian type XII collagen: α1(XII) chains contain 190 kDa non-triple helical amino-terminal domains and form homotrimeric molecules. J. Biol. Chem. In press.