Absence of the α1(IX) Chain Leads to a Functional Knock-out of the Entire Collagen IX Protein in Mice*

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Cartilage fibrils contain collagen II as well as smaller amounts of collagens IX and XI. The three collagens are thought to co-assemble into cartilage-specific arrays. The precise role of collagen IX in cartilage has been addressed previously by generating mice harboring an inactivated Col9a1 gene encoding the α1(IX) chain, i.e. one of the three constituent chains of collagen IX (Fässler, R., Schneegolsberg, P. N. J., Dausman, J., Shinya, T., Muragaki, Y., McCarthy, M. T., Olsen, B. R., and Jaenisch, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5070–5074). The animals did not produce α1(IX) mRNA or polypeptides and were born with no conspicuous skeletal abnormality but post-natally developed early onset osteoarthritis. Here we show that the deficiency in α1(IX) chains leads to a functional knock-out of all polypeptides of collagen IX, whereas the Col9a2 and Col9a3 genes were normally transcribed. Therefore, synthesis of α1(IX) polypeptides is essential for the assembly of heterotrimeric collagen IX molecules. Surprisingly, cartilage fibrils of all shapes and banding patterns found in normal newborn, adolescent, or adult mice were formed in transgenic animals, although they lacked collagen IX. Therefore, collagen IX is not essential, and may be functionally redundant, in fibrillogenesis in cartilage in vivo. The protein is required, however, for long term tissue stability, presumably by mediating interactions between fibrillar and extrafibrillar macromolecules.

The biomechanical properties of cartilage are intimately linked to the structure of its extracellular matrix that in essence consists of two suprastructural compartments. An extended three-dimensional fibril network penetrates an extracellular matrix that appears as an amorphous mass in the electron microscope, although there is a high degree of molecular organization. Aggrecan, the major cartilage proteoglycan, is immobilized by specific interactions with high molecular mass hyaluronan. Each aggrecan molecule comprises a large core protein highly substituted by polyanionic glycosaminoglycans. This confers to the extracellular matrix a very high negative charge density and, through osmotic binding of large amounts of water, generates a swelling pressure that is contained by the fibril network. Unlike the extracellular matrix, cartilage fibrils exhibit a characteristic cross-striation in the electron microscope. The fibrils are macromolecular aggregates that include but are not confined to three types of collagens, i.e. collagens II, IX, and XI, as major structural and functional components. The collagen molecules are longitudinally organized into quarter staggered arrays with a repeat gap-overlap period, called D, of 67 nm. In immature cartilage, such as the chick embryo sternum, fibrils have a uniform diameter of 17 nm, are randomly oriented (1), and contain large amounts of collagens IX and XI, each representing about 10% of the total collagen (2). In postnatal mammalian hyaline cartilage, the fibrils contain much less of the minor collagens, are heterogeneous in their diameters (up to 200 nm), and form specific patterns depending on the age of the animal and the precise location within the tissue (3). Thus, cartilage at early stages of development appears to contain uniform fibrils, whereas mature cartilage is characterized by fibril populations that are morphologically and biochemically distinct (4).

The surface of most thin cartilage fibrils is populated by D-periodically arranged molecules of collagen IX. This protein is a heterotrimer of genetically distinct α1(IX), α2(IX), and α3(IX) chains, each incorporated into a molecule with alternating triple helical (COL1-COL3) and nontriple helical (NC1-NC4) domains. The NC2 and the NC3 domains connect the triple helical domains and confer flexibility to the molecule. The domains NC1, COL1, NC2, and COL2 are incorporated into the fibril body and may not necessarily be situated at the fibril surface. However, the globular NC4 domain attached to a stalk formed by the COL3 domain projects from the surface of the fibrils outwards and is connected to the rest of the molecule by the flexible NC3 domain (4). The NC4 domain at the amino-terminal end of the α1(IX) chain in cartilage is rich in basic amino acids, which has invited the speculation that this part of the molecule may mediate interactions between fibrils and their polyanionic environment. In addition, collagen IX is a proteoglycan (5, 6) with a single chondroitin-dermatan sulfate chain attached to the NC3 region of the α2(IX) chain (7, 8). These glycosaminoglycan chains may also be involved in the contact between the fibrils and the extracellular matrix.

The precise function of collagen IX in situ still is elusive. Recently, the issue has been addressed by creating transgenic mice harboring an inactivated Col9a1 gene after homologous recombination (9). Unexpectedly in view of the notions discussed above, homozygous mice showed no obvious defects in their skeletal development. However, they developed osteoarthritis with advancing age. This pointed toward collagen IX as a crucial component in the long term integrity of cartilage rather than the formation and development of cartilage matrix as such. However, the expression of collagen IX in these ani-

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1 R. Hagg, P. Bruckner, and E. Hedbom, unpublished results.
mals was not examined in detail in the previous study. The tissues were devoid of α1(IX) mRNA or the corresponding polypeptides, but it remained unclear whether α2(IX) and α3(IX) mRNA or polypeptides were synthesized. Studies on in vitro reassociation of collagen IX fragments indicated that collagen IX trimers without α1(IX) chains may be viable (10, 11). Therefore, it remained possible that the animals produced variants of partly functional collagen IX molecules assembled from α2(IX) and/or α3(IX) chains only, which may largely substitute for normal collagen IX during endochondral bone formation. Here, we provide this missing information. Thereby, we have also gained further insight into the molecular assembly of collagen IX in mouse cartilage as well as the consequences of its integration into cartilage matrix suprastructures.

MATERIALS AND METHODS
Experimental Animals and Cartilage Preparation—Transgenic mice with an inactivated Col9a1 gene were described elsewhere (9). DBA2 mice (Charles River, Germany) were used as controls. Rib cages of newborn, adolescent (~4 weeks old), and adult (~1 year old) mice were freed from surrounding noncartilaginous tissue for further biochemical and morphological analysis.

RNA Extraction and RNA Hybridization—Day 17.5 embryos of wild-type, heterozygous, and homozygous mice were homogenized and whole embryo RNA was isolated by the LiCl/urea method described by Auffray and Rougeon (12). Approximately 20 μg of RNA were electrophoretically separated on agarose gels and transferred to nylon membranes. Hybridization was performed overnight at 65 °C with probes labeled by random priming using [32P]dCTP. The filters were washed two times with 0.5× sodium phosphate, 7% SDS, 1 mM EDTA, 0.1 mg/ml salmon sperm DNA, 1% bovine serum albumin, pH 7.2. Bound probes were detected by autoradiography at ~70 °C on Kodak X-Ar x-ray films. The probes used were EcoRI/HindIII cDNA fragments either for mouse α2(IX) mRNA or mouse α3(IX) mRNA (13). β-Actin cDNA was employed as control probe.

Antibodies to Collagens II and IX—Triple helical pepsin fragments of collagens II, IX, and XI were isolated from transplantable (Swarm) chondrosarcoma of rats by limited digestion with pepsin (Serva) and subsequent differential salt precipitation. This procedure yields two disulfide bonded triple helical fragments of collagen IX termed HMW and LMW (14). The purity was judged by SDS-PAGE.2 Antisera were raised by immunizing rabbits with a mixture of fragments HMW and LMW of rat collagen IX in Freund’s complete and incomplete adjuvant. Transgenic mice (Pic, Sweden) and wild-type (C57BL/6 J) mice were used as controls. Rib cages from mice of different ages were homogenized with a Potter-Elvehjem tissue homogenizer (15). The protein pellets were dissolved in 0.1 M Tris-HCl, pH 6.8, containing 0.5 M urea, 10% glycerol, and 2% SDS (SDS sample buffer) and were run on a 4.5–15% polyacrylamide gradient gels (18).

Extraction of Fibril Fragments, Collagens, and SDS Soluble Molecules—Rib cages from mice of different ages were homogenized with a Poltron (Kinematica, Littau, Switzerland) in 10 volumes of 2 mM sodium phosphate, pH 7.4, containing 150 mM NaCl, 100 mM 6-amino-hexanoic acid, 20 mM EDTA, 5 mM benzamidine, 5 mM N-ethylmaleimide, and 0.1 mM phenylmethylsulfonyl fluoride and were subsequently centrifuged at 27,000 × g for 30 min to obtain a clear supernatant. This procedure was repeated twice with fresh extraction buffer. The supernatants containing the fibril fragments were combined, and the pelleted material was divided into two portions. One portion was resuspended in 100 volumes of 0.2 M NaCl, 0.5 M acetic acid, adjusted to pH 2.5 with 12 N HCl, and subjected to digestion with pepsin (100 μg/ml) for 48 h at 4 °C. To complete solubilization of collagens, the digestion step was repeated. The digests were combined and centrifuged. Finally, total collagens in the neutralized supernatants were precipitated by adding cold NaCl to a final concentration of 4.5 M. A separate portion was digested for 3 h at 37 °C in 150 mM NaCl, 20

2 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

RESULTS
Expression of Col9a2 and Col9a3 Genes in Col9a1 Knock-out Mice—In mice with an inactivated Col9a1 gene, the expression of Col9a1 mRNA and α1(IX) polypeptides was reduced in heterozygotes and absent in homozygotes (9). To investigate the effect of this genetic alteration on the expression of Col9a2 and Col9a3 genes, mRNA levels in wild-type, heterozygous, and homozygous mice were examined by Northern blot analysis. Transcription of Col9a2 and Col9a3 remained unaffected by the knock-out of the Col9a1 gene because the corresponding transcripts were identical in homozygous or heterozygous transgenic animals and in control animals (Fig. 1).

Expression of α2(IX) and α3(IX) mRNA in α1(IX)-null mice. RNA from homozygous (−/−), heterozygous (+/−), and wild-type (+/+ ) fetal day 17.5 mice was subjected to Northern blotting with cDNA fragments representing Col9a2 (A) or Col9a3 (B) as probes (upper panels). After stripping, the filters were rehybridized with a β-actin cDNA (lower panels). 0.2 M NaCl, 20 mM EDTA, 5 mM benzamidine, 5 mM hexanoic acid, 20 mM EDTA, 5 mM benzamidine, 5 mM 1-naphtol (Sigma) as color reagent. Monoclonal antibodies CIID3 and CIID4, raised by immunizing rabbits with a mixture of fragments HMW and LMW of rat collagen IX in Freund’s complete and incomplete adjuvant. This procedure yields two disulfide bonded triple helical fragments of collagen IX termed HMW and LMW (14). The purity was judged by SDS-PAGE. Antisera were raised by immunizing rabbits with a mixture of fragments HMW and LMW of rat collagen IX in Freund’s complete and incomplete adjuvant as described (15). The specificity of the antisera was checked by immuno-electron microscopy—(Weil, Sweden) on carbon-coated copper grids (4). The grids were washed with 150 mM NaCl, 2 mM sodium phosphate, pH 7.4 (PBS), and were treated for 30 min with 2% (w/v) dried skim milk in PBS. The absorbed material was then allowed to react for 2 h with antibodies to collagen II and/or collagen IX in 0.2% (w/v) dried skim milk in PBS. After washing five times for 2 min with PBS, the grids were incubated for 2 h with a suspension of colloidal gold particles (12 or 18 nm) coated with goat antibodies to mouse or rabbit immunoglobulins (Dianova, Hamburg, Germany), rinsed 3 times with PBS containing 0.2% (w/v) dried skim milk. For double labeling experiments a mixture of gold particles of different sizes was used. Finally, the grids were washed with PBS and negatively stained with 2% uranyl acetate. Electron micrographs were taken at 80 kV with a Philips CM 10 electron microscope.

Measurement of Fibril Diameters and Labeled Fibrils—Electron micrographs were calibrated on the basis of the D = 67 nm banding pattern of the fibrils. Diameters of fibril fragments were measured with a Peak scale magnifying glass 10 × (Plano, Marburg, Germany) on micrographs at final magnifications of at least 6.2 × 106-fold. Gold particles associated with fibrils were counted. Fibrils were considered as labeled if they carried more than 1 average gold particle per 10 D periods.

The presence of collageneous polypeptides in rib cartilage extracts was analyzed. Hyaline cartilage collagens are extensively cross-linked but can be solubilized quantitatively by limited digestion with pepsin to remove small, nontriple

FIG. 1. Normal expression of α2(IX) and α3(IX) mRNA in α1(IX)-null mice. RNA from homozygous (−/−), heterozygous (+/−), and wild-type (+/+ ) fetal day 17.5 mice was subjected to Northern blotting with cDNA fragments representing Col9a2 (A) or Col9a3 (B) as probes (upper panels). After stripping, the filters were rehybridized with a β-actin cDNA (lower panels).

The expression of Col9a2 and Col9a3 genes in Col9a1 knock-out mice was analyzed. Hyaline cartilage collagens are extensively cross-linked but can be solubilized quantitatively by limited digestion with pepsin to remove small, nontriple

The expression of Col9a2 and Col9a3 genes in Col9a1 knock-out mice was analyzed. Hyaline cartilage collagens are extensively cross-linked but can be solubilized quantitatively by limited digestion with pepsin to remove small, nontriple
Although no polypeptides derived from the α2(IX) or α3(IX) chains were detected in cartilage of α1(IX)-null mice after pepsin extraction, the possibility still remained that such chains were synthesized but not incorporated into pepsin-resistant, triple helical collagen IX molecules. Therefore, rib cages of newborn, adolescent, or adult mice were extracted by boiling in SDS sample buffer containing 2% β-mercaptoethanol. This procedure yielded intact collagen IX polypeptides from chick cartilage (20). The crude extracts were subjected to SDS-PAGE and immunoblotting with the antisera to collagen IX. Polypeptides with apparent molecular masses of 84, 72, and 67 kDa corresponding to α1(IX), α2(IX), and α3(IX) chains, respectively, were detected in samples from normal (Fig. 2B, lane 7) but not α1(IX)-null mice (Fig. 2B, lane 8). The crude mixtures of proteins extracted with boiling SDS sample buffer from cartilage pieces of mutant and control mice were indistinguishable by SDS-PAGE (Fig. 2B, lanes 1–6). Thus, lack of collagen IX expression did not conspicuously alter the production of other matrix constituents.

Ultrastructure of Cartilage Fibrils—The consequences of the absence of collagen IX for fibrillogenesis in cartilage were studied by ultrastructural analysis of cartilage fibrils. Fibril fragments were prepared from rib cages of mutant and wild-type mice and characterized by electron microscopy after indirect immuno-gold labeling and negative staining. To verify their cartilage origin, the fibril fragments were double-labeled with monoclonal antibodies recognizing collagen II epitopes together with our antisera to rat collagen IX. Fibrils at different stages of cartilage development were analyzed to take into account the possibility that age-dependent differences may occur between normal and transgenic mice. Surprisingly, the shapes and ultrastructural details were not obviously different in fibril fragments isolated from wild-type and transgenic mice, respectively. The banding patterns (Fig. 3) and the diameter distributions (Fig. 4) of fibril fragments were the same in normal and α1(IX)-null mice. In agreement with the biochemical data, collagen II but not collagen IX could be localized on fibrils from transgenic mice. By contrast, most fibrils from control mice were labeled with both antibodies, regardless of their diameter and the age of the donor animal.

DISCUSSION

Here, we have demonstrated that a null mutation of the Col9a1 gene encoding α1(IX) chains leads to a functional knock-out of collagen IX in mice. Although the mRNAs for the α2(IX) and α3(IX) chains are normally transcribed, the corresponding polypeptides cannot be detected in cartilage of α1(IX)-null mice. Presumably, α2(IX) and α3(IX) chains are rapidly degraded, or their production is suppressed at the translational level in the absence of α1(IX) chains. Preceding studies on in vitro reassociation of short polypeptides containing the presumptive interaction sites within the carboxyl-terminal COL1 domain suggested that homotrimeric molecules composed of α2(IX) chains or heterotrimeric molecules containing α2(IX) and α3(IX) polypeptides could be formed in the absence of α1(IX) chains (10, 11). However, our data indicate that such molecules are not assembled in mouse cartilage in vivo.

Indirect evidence from several studies pointed toward a pivotal role of collagen IX in embryonic skeletal development. In mammals, collagen IX is most abundant in immature cartilages, including areas undergoing endochondral ossification (21). The fibrils of chick embryo sternal cartilage have a uniform diameter of ~17 nm and contain collagens II, IX, and XI in relative proportions of 8:1:1, respectively (2). Furthermore, in vitro reconstitution experiments with isolated chick cartilage collagens suggested that as much as 10% of collagen IX was required to generate fibrils morphologically similar to...
other mouse strain, with a transgene encoding only the NC4 domain of α1(IX) had a similar phenotype (24). Overexpression of this transgene led to osteoarthritis, and again the severity depended on the age and level of transgene expression. Consistently with these transgenic mouse models, multiple epiphyseal dysplasia, a human dominant heritable disorder characterized by mild skeletal malformations and early onset osteoarthritis, can be caused by heterozygous mutations in the Col9a2 gene (25). These observations have prompted the hypothesis that collagen IX acts as a tissue stabilizer through interactions between the fibril surface and the extracellular matrix, perhaps via its NC4 domain (26). This role of collagen IX also is consistent with the cartilage pathology of collagen IX-deficient mice.

Recent studies suggested that collagen XI, the other minor collagen of cartilage fibrils, is essential for skeletal morphogenesis, including the formation of thin cartilage fibrils. The phenotype of cho/cho mice, which includes abnormally thick cartilage fibrils, is caused by the absence of the α1 chain of collagen XI in extracellular matrices (27). Further, unusually thick fibrils are found in cartilage of patients with Stickler syndrome, where a genetic defect results in abnormally short α2(XI) chains which presumably compromises the molecular assembly of collagen XI (28). In addition, collagen XI is associated predominantly with fibrils less than 25 nm in diameter in human juvenile rib cartilage, whereas collagen IX is present on fibrils of various sizes in this tissue (29, 30). However, experiments on fibril reconstitution from soluble avian collagens II and XI in vitro showed that control of lateral fibril growth was incomplete. At least one further component was required, and collagen IX was shown to satisfy this need (22). Additional surface components of cartilage fibrils include the small proteoglycans decorin and fibromodulin, which alter fibrillogenesis of collagens I and II in vitro (31, 32). Our recent studies demonstrated that decorin and collagen IX coexist on some fibrils from fetal bovine epiphyseal cartilage. Therefore, these
molecules may participate in fibril formation and may thus be functionally redundant with collagen IX in controlling lateral aggregation of collagens. Similar to the mice with an α1(IX) knock-out, skeletal malformations were not observed in decorin-deficient mice (33). Targeted disruption of the decorin gene caused abnormal collagen fibril morphology and skin fragility. Studies on mice that are deficient in both collagen IX and decorin are in progress and may help to clarify the roles of fibril surface components in cartilage.

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