Collagen XXIV, a Vertebrate Fibrillar Collagen with Structural Features of Invertebrate Collagens

SELECTIVE EXPRESSION IN DEVELOPING CORNEA AND BONE*

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Manuel Koch‡, Friedrich Laubi, Peihong Zhoui, Rita A. Hahn‡, Shizuku Tanakai, Robert E. Burgeson, Donald R. Gerecke‡, Francesco Ramirez§, and Marion K. Gordon¶**

From the ‡Institute for Biochemistry II, University of Cologne, Joseph-Stelzmann Strasse 52, Cologne 50931, Germany, the §Laboratory of Genetics and Organogenesis, Hospital for Special Surgery, Weill Medical College of Cornell University, New York, New York 10021, the ¶Environmental and Occupational Health Sciences Institute and the Department of Pharmacology and Toxicology, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, New Jersey 08854, and the ¶Cutaneous Biology Research Center, Massachusetts General Hospital East, Harvard Medical School, Charlestown, Massachusetts 02129

Tissue-specific assembly of fibers composed of the major collagen types I and II depends in part on the formation of heterotypic fibrils, using the quantitatively minor collagens V and XI. Here we report the identification of a new fibrillar-like collagen chain that is related to the fibrillar α1(V), α1(XI), and α2(XI) collagen polypeptides and which is coexpressed with type I collagen in the developing bone and eye. The new collagen was designated the α1(XXIV) chain and consists of a long triple helical domain flanked by typical propeptide-like sequences. The carboxyl propeptide is classic, with 8 conserved cysteine residues. The amino-terminal peptide contains a thrombospondin-N-terminal-like (TSP) motif and a highly charged segment interspersed with several tyrosine residues, like the fibril diameter-regulating collagen chains α1(V) and α1(XI). However, a short imperfection in the triple helix makes α1(XXIV) unique from other chains of the vertebrate fibrillar collagen family. The triple helical interruption and additional select features in both terminal peptides are common to the fibrillar chains of invertebrate organisms. Based on these data, we propose that collagen XXIV is an ancient molecule that may contribute to the regulation of type I collagen fibrillogenesis at specific anatomical locations during fetal development.

Vertebrate collagens are a large family of extracellular proteins that provide mechanical stability to the connective tissue of virtually every organ system. There are at least 40 collagen chains that trimerize into 27 types (1–9), which, in turn, form a large variety of specialized macroaggregates. Structural considerations and the architecture of the resulting polymers have segregated individual collagen molecules into functionally distinct groups (10). Among them, the fibril-forming (fibrillar) collagens represent the most abundant product synthesized by connective tissue cells and include the highly expressed types I–III and the quantitatively minor collagen types V and XI. Types I, III, and V are distributed widely in non-cartilaginous tissues, and types II and XI collagen are found almost exclusively in cartilage and the eye. The pleiotropic manifestations of human fibrillar collagenopathies have dramatically underscored the importance of this ancient group of extracellular proteins in maintaining tissue integrity (1, 11–15).

Fibrillar collagens display a common molecular structure that consists of a long collagenous domain made of ~330 Gly-X-Y triplets flanked at both ends by non-collagenous propeptides. Extracellular removal of propeptides initiates the process of maturation facilitating trimer self-assembly into fibrils (for review, see Ref 16). Ultimately, fibers are organized into specific spatial arrays that are responsible for the properties of individual tissues. It follows that the regulation of fibril diameter is an important determinant of connective tissue function. For instance, large diameter fibrils, spatially arranged in unidirectional bundles, are appropriate for the integrity of tendons. However, such structures would impair the tissue integrity of the cornea, where transparency is dependent upon having thin diameter fibrils arranged in orthogonal layers. One method of controlling fibril diameter is by assembling heterotypic fibrils, incorporating either type V or XI collagen into fibrils with types I or II collagen, respectively (17, 18). Types V and XI collagen most likely serve as fibril diameter regulators because they retain a bulky amino-terminal portion attached to the triple helix after their final extracellular processing (17, 19). The retained N-peptide domain cannot be embedded in fibrils but instead, projects from the fibrillar surface inhibiting the lateral aggregation of additional molecules onto the fibril (20, 21). Heterotypic assembly of isolated fibrillar collagen molecules and loss-of-function mutations of minor fibrillar collagen types in mice have provided in vitro and in vivo support for this regulatory model of fibrillogenesis (17–24).

Here we report the identification of a novel fibrillar collagen chain, designated α1(XXIV), which contains an amino-terminal domain closely related to those of the types V and XI collagen subunits. We show that embryonic expression of the mouse gene (Col24a1) is confined to the developing eye and skeleton.
EXPERIMENTAL PROCEDURES

Identification and Full-length Cloning of \(\alpha(XXIV)\) Collagen cDNA—The strategy for isolating \(\alpha(XXIV)\) collagen is presented first, followed by descriptions of the methods employed. A BLAST search (25) of the data base of expressed sequence tags (dbEST)1 (26) looking for amino acid sequence homology with the COOH-terminal third of the \(\alpha(XXIV)\) collagen (Swiss-Prot accession number BAA14523) yielded one clone (GenBank accession number, AA331798) as a possible candidate for a novel human collagen cDNA. The clone, EST 356863, contained an insert about 2.4 kb in length. About 255 bp had been sequenced, encoding 85 amino acid residues with a perfect Gly-X-Y triplet structure. The EST was purchased from the American Type Culture Collection and sequenced in its entirety using the method described below. The sequence revealed codons for an additional 33 amino acid residues of Gly-X-Y structure (i.e., 11 more triplets) and 235 amino acid residues of a non-collagenous (NC) domain. This was followed by a stop codon and −1.4 kb of 3′-untranslated region (UTR). From the sequence of the EST clone, nested primers were designed for 5′-RACE, using human placental Marathon ready cDNA (Clontech), as template, as described previously (27). The method was adapted as follows. Because the collagen XXIV mRNA was rare in the pool of placental mRNA, a method was adapted as follows. Because the collagen XXIV mRNA was rare in the pool of placental mRNA, a method was adapted as follows. Because the collagen XXIV mRNA was rare in the pool of placental mRNA, a method was adapted as follows. Because the collagen XXIV mRNA was rare in the pool of placental mRNA, a method was adapted as follows. Because the collagen XXIV mRNA was rare in the pool of placental mRNA, a method was adapted as follows. 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RESULTS

Identification of Collagen XXIV cDNA—A search of the GenBank dbESTs for collagen domain homologies identified three cDNAs, each encoding a potentially unique collagen. These EST clones were purchased and sequenced. Full-length copies of each mRNA were obtained by 3′- and 5′-RACE using a commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent EST clones were purchased and sequenced. Full-length copies of each mRNA were obtained by 3′- and 5′-RACE using a commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template. Once it became clear from sequence analysis that the cDNAs did, indeed, represent commercial human library as a template.
FIG. 2. Amino acid sequence of human (h) α1(XXIV) collagen and homologous domains in related human fibrillar collagens. The signal peptide is 38 residues. The N-peptide domain is compared with those of vertebrate fibrillar collagen chains that regulate fibril diameter. The 931-residue triple helix, with the imperfection in bold, is shown without comparison with other collagens. The C-propeptide of α1(XXIV) is shown in alignment with various vertebrate and invertebrate collagens. In the N-peptide, the potential furin cleavage sites are indicated by cyan color and are dot overlined and underlined. The N-peptide bone morphogenetic protein-1 cleavage sites of α1(XXIV), α1(V), and α2(XI) collagens are highlighted in purple. The minor triple helix of the α1(XXIV) collagen N-peptide is indicated by a dotted underline. Cysteines throughout the N- and C-peptides are highlighted with red. Across species, the first 51 residues of the C-propeptide are the most variable. Positive and negative charges are indicated in green and cyan, respectively, to suggest where conservation exists. The chain association domain (9) is shown in yellow.
Collagenous domain are 54 bp in length or multiples of 54 bp (data not shown). Altogether, these data indicate that collagen XXIV is a new member of the fibrillar group of collagens and is most closely related to the α1(V) collagen polypeptide chain.

Collagen XXIV Contains Features of Invertebrate Collagens—Sequence analyses also revealed several features in the N-peptide, triple helix, and the C-propeptide of α1(XXIV) which are unique to invertebrate fibrillar collagen chains. The most distinctive of these characteristics is a single imperfection in the collagenous domain (residues 649–652) consisting of 4 inserted amino acid residues, STVL, in bold in Fig. 2B. The interruption is flanked by 216 perfect Gly-X-Y triplets on the N side and 93 perfect triplets on the C side. To rule out that the imperfection was an artifact, we used human corneal and hip mRNA as template and performed RT-PCR to produce cDNA fragments encoding the imperfection and its surrounding area. Sequence analysis of the amplified fragments, as well as analysis of the gene, confirmed the existence of the imperfection in the triple helix. Although imperfections have not been observed in vertebrate fibrillar collagen triple helices prior to the discovery of collagens XXIV and XXVII (8, 9), they are not rare in invertebrate fibrillar collagens (54–57). The kind of imperfection found in α1(XXIV) collagen is most like that of a sponge fibrillar collagen (57). This sponge imperfection has been referred to as a Gly-X-Y-Z insertion, however, it can also be viewed as a 4-residue insertion that coincidentally begins with a glycine residue. From this viewpoint, it becomes an analog of the STVL imperfection in α1(XXIV) collagen. Also, the sponge fibrillar collagen imperfection is located at a relatively similar position in the triple helix, being 81 triplets from the C-propeptide (57).

There is great diversity within the first ~50 amino acid residues of the C-propeptides of fibrillar collagens, as shown by the peppered appearance of green and cyan highlighting of charged residues in Fig. 2C. However, after this divergent region, the C-propeptides of all species of all fibrillar collagens show a high degree of conservation. The overall identity of the of the α1(XXIV) C-propeptide with the annelid (54) and sponge (57) counterparts (27.2% and 26.8%) is not very different from its 29.4% identity with the human α1(V) collagen chain (46, 47); conceivably, this closeness may reflect the necessity of maintaining particular residues for function. However, some regions in the α1(XXIV) collagen polypeptide are conspicuously invertebrate-like. There is an unusually high sequence identity (33 out of 56 residues) in C-propeptide residues 52–107 of collagen XXIV and the analogous region of EmF1α, the sponge fibrillar collagen (57); and, the chain selection sequence in the C-propeptide (9), which falls between the 5th and 6th cysteinyl residues (highlighted in yellow in Fig. 2C), is unusually short compared with the vertebrate fibrillar chains but is more consistent with the length of the invertebrate counterparts.

Another evolutionarily conserved feature that α1(XXIV) collagen shares with invertebrate, but not vertebrate chains, was noted in the N-peptide. The N-peptides of fibrillar collagen chains contain a variable number (one to three) of minor triple helical sequences just upstream of the major collagenous domain. Collagen XXIV contains only one minor triple helix separated from the major helix by 6 residues. Such an arrangement closely resembles the 8-amino acid spacer between the major and minor triple helices of the H. vulgaris collagen (58). Altogether, these observations suggest that collagen XXIV is of ancient origin. Similar conclusions have been reached independently for the closely related collagen, type XXVII (8, 9).

Collagen XXIV Expression Is Restricted in Adult and Embryonic Tissues—We attempted to examine the expression profile of the Col24a1 gene in embryonic, perinatal, and adult mouse tissues by Northern analyses, but the nonabundance of the collagen XXIV mRNA made such analyses difficult. However, preliminary results of a staged mouse embryo Northern blot suggested that the Col24a1 transcripts were first detectible around E15. Because the Northern analyses were not perfectly definitive and because commercial blots do not contain connective tissue mRNAs, we used other approaches to look at α1(XXIV) collagen RNA expression. RNA was isolated from adult mouse bone, cornea, retina, skin, and tendon for analysis by semiquantitative relative RT-PCR (Fig. 3). Not only did this show which of the tissues contained the mRNA, but it also allowed us to estimate the relative abundance of α1(XXIV) collagen transcripts compared with mRNAs for chains of fibrillar collagen types I, III, and V. The RT-PCR products are shown in Fig. 3A. The upper band is the normalizer, the reproducibly attenuated 18 S signal, whereas the lower band in each panel represents the product of a particular collagen mRNA amplification. The collagen XXIV primers (rightmost panel of Fig. 3A) robustly amplify mRNA from bone and retina, and, to a lesser degree, also amplify the mRNA from cornea, skin, and
tendon. (For a negative signal, see collagen III mRNA in cornea.) This radiograph indicates that, first, as expected, retina is not an abundant source of fibrillar collagen mRNAs. It contains the least type I, III, and V collagen mRNA compared with the other tissues. However, it is a significant source of \(\alpha_1(XXIV)\) collagen mRNA. Second, the radiograph shows that, as expected, \(\alpha_2(I)\) collagen mRNA is expressed very highly in bone, cornea, skin, and tendon. Also, \(\alpha_1(III)\) collagen mRNA is expressed very highly in skin, but, as mentioned, is not present in cornea. Finally, it demonstrates that \(\alpha_1(XXIV)\) collagen mRNA is a minor product compared with the fibrillar collagen mRNAs.

The RT-PCR data are represented in histogram form with arbitrary \(y\) axis units in Fig. 3B, showing the normalized relative abundance for each of the collagen chain mRNAs tested. Here again, it is clear that collagen XXIV mRNA is a nonabun-
dant species, representing only about 70% of the amount of type V collagen in bone. Fig. 3 presents the ratio of various fibrillar collagen mRNAs compared with the $\alpha_1$(XXIV) transcript. These data again emphasize the nonabundant nature of $\alpha_1$(XXIV) collagen mRNA, showing that $\alpha_2$(I) mRNA is about 10 times more abundant than $\alpha_1$(XXIV) collagen mRNA in bone and more than 90 times more abundant in tendon. To validate further the conclusions derived from these data, the $\alpha_2$(I):$\alpha_1$(V) collagen mRNA ratio was also calculated to show the expected higher percentage of collagen V in mouse cornea compared with bone, tendon, or skin. As indicated, when chain composition is taken into account, the total collagen I:collagen V mRNA ratio predicts that the type V collagen mRNA expression is 5.9% of that of type I collagen in adult mouse tendon but is 14.3% of type I in adult mouse cornea. The ultimate conclusion from this experiment is that, of the adult mouse connective tissues tested, collagen XXIV is most highly expressed in bone.

The seemingly restricted tissue distribution of collagen XXIV from Northern blots (not shown) and RT-PCR (Fig. 3) raised the possibility of a specialized role for this matrix product. Unfortunately, our attempts to generate antibodies suitable to analyze the tissue distribution of collagen XXIV in more detail have not yet been successful. As an alternative, we resorted to refining the expression profile of the Col24a1 gene by means of RNA in situ hybridization. This analysis was also undertaken to facilitate the future analysis of knockout mice deficient in collagen XXIV, the production of which are currently under way. To confirm genuine hybridization from background, in addition to sense controls, all sections were examined in darkfield and brightfield. In brightfield, silver grains are very clearly distinguishable, confirming positive signals.

**Fig. 7.** Darkfield micrographs of E16.5 sections hybridized with the antisense probe as above ($A$–$E$) and a sense probe ($A'$, $D'$, $E'$) to control for specificity of the signal. No signal was detected using the sense control. Arrows mark nonspecific signal caused by erythrocyte scatter. Shown are transverse sections through the developing lower molar ($A$), the upper incisor and maxilla ($B$), mandible ($C$), temporal bone ($D$), and a transverse section through the hind limb ($E$). $lm$, lower molar; $m$, mandible; $mt$, metatarsus; $mx$, maxilla; $t$, temporal bone; $ui$, upper incisor. Scale bar, 0.2 mm.

**Fig. 8.** Additional darkfield micrographs of E16.5 sections hybridized with the antisense $\alpha_1$(XXIV) riboprobe ($A$–$D$), and the sense riboprobe ($A'$–$D'$). Shown are transverse sections through the developing proximal fore limb ($A$ and $A'$), where the ossifying portion of the humerus is positive, and through the vertebral column at three progressively more caudal levels of the trunk ($B$–$D'$). Arrowheads indicate erythrocyte scatter. $h$, humerus; $pr$, proximal rib; $v$, vertebrae. Scale bar, 0.2 mm.
5A, at E14.5 the Col24a1 transcripts labeled the emerging skeletal elements of the head, namely the outer table of membranous primordial parietal bone, just outside of what will become the cerebral cortex, and the mesenchymal precursor of the parietal bone. The embryonic brain itself showed no detectable signal. Positive signals were also present within intramembranous ossification centers of the maxilla and mandible around Meckel’s cartilage, but Meckel’s cartilage itself, circled by a dotted line in C, was negative (Fig. 5, B and C). The basihenoid bone and femur are positive as well (Fig. 5, D and E).

At E15.5, collagen XXIV expression in areas of ossification continues and was again identified in the body of the mandible (Fig. 7A, B, and C). Expression in the eye (Fig. 4B and Fig. 6A) was strongly localized to the embryonic cornea. (The lens expression is artifactual from probe accumulating in a ruptured area of tissue.) In the retina, if a signal is present at all, it is a very weak one and is limited to the nerve fiber layer of the optic cup and the inner nuclear layer. The expression in the E15.5 skeleton coincided with the formation of primary ossification centers, but, additionally, transcripts were detected in the developing otic capsule (see the area indicated by e in Fig. 6B). For the most part, collagen XXIV expression occurred in sites of robust type I collagen fibril assembly and deposition.

At E16.5, ossification sites continue to be the predominant site of hybridization, where transcripts are detected in the teeth (Fig. 7, A and B), mandible (Fig. 7C), and other developing bones, such as the temporal bone in the head (Fig. 7D). Moreover, α1(XXIV) collagen mRNA accumulated in cells of the ossifying metatarsal bones (Fig. 7E) and mid-shaft region of the humerus (Fig. 8A). Furthermore, the mRNA is found in ossifying regions of cervical bodies, i.e. around the foramen transversarium (Fig. 6B), and lower regions of the vertebral column (Fig. 8, C and D).

Taken together, collagen XXIV appears to be a marker for the process of bone formation in the embryonic mouse. It is first expressed at E14.5, concomitant with the appearance of the first ossification centers, and remains restricted to these, as well as ossification centers that emerge later during subsequent stages of development. The embryonic cornea is the main exception. Nevertheless, the expression pattern of collagen XXIV in the fully developed skeleton and other tissues remains to be determined. Likewise, the regional localization of Col24a1 in the cornea has yet to be fully determined.

**DISCUSSION**

The α1(XXIV) polypeptide represents a novel collagen chain with structural features of both vertebrate and invertebrate fibrillar chains. Together with the newly described chain of collagen XXVII (8, 9), collagen XXIV represents a separate clade within the fibrillar group of collagens. Structural properties unique to these invertebrate-like fibrillar chains, such as the size of the triple helix and the triple helical perfection, suggest that trimeric molecular assembly with polypeptides of types I, II, III, V, and XI collagen does not occur. Additionally, the distinct expression patterns of the Col24a1 and Col27a1 genes during mouse embryogenesis strongly indicates that they, too, do not coassemble into heterotrimeric molecules. Lacking additional evidence, we therefore surmise that type XXIV collagen is probably a homotrimeric collagen molecule with specialized functions at specific anatomical sites of fibrillogenesis.

Collagen XXIV is expressed during development at sites undergoing intense type I collagen fibrillogenesis. Of major interest is whether collagen XXIV is assembled into growing fibrils of type I collagen, i.e. forming a heterotypic fibril. If this turns out to be true, it is likely that lateral growth of the fibril will be terminated. The shortness of the triple helix and the interruption within the α1(XXIV) collagen would probably not be conducive to further fibrillar assembly. Additionally, collagen XXIV most likely retains a N-peptide domain that would inhibit lateral growth, regulating the fibril diameters as the retained N-peptides of collagens V and XI do. The question of whether or not collagen XXIV assembles into fibrils with collagen I will be easier to dissect when specific antibodies are generated.

The sum of our in situ hybridization and RT-PCR data also suggests that, although bone expresses collagen XXIV during development as well as during adult life in the mouse, the cornea and retina temporally regulate the expression of the molecule. The cornea contains robust amounts of the mRNA primarily during development, whereas the enhanced retinal expression is postnatal. The possibility that samples had been reversed in performing RT-PCR led us to verify the adult expression pattern using multiple adult corneal (three total) and retinal (four total) mRNA preparations. Little is known about collagen expression in the retina, but a large reduction in corneal expression after maturity is not surprising: a dramatic decrease in fibrillar collagen mRNAs is observed after the cornea matures.

Corneal expression of collagen XXIV is also consistent with the idea that the molecule may be a fibril diameter regulator. Within the human cornea is a stroma composed of many orthogonal layers of thin diameter fibrils (~25 nm). More anteriorly, under the corneal epithelial basement membrane, is Bowman’s layer, a region of even smaller diameter fibrils (~18–20 nm). It is well documented that collagen V plays a large role in regulating the diameter of the fibrils assembled in the stroma by being synthesized as 15–20% of the total fibrillar collagen (17, 59, 60). Further experiments will determine whether collagen XXIV also plays a role in this regulation.

Although our results show that the two major producers of collagen XXIV during development are cornea and bone, it is also possible that the molecule may play a structural role in other tissues such as skin and tendon, where the α1(XXIV) collagen mRNA is expressed at very low levels.

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