Wittaya Pimtong

Suprastructure Interaction in Cartilage Matrix: Interaction of Collagen VI Microfibrils with Banded Fibrils Containing Collagens II, IX, and XI

2007

Biochemie

Suprastructure Interaction in Cartilage Matrix: Interaction of Collagen VI Microfibrils with Banded Fibrils Containing Collagens II, IX, and XI

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften im Fachbereich Chemie und Pharmazie
der Mathematisch-Naturwissenschaftlichen Fakultät
der Westfälischen Wilhelms-Universität Münster

vorgelegt von
Wittaya Pimtong
aus Yasothon, Thailand

- 2007 -

Dekan: Prof. Dr. F. Ekkehardt Hahn

Erster Gutachter: Prof. Dr. Hans-Joachim Galla

Zweite Gutachter: Prof. Dr. Peter Bruckner

Tag der mündlichen Prüfung: 15.03.2007

Tag der Promotion: 15.03.2007

จงละความกระวนกระวายของท่านไว้กับพระองค์
เพราะว่าพระองค์ทรงห่วงใยท่านทั้งหลาย
๑ เปโตร ๕:๗

Alle eure Sorge werft auf ihn; denn er sorgt für euch.
1 Petrus 5:7

Cast all your anxiety on him because he cares for you.
1 Peter 5:7

Acknowledgments

This work was carried out at the Institute of Physiological Chemistry and Pathobiochemistry, Westfälischen Wilhelms-Universität Münster, during the years 2003-2007. I memorize the late Prof. Dr. Hans Kresse for allowing me to work in the Institute.

I am indepted to Prof. Dr. Peter Bruckner, the Head of our extracellular matrix team and the advisor of this thesis, for giving me a chance to become a member of such a multi-talented and passionate research group. His understanding and broad knowledge of this research area has enabled me to gain the basic principles for deeper comprehension of biochemical events in the cartilage matrix. Also his constructive criticism has greatly helped me in the improvement of my manuscripts.

I give my most appreciated thanks to Dr. Uwe Hansen, the main supervisor of this study, for his valuable advice, his helpful guidance, his supervision, and his encouragement. Also his great help to improve the manuscripts.

I am grateful to Prof. Dr. Hans-Joachim Galla, the official advisor of this thesis, for his help that I could be a Ph. D. student and his valuable comments and criticism to make the thesis complete. I express my gratitude to Prof. Dr. Karl-Heinz Klempnauer, to be my third referee of this thesis.

I am appreciated for the kindness from Prof. Dr. Matt Paulsson and Dr. Raimund Wagener for the discussion in this study. I gratefully acknowledge them for some material and also from Dr. Daniela Seidler, Dr. Bastian Budde.

I also thank my colleagues in the matrix research group, Dr. Rita Dreier, Dr. Johannes Eble, Dr. Stephan Niland, Dr. Alexej Navdaev, Daniela Villone, Philipp Uhlig, Ferda Cevikbas, Sandra Kroppen, Birgit Günther, Tobias Steens, Karla de Santana Evangelista, and Filipe Andrich for creating such a pleasant social atmosphere in the lab and during the leisure time.

I owe my special thanks to Dr. Zerina Lokmic for her kindness to revising the language of the manuscripts. For excellent technical assistants, Gerburg Piltrup, Magret Bahl, Alletta Schmidt-Hederich, Marianne Ahler, Anne Forsberg, and the whole personnel of the Institute are acknowledged. Importantly, the

Leibniz-Institute of Arteriosclerosis Research: Cell Biology and Ultratructure Research for the electron microscope was accessible through Prof. Dr. Horst Robenek, Karin Schlattmann, Christina Köppler, and Marianne Jansen-Rust. In addition my thanks are given to my friends for their encouragement.

My dearest thanks are addressed to my parents and my big family. Their love and patience during the years have been an essential part of this thesis.

Finally, I honestly thank God for this opportunity and for seeing me through some truly difficult times, especially through the study of working this thesis.

Münster, February 2007

Wittaya Pimtong

Abstract

The biogenesis of functional extracellular matrices necessitates the appropriate combination and mutual association of matrix suprastructures, each comprising more than one molecular constituent. Collagen VI-containing aggregates are prominent examples of the suprastructural plasticity of extracellular matrix aggregates, depending on the exact composition.

In order to gain more insight into the organization and molecular compositions of matrix suprastructures we examined fibrilar fragments and collagen VI microfibrils from articular bovine cartilage. Authentic suprastructures were extracted by mechanical disruption of the tissue in PBS. Thereafter, fibrillar fragments were investigated by transmission and immuno-electron microscopy. We found collagen VI microfibrils are firmly associated by twisting around thin banded fibrils containing collagens II, IX, and XI. We also found that matrilin-1, biglycan and decorin are structural components of collagen VI-containing suprastructures. Moreover, we found that COMP is a novel component of collagen VI-containing suprastructures. Interestingly, we found biglycan bound to the globular domain of collagen VI microfibrils in a regular pattern that twisted around banded fibrils. Moreover, after treatment with 5 M guanidine hydrochloride, collagen VI microfibrils are still associated with banded fibrils. From these data we deduce that collagen VI microfibrils are covalently cross-linked to banded fibrils. Further, we investigated rib cages of newborn collagen IX- and biglycan-knockout mice to substantiate the role of these components in tethering collagen VI microfibrils to cartilage banded fibrils. We found a regular binding pattern of collagen VI microfibrils on banded fibrils in wild type as well as in biglycan knockout mice. This binding is disrupted in collagen IX knockout mice. Although, by using a binding assay, we found that collagen VI directly interacts with the NC4-domain of collagen IX with a relatively weak binding constant (Kd = 3×10^{-7} M). Nevertheless, the repetitive projection of NC4-domain of collagen IX present a substrate with multiple binding sites and, hence, an extremely high avidity for collagen VI microfibril binding. This data are supported by the result from *in vitro* fibrillogenesis experiment in which collagen VI microfibrils bound to reconstituted fibrils

containing collagens II, IX and XI but not to fibrils without collagen IX. Therefore, these data suggest that collagen IX serves as an adaptor between collagen VI microfibrils and the banded collagen fibrils.

Contents

Abbreviation

BSA	bovine serum albumin
COL	collageneous domain
Col9α1	α1-chain of collagen IX
COMP	cartilage oligomeric matrix protein
CR3	complement receptor type 3
CS	chrondroitin sulphate
DEAE	diethylaminoethyl
DMEM	dulbecco's modified eagle medium
DS	dermatan sulphate
EBNA	Ebstein-Barr nuclear antigen
ECM	extracellular matrix
EDTA	ethylenediamine tetra acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
ES	embryonic stem cell line
FACIT	fibril-associated collagens with interrupted triple helices
GAG	glycosaminoglycan
GuHCl	guanidine hydrochloride
HS	heparin/heparan sulphate
K_d	association constant
kDa	kilodalton
KS	keratan sulphate
kV	kilovolt
LRR	leucine-rich repeat
mA	milliampere
MAGP	microfibril-associated glycoprotein
MED	multiple epiphyseal usdysplasia
NC	non-collagenous domain
NEM	N-ethylmaleimide
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PG	proteoglycan
Pgk-1	phosphoglycerate kinase-1
PM	plasma membrane
PMSF	phenylmethanesulfonylfluoride
PSACH	pseudoachondroplasia
Pu	puromycin
rER	rough endoplasmic reticulum
SDS	sodium dodecyl sulphate
TBS	tris buffered saline
TBST	tris buffered saline + tween-20
TEM	transmission electron microscope
TEMED	N,N,N',N'-tetramethylethylenediamine
VWA	von Willebrand factor A-domain

1. INTRODUCTION

The extracellular matrix (ECM) is a component of all mammalian tissues and consists of a network of fibrous proteins, elastin, and collagens. The network is embedded in a viscoelastic gel rich in high molecular weight anionic polymers known as proteoglycans. This structure, which is quantitatively a major component in tissues such as cartilage, intervertebral disc, and blood vessels, endows tissues with the requisite mechanical properties and regulates the movement of water, nutrients, and other solutes. There is strong evidence that changes in these functions are associated with diseases such as arthritis, atherosclerosis, and cancer. The basic information of the molecular components and their suprastructures in extracellular matrix is requied for diagnosis and healing.

Collagen VI-containing aggregates are prominent examples of the suprastructural plasticity of extracellular matrix aggregates. Matrilin-1, biglycan, and decorin are components of collagen VI-containing suprastructure (Wiberg et al., 2003). However, the interactions of collagen VI-containing supra-structures with collagen banded fibrils and network-like structures are only partially known. In the present study, therefore, these interactions were investigated.

2. REVIEW OF THE LITERATURE

2.1. Cartilage

Cartilage is a type of connective tissue characterized by firmness and resilience. It is composed of cells called chondrocytes and an extracellular matrix which is produced by these cells. Unlike other connective tissues, cartilage is an avascular tissue. Therefore, the nutrition of cartilage occurs by diffusion of substances from capillaries in adjacent connective tissue or via synovial fluid from joint cavities and the chondrocytes (Stockwell, 1979). Further characteristics are the lack of nerve fibres and absence of a lymphatic system. Most properties of cartilage tissue are not dependent on the chondrocytes themselves, but rather on what the chondrocytes secrete and deposit in the extracellular space. There is a highly complex but well organized network of diverse suprastructures in the extracellular matrix. Most of the extracellular volume, however, is made up of water (~80%) (Miller, 1996). The important biomechanical properties of cartilage are elasticity and resistance to compression and deformation. The major factors determining these biomechanical are the physical characteristics of the individual matrix molecules and their interactions (Ratcliffe and Mow, 1996).

Cartilage is classified into elastic, fibrous, and hyaline cartilage based on its morphological appearance and the composition of its extracellular matrix (Horton, 1993).

2.1.1 Elastic cartilage

Elastic cartilage contains elastic fibrils in addition to the collagen fibrils. It is consequently more resilient than either the hyaline or fibrous cartilage. Elastic cartilage is found in structures that are subjected to continual bending, such as parts of the external ear and epiglottis.

2.1.2 Fibrous cartilage

Fibrous cartilage contains a large number of fibrous bundles containing collagen I embedded in its matrix and very few cells arranged in rows between

the bundles. The fibrous bundles provide greater tensile strength than in the other types of cartilage. The cushioning discs of fibrous cartilage between the vertebrae and the internal cartilages of the knee joints are enabled by their structure to stand up under the continuous heavy pressure to which they are subjected.

2.1.3 Hyaline cartilage

Hyaline cartilage is the most abundant type of cartilage and plays a central role in the formation and growth of the vertebrate skeleton (for review see Morris *et al*., 2002). It is semi-transparent and appears bluish-white in colour. Hyaline cartilage is strong, but flexible and elastic. It is derived, like other types of connective tissue, from mesenchymal cells. At the begining of about the fifth foetal week, the precursor cells become rounded and form densely packed cellular masses, called centres of chondrification (Stockwell, 1979). The cartilage-forming cells, chondroblasts, begin to secrete components of the extracellular matrix of cartilage (Stockwell, 1979). The extracellular matrix consists of ground substance (hyaluronan, chondroitin sulphate and keratin sulphate) and tropocollagen which polymerise extracellularly into fine collagen fibrils (Poole et al., 1982). Hyaline cartilage lines the bones in joints, articular cartilage, and is also present inside bones, serving as a centre of ossification or bone growth (Arey, 1974).

As the amount of matrix increases, the chondroblasts become separated from each other and become, from this time on, isolated in small cavities within the matrix. These cavities are called the lacunae. Concurrently the cells differentiate into mature chondrocytes.

Cartilage growth occurs by two mechanisms.

i) Interstitial growth where the chondroblasts within the existing cartilage divide and form small groups of cells called isogenous groups. These groups produce matrix to become separated from each other by a thin partition of matrix. Interstitial growth occurs mainly in immature cartilage.

ii) Appositional growth where mesenchymal cells surrounding the cartilage in the deep part of the perichondrium or the chondrogenic layer differentiate into chondroblasts. Appositional growth also occurs in mature cartilage.

The matrix near the isogenous groups of chondrocytes, territorial matrix, contains larger amounts and different types of glycosaminoglycans than the matrix more remote, interterritorial matrix, from the isogenous groups. An idealized chondrocyte and its matrix compartments are illustrated in Figure 2-1.

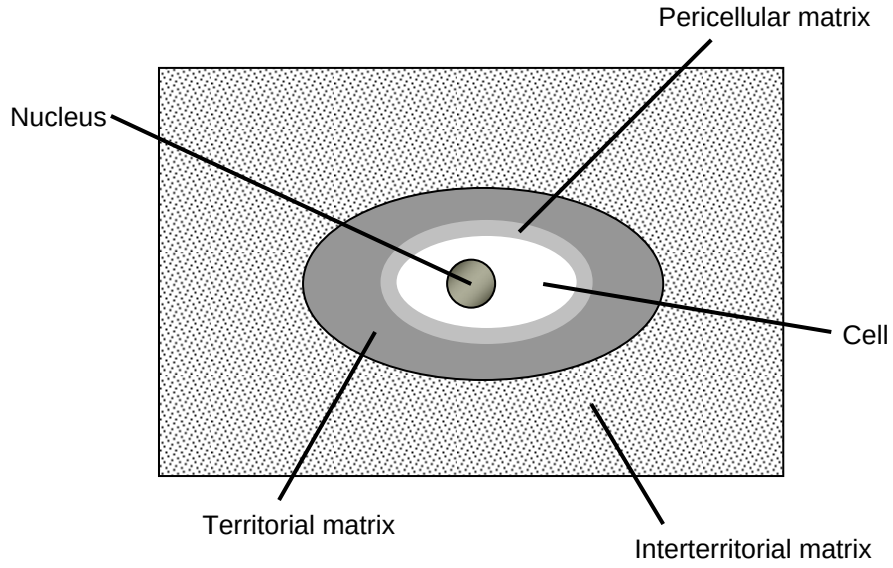

Figure 2-1. Idealized chondrocyte, depicting surrounding matrix compartments and other landmarks. Modified from Morris et al., 2002.

2.1.4 Articular cartilage

Articular cartilage is a specialised form of hyaline cartilage. It is usually divided into four zones: superficial, intermediate, deep, and calcified zone depending on the arrangement of chondrocytes and collagen fibrils (Figure 2-2). As with other cartilage types, there are differences in cellular and matrix morphology depending on joint location, degree of weight bearing, the age and species of cartilage. The articular cartilage of a growing bone does not have a calcified zone. It blends into epiphyseal cartilage that resides between the articular surface and the growth plate. This is more evident in species with large bones and large epiphyseal cartilages, such as human. However, it becomes

less apparent as secondary ossification centres in the epiphyseal cartilage encroach upon the articular region. Despite these differences, articular cartilage shares many features such as the suprastructure of the chondrocytes and matrix, the organization of matrix into compartment, and cell-matrix relationships, with each other as well as with other cartilage types.

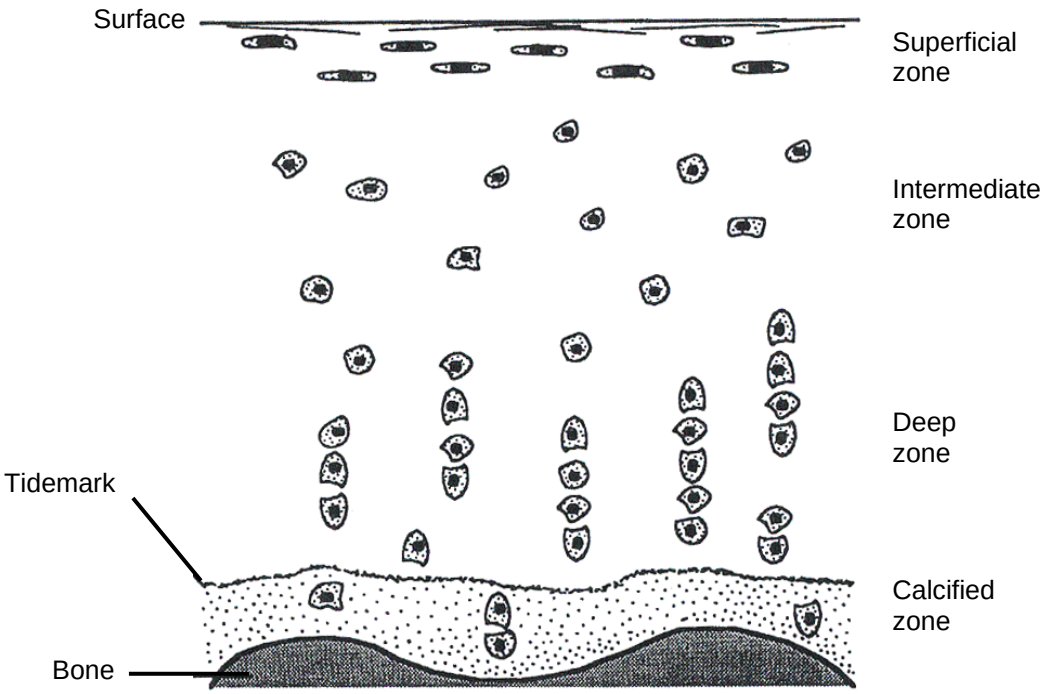

Figure 2-2. Schematic representation of adult articular cartilage showing the zones and organization of articular chondrocytes (according to Morris et al., 2002).

The superficial zone is the thinnest along the articular surface and widest at the articular margin, where it merges with the perichondrium. The articular surface is comprised of a thin layer of densely packed, fine collagen fibrils running parallel to the surface of cartilage (Weiss *et al.*, 1968). These fibrils have characteristic periodic banding, and many form bundles (Poole *et al.*, 1984; Cameron and Robinson, 1958). There are no cells in this fibrous layer. However, the lower part of the superficial zone contains flattened cells aligned parallel to the surface of cartilage. These chondrocytes display a cell-surface polarity (Schenk *et al.*, 1986; Weiss *et al.*, 1968). The cell membrane facing the surface tends to contain prominent invaginations, or caveolae, while many

cytoplasmic processes are found on the other side of the cell. Rough endoplasmic reticulum and Golgi complexes are sparse (Weiss *et al.*, 1968). The typical basket-like pericellular capsule and territorial matrix compartment are not prominent in this zone. Cationic dyes display less proteoglycan than in deeper zones. However, proteoglycans and proteoglycan link proteins can be detected by immunostaining (Schenk *et al.*, 1986; Poole *et al.*, 1982). The chondrocytes of the intermediate zone are more spherical, and the surface polarity seen in the superficial zone is much less apparent (Schenk *et al.*, 1986; Weiss *et al.*, 1968). The cells have more prominent perinuclear filaments, and rough endoplasmic reticulum and Golgi complexes are more abundant. There is more organization of matrix compartments, and chondrons with concentric capsular layers are apparent (Poole *et al.*, 1987). Collagen fibrils are thinner and more widely spaced, and display a faint periodic banding. Their orientation tends to be random, and bundles are rarely remarkable (Cameron and Robinson, 1958). The matrix of the intermediate zone stains intensely with cationic dyes for glycosaminoglycans (Stockwell, 1979).

The deep zone is distinguished by the tendency of the cells to align themselves perpendicularly to the joint suface. The collagen fibrils of the interterritorial compartment are thicker than in the intermediate zone. Furthermore, they exhibit the same orientation (Schenk *et al.*, 1986; Poole *et al.*, 1982). Rough endoplasmic reticulum and Golgi complexes are prominent in the chondrocytes (Weiss *et al.*, 1968; Eggli *et al.*, 1988). Cationic stains and immunostaining indicate abundant proteoglycan in this zone (Poole *et al.*, 1982). Schenk and colleagues have observed that the cells of the upper deep zone are rich in intermediate filaments and glycogen deposits compared with the cells of the lower deep zone. The border of this zone and the calcified cartilage zone is called tidemark (Schenk *et al.*, 1986). Matrix vesicles can be identified in the interterritorial matrix of the radial, as well as the superficial and intermediate zones, but they are not mineralized (Poole, *et al.*, 1984).

2.2 Molecular constituents of hyaline cartilage

The extracellular matrix of hyaline cartilage contains two major supramolecular elements: a framework formed by collagen II-rich fibrils, and a

hydrated substance with a high content of the cartilage-specific proteoglycan aggrecan. The proteoglycan-rich gel has a high osmotic swelling pressure. Fibrils strongly resist tension, and the fibrillar network contains the swelling pressure. As a consequence, the tissue is provided with mechanical stiffness and elasticity, which is essential for its capacity to withstand shearing and compressive forces. These properties are required particularly in mature joint cartilage where mobility is combined with tolerance for extremely high loads. The extracellular matrix of adult articular cartilage shows a complex suprastructural pattern. Fibril diameters and the preferential orientation of fibrils vary with the location within the tissue (Lane and Weiss, 1975; Poole *et al.*, 1984; Hunziker and Herrmann, 1990). The interfibrillar proteoglycan organizations also vary in the four zones within the cartilage (Poole *et al.*, 1982).

Over recent years, our understanding of cartilage matrix biology has been substantially extended. A number of novel, less-abundant matrix proteins have been identified any characterized. Furthermore, some cases of heriditary cartilage diseases do not show abnormalities with respect to the major gene products. This additional information made it clear that cartilage matrix organization and function is much more complex than originally thought. Most of these proteins are introduced in more detail below.

2.2.1 Collagens

The most abundant proteins in the extracellular matrix are members of the collagen family. They play a dominant role in maintaining the structure of various tissues and also have many other important functions. For example, collagens are involved in cell adhesion, chemotaxis and migration. Furthermore, the dynamic interplay between cells and collagens regulates tissue remodeling during growth, differentiation, morphogenesis, wound healing, and many pathologic states.

All collagen molecules compose three polypeptide chains, called α chains, and contain at least one domain composed of repeating Gly-X-Y sequences in each chain (for review see Ricard-Blum *et. al*., 2005). In some collagens all three α chains are identical whereas, in others, the molecules

contain two or even three different α chains. The three α chains are each coiled into a left-handed helix and are then twisted around a common axis to form a triple helix with a shallow right-handed superhelical pitch, so that the final structure is a rope-like rod (Fleischmajer *et al*., 1990; Engel and Prockop, 1991; van der Rest and Garrone, 1991; Hulmes, 1992; Kielty *et al.*, 1993). The presence of glycine, the smallest amino acid, in every third position is essential for the packing of this coiled-coil structure. The X and Y position can be occupied by any amino acid other than glycine, but proline is often found in the X position and 4-hydroxyproline in the Y position (for review see van der Rest and Garrone, 1991). The 4-hydroxyprolines are essential for the thermodynamic stability of the triple-helix (Ramachandran and Ramakrishnan, 1976). The conformation of the triple helix places the side chains of amino acids in the X and Y positions on the surface of the molecule. This arrangement enables many collagens to polymerize, since the multiple clusters of hydrophobic and charged side chains direct self-assembly into precisely ordered structures. The triple helix is relatively rigid. In some contexts, the resistance of the molecule to extension or compression is important for the biological function of the protein.

Vertebrates have at least 28 different collagen types with 43 distinct α chains in total, while more than 20 additional proteins have collagen domains. Based on their structure and supramolecular organization, collagens can be grouped into

i) fibril-forming collagens
ii) fibril-associated collagens with interrupted triple helices (FACIT)
iii) network-forming collagens
iv) basement membrane collagens
v) others with unique functions.

Collagens I-XIX have been thoroughly reviewed previously (Figure 2-3) (Kivirikko and Prockop, 1995; Kadler, 1995; Myllyharju and Kivirikko, 2001; Kielty and Grant, 2002), whereas collagens XX-XXVIII (Table 2-1) have been reported only in the past five years (Koch, *et al.*, 2001; Fitzgerald and Bateman, 2001; Banyard, *et al.*, 2003; Koch, *et al.*, 2003; Hashimoto, *et al.*, 2002; Sato, *et al.*, 2002; Pace, *et al.*, 2003; Boot-Handford, *et al.*, 2003; Veit, *et al.*, 2006).

The different collagens are characterized by considerable complexity and diversity in their structures, their variants, the presence of additional, non-helical domains, their assembly, and their functions. The most abundant and widespread family of collagens with about 90% of the total collagen is represented by the fibril-forming collagens. Fibrils containing collagens I and V contribute to the structural component of bone while collagens II and XI are found predominantly in the fibrillar matrix of articular cartilage. Their torsional stability and tensile strength lead to the stability and integrity of these tissues (von der Mark, 1999; Birk, *et al.*, 1988; Mayne, 1989). Collagen IV, with a more flexible triple helix, assembles into networks restricted to basement membranes. The collagen VI microfibril is highly disulfide cross-linked and contributes to a network of beaded filaments interwoven with other collagen fibrils (von der Mark, 1984). Fibril-associated collagens with interrupted triple helices (FACIT) such as collagens IX, XII, and XIV are associated with banded fibrils and they can be integral parts and important organizers of the overall fibril structure rather than optional addition to preexisting aggregates (Birk and Bruckner, 2005). Collagens VIII and X form hexagonal networks while collagens XIII and XVII even span cell membranes (van der Rest and Mayne, 1987). Transmembrane collagens are collagens XIII, XVII, XXIII, and XXV, each containing a single-pass hydrophobic transmembrane domain (Peltonen *et al.*, 1999; Schäcke *et al.*, 1998; Banyard *et al.*, 2003; Hashimoto *et al.*, 2002).

Given the diversity of the collagen superfamily, it is not surprising that mutations in collagen genes or deficiencies in the activities of specific post-translational enzymes of collagen synthesis have been discovered in many heritable disorders such as osteogenesis imperfecta (Roughley *et al.*, 2003), chondrodysplasias (Cohn, 2001; Vikkula, 1993, 1995), several subtypes of the Ehlers-Danlos syndrome (Eyre *et al*., 2002; Nuytinck *et al*., 2000), and several forms of epidermolysis bullosa (Bruckner-Tuderman *et al.*, 1999). Intriguingly, collagen gene defects have also been found in certain forms of common disease, such as osteoporosis (Laitinen, 1976; Knott *et al*., 1995), osteoarthritis (Hollander *et al*., 1994) and aortic aneurysm (Kuivaniemi *et al*., 1991).

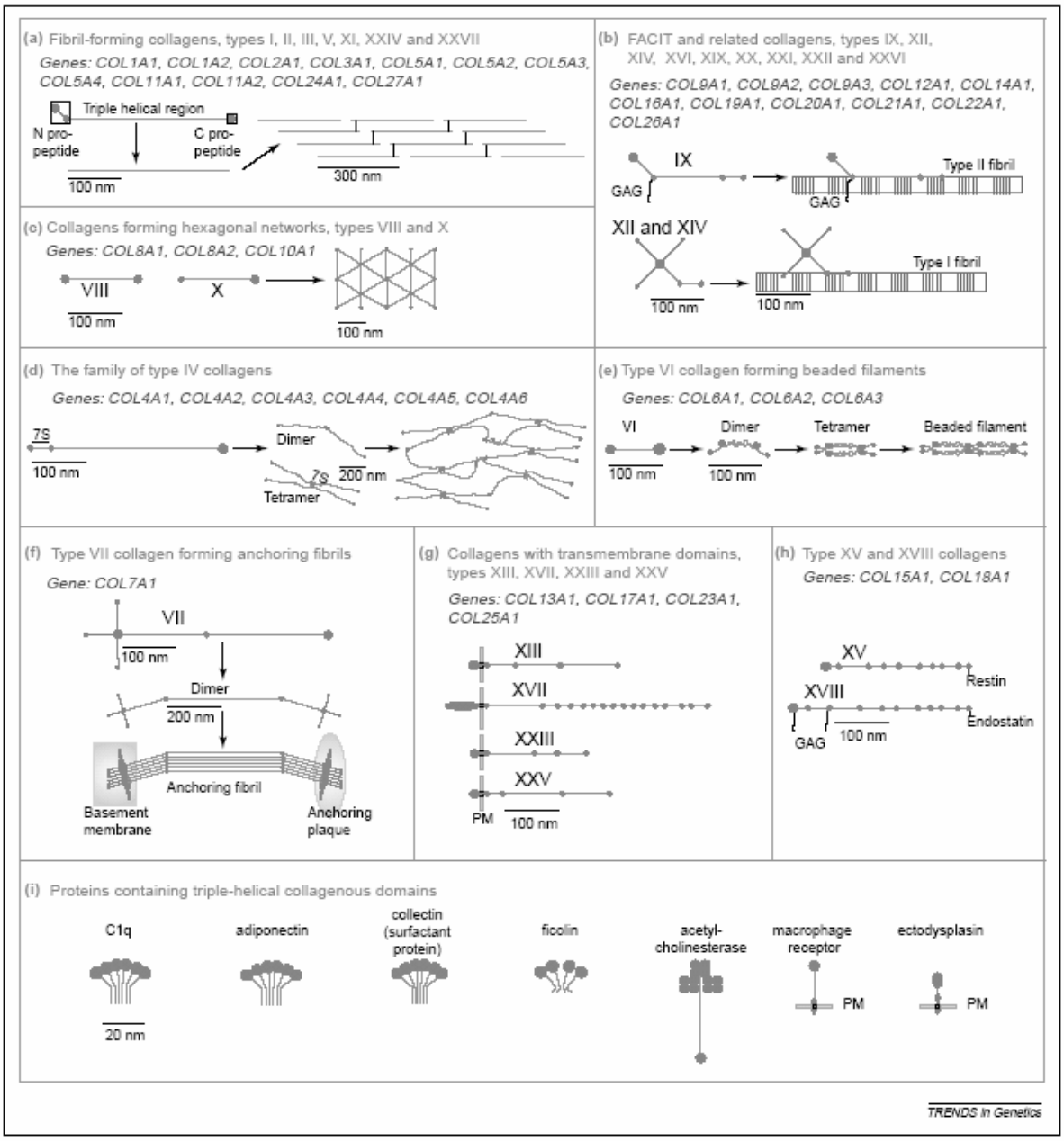

Figure 2-3. Members of the collagen superfamily and their known supramolecular assemblies. The collagen superfamily can be divided into nine families on the basis of the supramolecular assemblies and other features of its members: (a) fibril-forming collagens; (b) fibril-associated collagens with interrupted triple helices (FACITs) located on the surface of fibrils, and structurally related collagens; (c) collagens forming hexagonal networks; (d) the family of collagen IV located in basement membranes; (e) collagen VI, which forms beaded filaments; (f) collagen VII, which forms anchoring fibrils for basement membranes; (g) collagens with transmembrane domains; and (h) the family of collagens XV and XVIII. The supramolecular assemblies of families (g) and (h) are unknown and are therefore not shown in this figure. The polypeptide chains found in the 27 collagen types are coded by 42 genes in total, each molecule consisting of three polypeptide chains that can be either identical or different. An additional highly heterogenous group (i) within the superfamily comprises proteins that possess collagenous domains but have not been defined as collagens. Some of the group (i) proteins could also be defined as collagens, although some of the collagens might also belong to this group because there are no distinct criteria for distinguishing between a collagen and a protein containing a collagen domain(s). Abbreviation: PM, plasma membrane. (according to Myllyharju and Kivirikko, 2004).

Table 2-1. Collagen types and their expressions, modified and updated from Gelse *et al*. 2003.

Type	Chain	Location
I	α1(I), α2(I)	Most connective tissues
II	α1(II)	Cartilage, vitreous humour
III	α1(III)	Extensible connective tissues, e.g. skin, lung, vascular system
IV	α1(IV), α2(IV), α3(IV), α4(IV), α5(IV), α6(IV)	Basement membranes
V	α1(V), α2(V), α3(V)	Tissues containing collagen I, quantitatively minor component
VI	α1(VI), α2(VI), α3(VI)	Most connective tissues
VII	α1(VII)	Anchoring fibrils
VIII	α1(VIII), α2(VIII)	Many tissues, especially endothelium
IX	α1(IX), α2(IX), α3(IX)	Tissues containing collagen II
X	α1(X)	Hypertrophic cartilage
XI	α1(XI), α2(XI), α1(II)[a]	Tissues containing collagen II
XII	α1(XII)	Tissues containing collagen I
XIII	α1(XIII)	Many tissues
XIV	α1(XIV)	Tissues containing collagen I
XV	α1(XV)	Many tissues
XVI	α1(XVI)	Many tissues
XVII	α1(XVII)	Skin hemidesmosomes
XVIII	α1(XVIII)	Many tissues, especially liver and kidney
XIX	α1(XIX)	Rhabdomyosarcoma cells
XX	α1(XX)	Corneal epithelium, skin, cartilage and tendon
XXI	α1(XXI)	Many tissues
XXII	α1(XXII)	Tissue junctions
XXIII	α1(XIII)	Metastatic tumour cells
XXIV	α1(XXIV)	Developing bone and cornea
XXV	α1(XXV)	Neurons
XXVI	α1(XXVI)	Testis, ovary
XXVII	α1(XXVII)	Cartilage, eye, ear and lung
XXVIII	α1(XXVIII)	Sciatic nerve

[a]The α3(XI) chain of collagen XI is encoded by the same gene as the α1(II) chain of type.

2.2.1.1 Cartilage collagens

Six collagens (collagens II, VI, IX, X, XI, and XXVII) have been identified in articular cartilage. Collagen II, a homotrimer composed of α1(II) chains, is the most abundant fibrillar protein found in articular cartilage and constitutes 80-85% of the total collagen content (for review see Cremer et al., 1998). Collagens IX and XI are present in smaller quantities than collagen II, ranging between 3 and 10% each, depending on the cartilage species and age. Unlike collagen II, collagens IX and XI are heterotrimers. Collagen IX has a large globular non-collageneous (NC) domain N-terminus from α1(IX) and is further characterized by two short NC segments that interrupt the helix. One of these short NC segments creates a "kink" in the molecule which is a site of a chondroitin sulphate rich glycosaminoglycan chain extending from α2(IX) (van der Rest and Mayne, 1988). Cartilage collagen IX differs from vitreous collagen IX, because the former retains a large NC4 domain (Brewton *et al.*, 1991) and has a tenfold shorter chondroitin sulphate chain (Yada *et al.*, 1990). Collagen XI resembles collagen II in structure because the helices of both molecules are uninterrupted by NC domains; some collagen XI molecules, however, retain an extractable α3(XI) N-propeptide which projects from the fibril (Thom and Morris, 1991).

Collagens XI and II are quite similar with respect to their α3(XI) and α1(II) chains which are mostly encoded by the same gene. A higher degree of glycosylation of α3(XI), however, indicates differences in posttranslational processing. Collagen XI also share similarity with collagen V, as demonstrated by significant sequence homology between α1(XI) and α1(V) and between α2(XI) and α2(V) (Seyer and Kang, 1990; Mayne *et al.*, 1993). Studies of the vitreous body of the eye suggest that, at least in this environment, collagen V and collagen XI are not separate collagens (Mayne *et al.*, 1993). The possibility that α1(XI) and α2(XI) can be expressed in non-cartilaginous tissues, in the absence of α3(XI), is suggested by the detection of mRNA for both α-chains in embryonic tissue. It is presently unknown whether α1(XI) and α2(XI) can combine to create homotrimers or heterotypic collagen XI (Lui *et al.*, 1995).

Collagen X is also cartilage-specific and a homotrimer, although considerably shorter than collagens II and XI. It is synthesized by terminally differentiating chondrocytes, i.e. hypertrophic chondrocytes (Schmid and Linsenmayer, 1985). It is thought that the biological function of collagen X is to facilitate the process of calcification possibly through matrical organization changes (Kwan *et al*., 1991). The expression of collagen X is restricted in the matrix of hypertrophic chondrocytes, suggesting that it is of major importance in endochondral bone growth and development. Furthermore, the mutations in collagen X may be responsible for some chondrodysplasias in humans and mice (Kwan *et al.*, 1989).

Other collagens have also been identified which are collagen VI and collagen XXVII in cartilage. However, these collagens are also present in non-cartilaginous tissues. Collagen VI is well characterized. Homotrimeric and heterotypic forms of collagen VI exist which contain helical domains shorter than those found in collagen X and large globular termini which make up two-thirds of the molecule (von der Mark *et al.*, 1984). Collagen VI is addressed in more detail in the next section.

Collagen XXVII is a novel vertebrate fibrillar collagen that is highly conserved in human, mouse and fish (*Fugu rubripes*). The pro-α1(XXVII) chain has a domain structure similar to that of α1(V), α3(V), α1(XI), and α2(XI). However, compared with other vertebrate fibrillar collagens, collagen XXVII has unusual molecular features. Collagen XXVII has a short chain selection sequence within the NC1 domain (Pace *et al*., 2003; Boot-Handford *et al.*, 2003).

2.2.1.2 Supramolecular assemblies of cartilage collagens

The biogenesis of functional extracellular matrices necessitates the appropriate combination and mutual association of matrix suprastructures, each comprising more than one molecular constituent. The following topics are prominent examples of supramolecular assemblies of collagens in cartilage matrix.

(i) Organization of collagens in cartilage banded fibrils

The prototypic cartilage collagen fibril, original form, is an assembly of collagens II, IX, and XI (Mendler *et al*., 1989). Collagen II represents about 80% of the collagens of the cartilage fibrils. The remaining 20% are contributed to an equal extent by collagens IX and XI (Eyre et al., 1987). Collagens II and XI molecules are arranged in longitudinally staggered arrays of molecules of a length that is being a non-integer multiple of the stagger between next neighbors. Therefore, a gap occurs sequentially between neighboring molecules giving rise to a gap-overlap structure in the collagen fibrils with a D-periodic banding (Figure 2-4, Birk and Bruckner, 2005).

In addition, depending on their precise tissue origin cartilage fibrils also contain the FACIT collagens IX (Müller-Glauser *et al.* 1986; Eyre *et al.*, 1987, 2004; van der Rest and Mayne, 1988) or XVI (Kassner *et al.*, 2003) or are associated with the small leucine-rich proteoglycan (SLRP) decorin (Hagg *et al.*, 1998). Therefore, some composite fibrils comprise a heterotypic fibril body encompassing parts of all three collagens from which collagen IX can project to the exterior with its aminoterminal globular NC4-domain (Vaughan et al., 1988). However, it is possible to distinguish between different populations of fibril. Thin fibrils containing collagens II, IX, and XI with uniform diameters coexist with large fibrils with heterogeneous diameters containing collagens II and XI. Notably, collagen IX is always absent from the large fibrils (Figure 2-5, Hagg *et al.*, 1998). Thin collagen fibrils are particularly enriched in the territorial matrix embedding the chondrocytes by forming basket-like structures (Poole, 1992) whereas larger collagen fibrils were exclusively found in the interterritorial region more remote from the chondrocytes.

Cross-linking studies have identified at least six sites of cross-linking within the collagen IX molecule where covalent bonds form with either collagen II molecules or with other collagen IX molecules (Wu *et al.*, 1992; Ichimura *et al.*, 2000; Diab *et al.*, 1996) (Figure 2-6). The cross-linking residues are either trivalent pyridinolines or divalent borohydride-reducible intermediates formed by the same lysyl oxidase-mediated mechanism as occurs in the major fibril-forming collagens (Eyre *et al*., 2004). Figure 2-6 shows how collagen IX

molecules can be accommodated on a fibril surface and can satisfy all the covalent interactions so far identified. In the model, the COL1/NC1-domain docks in the space region, oriented as shown in Figure 2-6, and the molecule hinges back on itself at the NC2-domain.

Collagen XI is located largely within the fibrils, where it is covalently linked with collagen II via hydroxylysine-based aldehyde cross-links. Collagen XI is found in greatest quantities in small fibrils. Cross-linked peptide analyses have shown that collagen XI molecules are cross-linked to each other through their N-telopeptide-to-helix interaction sites (Wu and Eyre, 1995). Interestingly, the N-telopeptide cross-linking lysines are located externally to candidate metalloproteinase cleavage sites, in α1(XI), α1(V), and α2(XI). It implies that any of such cleavages could selectively depolymerise collagen XI (Wu and Eyre, 1995). The N-terminal helical cross-linking site of collagen XI molecules is occupied by α1(II) C-telopeptide in α1(XI). By analogy to findings with the collagen I/V copolymeric fibrils of bone (Niyibizi and Eyre, 1994), this is consistent with the formation of lateral cross-links between collagens II and XI molecules at this locus. Together, these findings can be interpreted as collagen XI initially forming a head-to-tail self-cross-linked filament. This filament becomes integrated and cross-linked laterally onto or within the body of collagen II fibrils. Collagen XI could conceivably form an interconnecting, secondary filamentous network that provides links between fibrils as well as running within fibrils, not inconsistent with the concept that collagen XI restricts the lateral growth of collagen II-containing fibrils (Blaschke *et al.*, 2000).

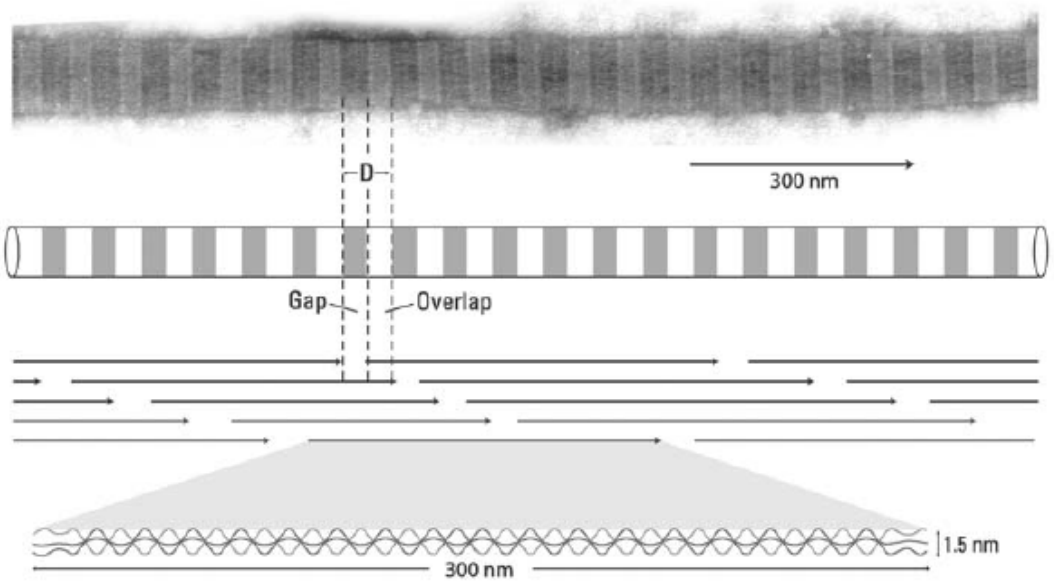

Figure 2-4. Structure of a generic collagen fibril. A D-periodic collagen fibril from tendon is presented at the top of the panel. The negative stained fibril has a characteristic alternating light/dark pattern representing the gap (dark) and overlap (light) regions of the fibril. The diagram represents the staggered pattern of collagen molecules giving rise to this D-periodic repeat.The collagen molecules (arrows) are staggered N to C. The fibrillar collagen molecule is approximately 300 nm (4.4 D) in length and 1.5 nm in diameter. (according to Birk and Bruckner, 2005).

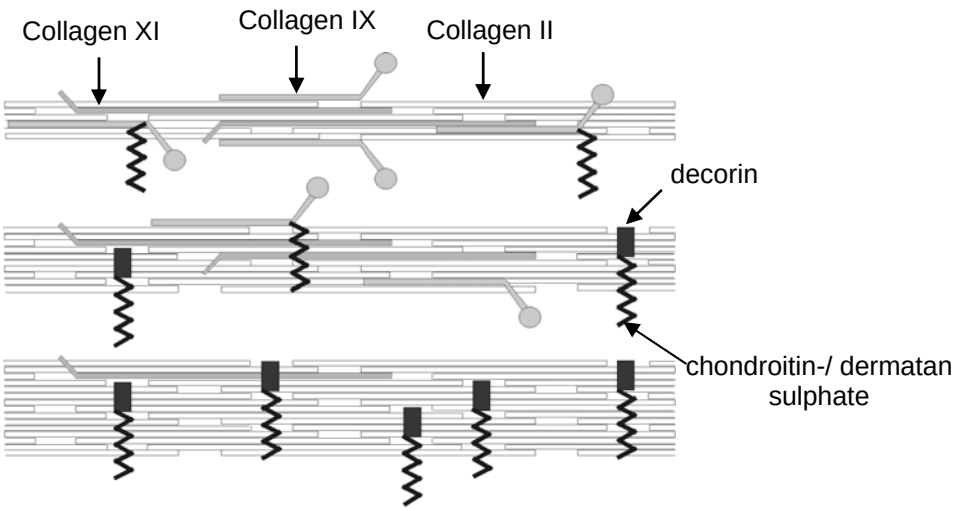

Figure 2-5. Schematic representation of the biochemical heterogeneity of D-periodic fibrils in mature articular cartilage. The thinnest fibrils are enriched in collagens IX and XI. Fibrils of intermediate size have lower contents of collagen IX, and the small proteoglycan decorin can occur as an additional component. The coexistence of collagen IX and decorin on the fibril surface is a notable feature. The thickest fibrils lack collagen IX but they contain some collagen XI and frequently decorin. Modified and up-dated from Hagg *et al.*1998.

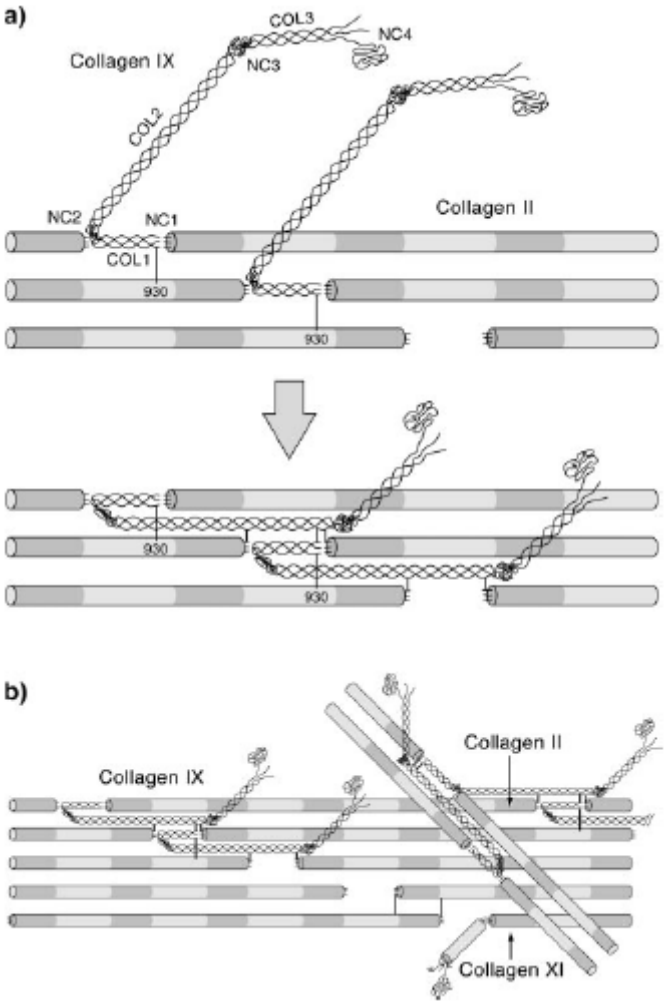

Figure 2-6. Molecular model showing how collagen IX molecules might initially interact with nascent collagen II molecules and fold to accommodate known cross-linking interactions. *a*) the NC1(IX) to Lys-930(II) cross-link is presented as an early event with folding through the NC2 domain to accommodate the other, and cross-links between collagens II and IX molecules. *b*) the *bottom* structure indicates the potential for interfibrillar bonds between collagen IX and IX molecules on intersecting collagen II/XI-containing fibrils. (according to Eyre *et al.*, 2004).

(ii) Collagen VI networks

Collagen VI has a ubiquitous distribution throughout connective tissues. It is assembled into different tissue forms, including hexagonal networks and broad banded structure (von der Mark et al., 1984; Bruns et al., 1986). Moreover, it is found as an extensive microfibrillar network (Kielty and Grant, 2002). Collagen VI is important in maintaining the integrity of tissues such as

blood vessels, lung, and skin. Furthermore, the linkage of mutations in the collagen VI genes forms leads to muscle weakness and wasting such as observed in Bethlem myopathy (Scacheri *et al.*, 2002) and Ullrich syndrome (Camacho *et al.*, 2001).

Collagen VI is a large collagenous glycoprotein composed of three different α-chains, the α1(VI), α2(VI), and α3(VI) (Chu *et al.*, 1987). Each chain consists of a short collagenous domain and large globular domains at the N- and C-termini. The majority of the globular domains of collagen VI have a homology to von Willebrand factor A-domains (VWA) with a molecular mass of approximately 21 kDa. Varying numbers of these domains are found at the N- and C-termini of all three chains [Figure 2-7(a)] (Chu *et al.*, 1990). Structural studies on the A-domain of integrin CR3 have shown A-domains to be composed of a central β-sheet flanked on each side by three α-helices which is highly reminiscent of the Rossmann dinucleotide-binding fold (Huizinga, 1997).

By selective reduction of microfibril interchain disulphide bonds, monomers, dimers, and tetramers could be formed (Furthmayr *et al.*, 1983). By a combination of pepsin digestion, analytical ultracentrifugation, selective reduction and rotary shadowing transmission electron microscopy, a model of assembly of collagen VI has been proposed. This is shown in Figure 2-7(b). The model proposes that dimers are formed by the laterally staggered alignment of two monomers in an anti-parallel manner, giving rise to a 75 nm long region of two inter-twisted triple helices [Figure 2-7(b)] (Furthmayr *et al.*, 1983). The globular domains lie at the end of the triple-helical region corresponding to a left-handed superhelix with a pitch of 37.5 nm (Knupp and Squire, 2001). Discontinuities in the Gly-X-Y sequence are predicted to break the supercoil into segments which is called the collagen-segmented supercoil. Still within the cell, collagen VI dimers align with their ends in register to form tetramers (Furthmayr *et al.*, 1983) which are the secreted form of the molecule [Figure 2-7(b)]. The C2-domain is required for dimer and tetramer formation, and thus, the non-collagenous sequences play a central role in these assembly steps. Once in the extracellular space, end-to-end accretion of tetramers leads to microfibril formation. Collagen VI tetramers associate to form microfibrils in a non-covalent manner which is presumed to be mediated through A-domain interactions.

Collagen VI interacts with a spectrum of extracellular matrix molecules including collagens I, II, IV, XIV, microfibril-associated glycoprotein (MAGP-1), perlecan, decorin, biglycan, hyaluronan, heparin, and fibronectin as well as integrins and the cell-surface proteoglycan NG2 (Birk and Bruckner, 2005). For example, complexes of collagen VI microfibrils with matrilin-1, -3, -4, biglycan, and decorin are found in isolated Swarm rat chondrosarcoma cells as well as in the reconstitution products of isolated proteins (Figure 2-8, Wiberg *et al*., 2003). In these complexes, decorin and biglycan interact with VWA domains in the N-terminal region of collagen VI and that matrilins were in turn bound to these small leucine-rich repeat proteoglycans. At the periphery of these assemblies, matrilins could be seen to connect the collagen VI-containing microfibrils to aggrecan core proteins or to collagen II-containing fibrils. Therefore, supramolecular aggregates of collagen VI are composite structures with other integrated molecules modulating the functional properties of the collagen VI-containing suprastructure (Birk and Bruckner, 2005). In addition, collagen VI microfibrils have been proposed to play an important role in matrix organization due to their interactions with several matrix components (Kielty and Grant, 2002).

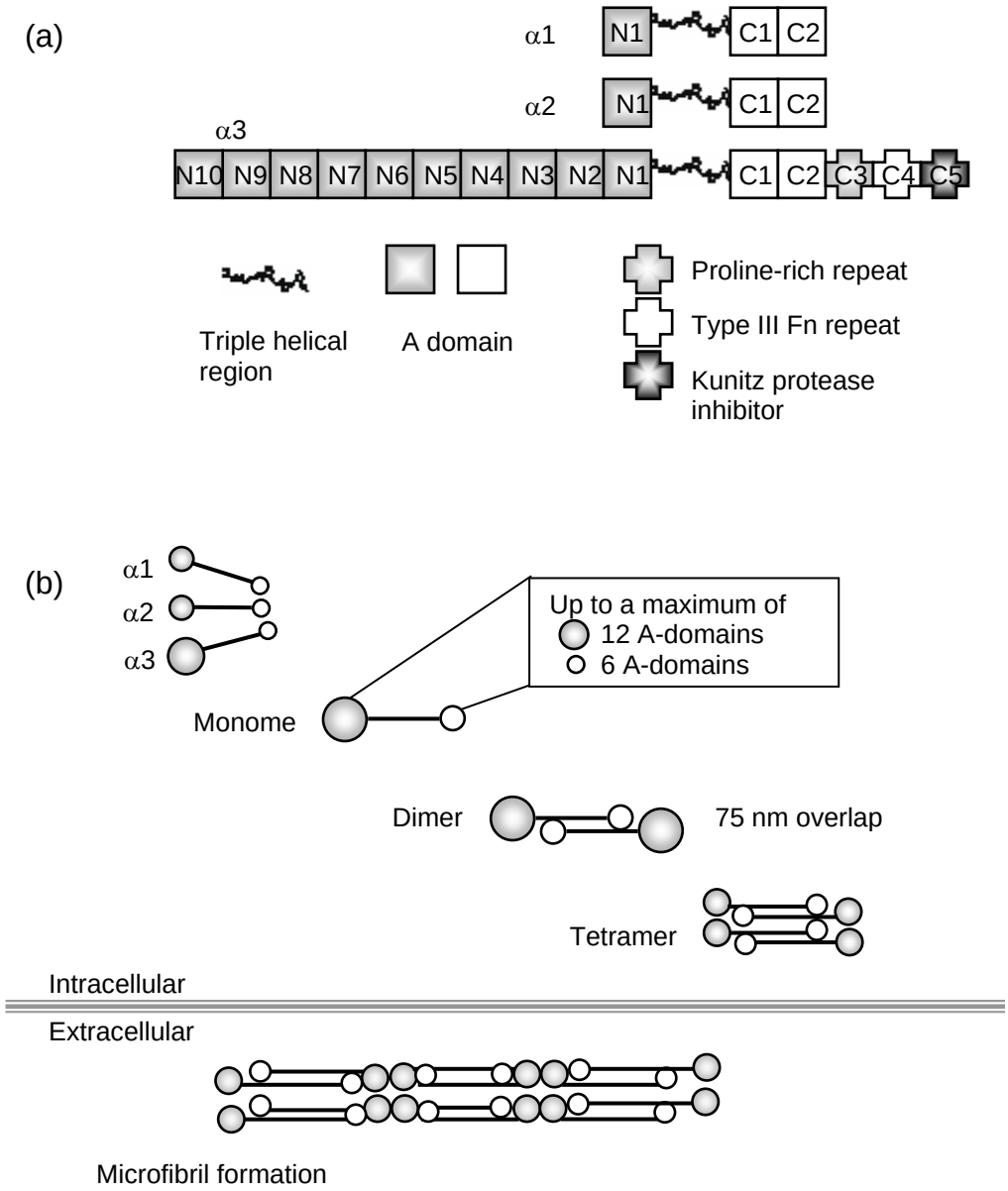

Figure 2-7. Domain organisation and assembly of the alpha chains from collagen VI: (a) A diagram showing the organisation of domains in the α1, α2 and α3 chains of collagen VI. Gray and white rectangles represent N- and C-terminal VWA domains, respectively. The gray, white and black crosses represent the proline-rich region, Fibronectin III repeat and Kunitz protease inhibitor domain, respectively. The collageneous region is shown in black as a molecular structure. (b) A diagram showing the assembly of collagen VI microfibrils from the three α chains. The formation of monomers, dimers and tetramers occurs intracellularly, while microfibril assembly occurs in the extracellular space (modified from Baldock *et al.*, 2003).

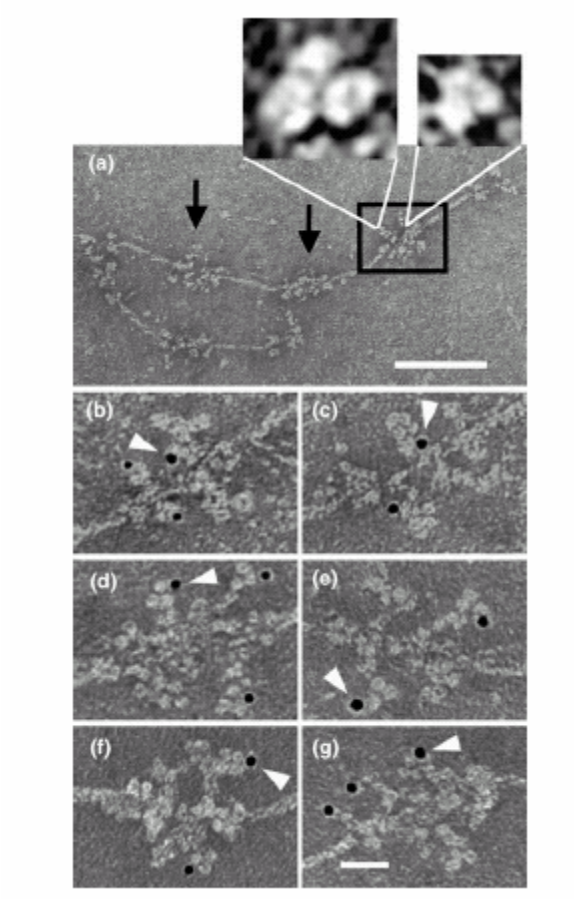

Figure 2-8. Structure of intact collagen VI from the Swarm rat chondrosarcoma as seen by electron microscopy after negative staining. (a) Collagen VI microfibrils with different molecules bound close to the N-terminal parts of the collagen VI tetramers are visible (arrows). Representative particles exhibiting multidomain structures of different sizes are shown at higher magnification (insets). Using specific gold-labelled antibodies these molecules are identified as biglycan (b), decorin (c), matrilin-1 (d), matrilin-3 (e), matrilin-4 (f) and chondroadherin (g). Thus, complexes of matrilin-1, -3, or -4 and the leucine-rich repeat proteoglycans biglycan or decorin, binding close to the collagen VI N-termini, are identified. The bar represent 100 nm (a) and 20 nm [(b) - (g)] (Wiberg *et al.*, 2003).

2.2.2 Proteoglycans

Proteoglycan (PG) is a group of biomolecules consisting of a core protein and one or more covalently attached glycosaminoglycan chains. PGs are characterized as having a polyanionic nature conferred for the most part by sulphate substitutents on carbohydrate residues and by the carboxyl group of the hexuronic acid moieties (for review see Iozzo, 1998). These glycosaminoglycan chains define the class of the PG by forming a generaly repeating disaccharide backbone. The PGs heparin, heparan sulphate (HS),

chrondroitin sulphate (CS), and dermatan sulphate (DS) backbones compose of [hexuronic acid-N-acetylhexosaminel]$_n$ which can have sulphate ester substituents on either the uronic acid or hexosamine or both (Price *et al.*, 1992). For keratan sulphate (KS) PGs, [galactose-N-acetylglucosamine]$_n$ defines the carbohydrate backbone with the polyanionic character conferred by the presence of sulphate groups on the galactose and hexosamine residues. These GAG modifications result in the production of mature PGs whose biophysical properties would be expected to differ. These are based upon the extent of sulphate substitutions and hexuronic acid epimerization.

The PGs such as biglycan, decorin, and fibromodulin contain core proteins whose structure (a duplicated leucine-rich repeat) confers an amphipathic nature to the intact PGs (Fisher *et al.*, 1989; Krantz *et al.*, 1991; Neame *et al.*, 1989; Oldberg *et al.*, 1989). Other PGs, such as aggrecan or versican, consist of peptide sequences that confer lectin-like properties and have epidermal growth factor (EGF)-like repeats. These EGF-like repeats have the potential to influence cellular metabolism (Baldwin *et al.*, 1989; Doege *et al.*, 1987; Krusius *et al.*, 1987). These large CSPGs also have very large core proteins (M_r > 200,000 daltons) which affect their behaviour in solution and isolation yields. The core protein sequence that determines the identity, and hence the name of a PG, such as for the large CSPGs aggrecan (Doege *et al.*, 1991) and versican (Zimmermann and Ruoslahti, 1989), the chondroitin sulphate proteoglycan IX collagen (Huber *et al.*, 1988; Yada *et al.*, 1990), the HSPGs syndecan (Saunders *et al.*, 1989), perlecan (Noonan *et al.*, 1991), betaglycan (Andres *et al.*, 1989), glypican (David *et al.*, 1990) fibroglycan (Marynen *et al.*, 1989), the small CS/DSPGs biglycan (Fisher *et al.*, 1989) and decorin (Ruoslahti, 1988). In the following section, biglycan and decorin, which were studied in the present work, are briefly discussed.

2.2.2.1 Biglycan

Biglycan is a member of the leucine-rich repeat (LRR) protein family. It is composed of a 38 kDa core protein that is substituted with two glycosaminoglycan chains on the N-terminal Ser-Gly sites. The core protein contains ten leucine rich repeats flanked by disulphide bond stabilized loops on

both sides and additional sites for glycosylation (N-linked glycosylation sites) within the leucine-rich repeats (Fisher *et al.*, 1991, Neame *et al.*, 1989). The quality of the glycosaminoglycans varies both in regard to the length and composition. The backbone of the glycosaminoglycan chain is composed of repeating disaccharide units of N-acetylgalactosamine and glucuronic acid. Glucuronic acid is often converted into iduronic acid through epimerization at carbon 5. As the chains are elongated they are modified by sulphation resulting in chondroitin sulphate and dermatan sulphate, respectively.

The degree of epimerization and sulphation varies between tissues (Cheng *et al.*, 1994). An isoform of biglycan with a single glycosaminoglycan substitution has been found (Jarvelainen *et al.*, 1991). Biglycan interacts with collagen VI and the complement component C1q (Krumdieck *et al.*, 1992, Hocking *et al.*, 1996). Conflicting data exists whether biglycan interacts with fibrillar collagens (Wiberg *et al.*, 2001; Bidanset *et al.*, 1992). Thus biglycan binds to the immobilized collagen but appears not to precipitate with collagen fibrils (Svensson *et al.*, 1995). It is also a Zn^{2+}-binding protein (Yang *et al.*, 1999). A distinct tight interaction of the core protein of biglycan with collagen VI at the border between the N-terminal non-triple helical and triple helical domains has been shown (Wiberg *et al.*, 2001, 2002, 2003). The interaction with the collagen molecule is not modulated by the GAG chains. Furthermore, biglycan also interacts with transforming growth factor-β (Hildebrand *et al*., 1994). Biglycan is dimeric in solution and the crystal structure of the glycoprotein core of biglycan has an appositon of the concave inner surface of the leucin-rich repeat domains (Scott *et al.*, 2006).

2.2.2.2 Decorin

Decorin is also a member of the leucine-rich repeat (LRR) protein family and is composed of a 36.5 kDa core protein substituted with one glycosaminoglycan chain on the N-terminal Ser-Gly site (Krusius and Ruoslahti, 1986). The core protein contains ten leucine rich repeats flanked by disulphide bond stabilised loops on both sides and additional sites for glycosylation (N-linked glycosylation sites) within the leucine-rich repeats (Krusius and Ruoslahti, 1986). The glycosaminoglycan chain backbone is composed of repeating

disaccharide units of N-acetylgalactosamine and glucuronic acid, the latter often being converted into iduronic acid through epimerization at carbon 5. As the chains are elongated they are modified by sulphation resulting in chondroitin sulphate and dermatan sulphate, respectively. The degree of epimerization and sulphation varies between tissues (Cheng *et al.*, 1994). Decorin can also exist without glycosaminoglycan substitutions or with two glycosaminoglycan substitutions (Fleischmajer *et al.*, 1991, Sampaio *et al.*, 1988).

Decorin was shown to interact with collagens I and II via its core protein and influence collagen fibrillogenesis (Vogel *et al.*, 1984). In addition, it was shown to decorate the surface of collagen fibrils at the 'd' and 'e' bands (Pringle and Dodd, 1990). Therefore, it was given the name decorin (Krusius and Ruoslahti, 1986). Decorin interacts with collagen I fibrils and effects fibril diameter *in vitro* resulting in thinner fibrils (Vogel and Trotter, 1987). The interaction occurs mainly via the leucine-rich repeats 4-5 of the decorin core protein (Svensson *et al.*, 1995). In addition to the fibrillar collagens I, II, III, and V (Bidanset *et al.*, 1992, Hedbom and Heinegård, 1993, Whinna *et al.*, 1993) decorin also interacts with collagens VI, XII, and XIV (Bidanset *et al.*, 1992, Font *et al.*, 1993, Font *et al.*, 1996) as well as fibronectin, thrombospondin, the complement component C1q, epidermal growth factor receptor (EGFR) and transforming growth factor-β (TGF-β) (Hildebrand *et al.*, 1994, Iozzo *et al.*, 1999, Krumdieck *et al.*, 1992, Schmidt *et al.*, 1987, Winnemoller *et al.*, 1992). Furthermore, decorin is also a Zn^{2+}-binding protein (Yang *et al.*, 1999).

2.2.3 Matrilins

Matrilins are a family of non-collagenous extracellular matrix proteins that form a subbranch of the superfamily of proteins containing the von Willebrand factor A (VWA) domains (for review see Wagener *et al*., 2005). VWA domains are present in a large number of extracellular and intracellular proteins and often participate in protein-protein interactions leading to the formation of multiprotein complexes (for review see Whittaker and Hynes, 2002). All four members of the matrilin family have been shown to be present in cartilage matrix, even though it is mainly matrilin-1 and -3 that are prominent in this tissue. Matrilins have emerged with the discoveries of matrilin-2 (Deak *et al.*,

1997), matrilin-3 (Belluoccio and Trueb, 1997; Wagener *et al.*, 1997) and matrilin-4 (Wagener *et al.*, 1998). The prototype member of this family is matrilin-1, which was initially identified as an abundant, proteoglycan-associated protein present in many forms of cartilage (Paulsson and Heinegard, 1979, 1981, 1982). Four members of the matrilin family have a closely similar domain structure (Figure 2-9). Two VWA domains are connected by a varying number of epidermal growth factor (EGF)-like domains. These are followed by a C-terminal α-helical coiled-coil domain which allows the oligomerisation of the single subunits in a bouquet-like fashion (Deak *et al.*, 1999). Only matrilin-3 lacks the second VWA domain and here the EGF-like domains are directly connected to the coiled-coil domain (Klatt *et al*., 2000). In addition, matrilin-2 and -3 contain a stretch of amino acid residues at the N-terminus with a high frequency of positively charged side chains. Uniquely, matrilin-2 contains a module between the second VWA domain and the oligomerisation domain that has no homology to any other known protein sequence (Piecha *et al*., 1999).

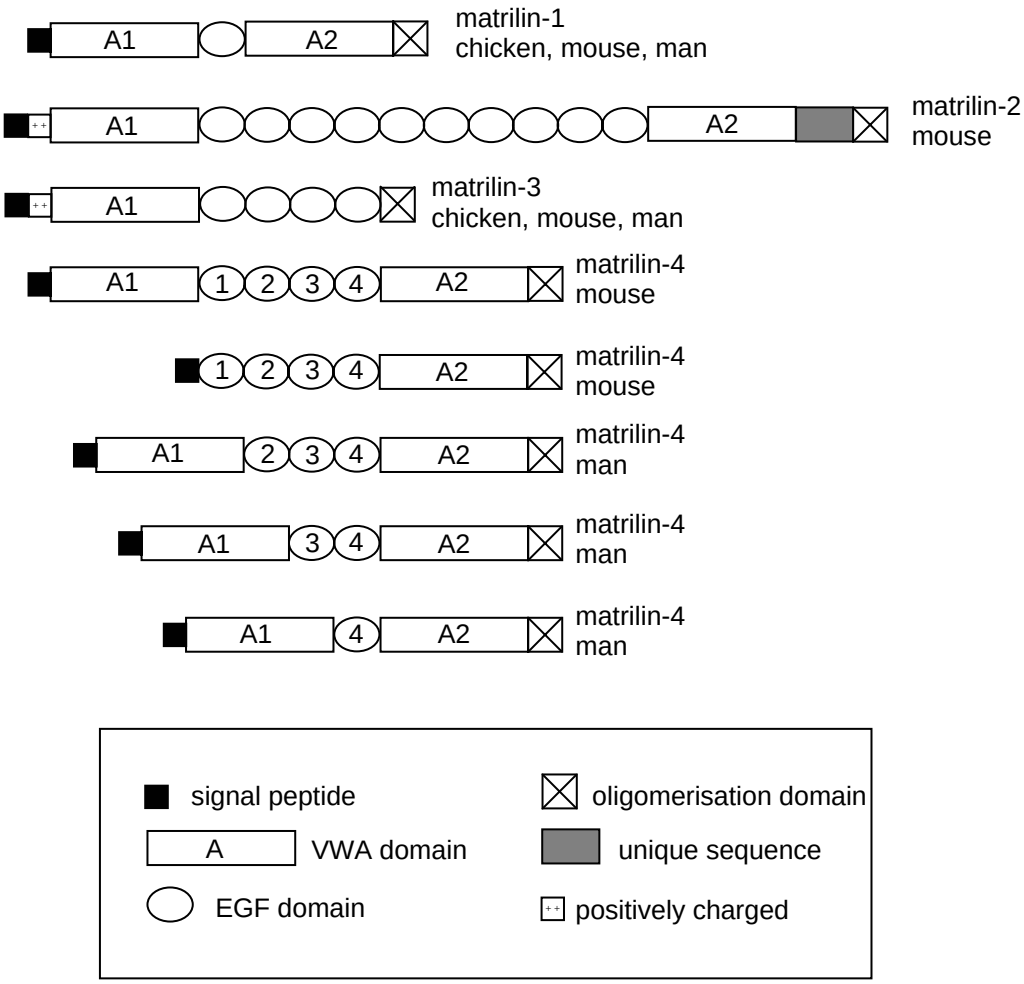

Figure 2-9. Comparison of the domain structures of the members of the matrilin family. Mouse matrilin-4 occurs in two, and human matrilin-4 in three, alternatively spliced forms. (modified from Deak *et al.*, 1999)

2.2.3.1 Interaction and potential functions of matrilins

Matrilin-1 was first recognised as a protein tightly bound to aggrecan and co-purified with aggrecan in a variety of separation methods (Paulsson and Heinegard, 1979). The complexes appear to be formed by protein-protein interactions occurring along the extended chrondroitin-sulphate-attachment region of aggrecan. The bound matrilin-1 molecules become covalently cross-linked to the aggrecan core protein, with at least some of the crosslinks insensitive to reduction of the interchain disulfide bonds (Hauser *et al.*, 1996). An association between matrilin-1 and cartilage collagen fibrils was also found

(Winterbottom *et al.*, 1992). Chondrocyte cell culture experiments showed matrilin-1 present in two types of pericellular filaments, where one type was collagen-dependent, as it required ascorbate for formation, and the other was not (Chen *et al.*, 1995). Similar staining of filaments in the pericellular matrix of cultured cells has been observed for each of the other matrilins and, in case of matrilin-2 and -4, not only around chondrocytes, but also in cultures of other cell types that express these matrilins (Klatt *et al.*, 2001; Piecha *et al.*, 1999; Klatt *et al.*, 2000).

Experiments have been performed to identify molecules that interact with matrilins to form such matrix assemblies. In studies of all four matrilins, a variety of collagens were found to bind to matrilins in solid phase binding assays (Mann *et al.*, 2004; Piecha *et al.*, 2002). However, some non-collagenous molecules, in particular cartilage oligomeric matrix protein (COMP) and decorin, bind to matrilins with higher affinity (Mann *et al.*, 2004). COMP and decorin are known to interact with collagens. Therefore, matrilins may be attached to collagen fibrils as part of a "sandwich" where other components may be the direct and high-affinity collagen binders (Wagener et al., 2005). Evidence in this direction comes from studies where the composition and assembly of molecular complexes attached to collagen VI microfibrils were studied (Wiberg *et al.*, 2003). With the use of immuno-gold electron microscopy, it could be shown that decorin and biglycan attach to VWA domains in the N-terminal region of collagen VI and that matrilins were in turn bound to these small leucine-rich repeat proteoglycans (Figure 2-8). At the periphery of these assemblies, the matrilins attached the collagen VI microfibrils to aggrecan core proteins or to collagen II-containing fibrils.

2.2.4 Cartilage oligomeric matrix protein (COMP)

Cartilage oligomeric matrix protein (COMP) is a non-collagenous glycoprotein initially found in articular, nasal, and tracheal cartilage (Hedbom *et al.*, 1992), but has later been isolated from tendon and ligament (DiCesare *et al.*, 1994; Smith *et al.*, 1997; Müller *et al.*, 1998), where also the corresponding mRNA was demonstrated (Smith *et al.*, 1997). In the growth plate, COMP is primarily observed in the proliferative region, where it is prominent pericellularly

(Shen *et al.*, 1995; Ekman *et al.*, 1997), indicating a role in cell growth and matrix development. In more developed articular cartilage, COMP is primarily located interterritorialy, especially in the more superficial part of the tissue. This high expression of COMP in mature articular cartilage may be induced by the high mechanical load on the tissue (Smith *et al.*, 2002).

COMP was first isolated from bovine articular cartilage under denaturing conditions with 4 M guanidine HCl (Hedbom *et al.*, 1992). Native COMP has been isolated from Swarm rat chondrosarcoma (Mörgelin *et al.*, 1992), bovine cartilage (DiCesare *et al.*, 1994), and human articular cartilage (DiCesare *et al.*, 1995) under mild nondenaturing conditions by extraction with 10 mM EDTA. This extraction indicates that the interaction of COMP with components of the ECM depends on divalent cations. Structurally, COMP is related to the thrombospondins. They have the same molecular domain arrangement of a series of four type-2 (epidermal growth factor) repeat domains followed by seven type-3 domains (calcium binding) (Oldberg *et al.*, 1992). COMP is assembled into a pentameric bouquet-like structure (Mörgelin *et al.*, 1992). The interactions involve the formation of a five-stranded coiled coil from an α-helical domain at the N-terminus (Efimov *et al.*, 1994). These interactions are stabilized by two disulfide bonds. The α-helical oligomerization domain is followed by a flexible strand and, at the C-terminal, a globular domain (Mörgelin *et al.*, 1992) that shows homology to the thrombospondins (Oldberg *et al.*, 1992). It is possible that the globular C-termini form multimeric binding sites for the interaction with other ECM components.

Rat COMP does not contain any RGD sequence (Oldberg *et al.*, 1992). In contrast the bovine (Oldberg *et al.*, 1992) and human (Newton *et al.*, 1994) COMP contain an RGD sequence although at different locations, indicating that COMP may mediate cell binding (DiCesare *et al.*, 1994). The primarily interterritorial localization of the protein in adult human cartilage suggests that the promotion of cell binding is not a primary function of COMP. It is possible that the adhesive properties of COMP are cell surface receptor for COMP has been isolated.

That COMP may have important functions in the ECM is illustrated by the fact that a mutation in the potentially Ca^{2+}-binding domain has been shown to lead to impaired secretion and severe multiple epiphyseal dysplasia (MED) (Briggs *et al.*, 1995). Moreover, mutations in the COMP gene are also responsible for the human genetic disorders pseudoachondroplasia (PSACH) (Briggs *et al.*, 1995, 1998; Chen *et al.*, 1994). Recombinant COMP carrying PSACH or MED mutations binds fewer divalent cations and exhibit a slightly altered affinity for collagen in the presence of zinc (Thur *et al.*, 2001). PSACH and MED are autosomal dominant chondrodysplasias causing mild to severe short-limb dwarfism and early-onset osteoarthritis. Suprastructural analysis of chondrocytes from patients with PSACH and MED show accumulation of material in the rough endoplasmic reticulum (rER) (Maynard *et al.*, 1972; McBurney *et al.*, 1991), which have a unique lamellar appearance. The accumulated material in rER consists mainly of COMP and collagen IX (Delot *et al.*, 1998; Maddox *et al.*, 1997).

3. AIM OF THE PRESENT STUDY

This present study has the aim to understand the relationships between the physical properties of the macromolecular constituents of the extracellular matrix and their supramolecular assemblies. Collagen VI-containing aggregates are prominent examples of the suprastructural plasticity of extracellular matrix aggregates. Therefore, it is interesting to investigate the collagen VI supramolecular assemblies in cartilage. The objectives of this study were the following:

(i) To prepare the discrete fragments of suprastructures from cartilage matrix from articular bovine cartilage and rib cages of newborn mice.

(ii) To determine macromolecular composition of collagen VI-containing suprastructures by transmission- and immuno-electron microscopy.

4. MATERIALS AND METHODS

4.1 Chemicals

The commonly available reagents used in these experiments are given in Table 4-1.

Table 4-1. List of used chemicals in the present work

Chemicals	Company: City, Country
6-aminohexanoic acid	Sigma: Steinheim, Germany
bovine serum albumin, BSA	Serva: Heidelberg, Germany
benzamidine hydrochloride	Sigma: Steinheim, Germany
calcium chloride	Merck: Darmstadt, Germany
coomassie blue	Serva: Heidelberg, Germany
dry skim milk	Fluka: Buchs, Switzerland
ethylenediamine tetra acetic acid, EDTA	Merck: Darmstadt, Germany
glycine	Roth: Karlsruhe, Germany
guanidine hydrochloride, GuHCl	Fluka: Buchs, Switzerland
hydrogen peroxide	Merck: Darmstadt, Germany
N-ethylmaleimide, NEM	Sigma: Steinheim, Germany
pepsin	Serva: Heidelberg, Germany
phenylmethanesulfonyl fluoride, PMSF	Merck: Darmstadt, Germany
polyacrylamide	Roth: Karlsruhe, Germany
potassium carbonate	Merck: Darmstadt, Germany
sodium chloride	Roth: Karlsruhe, Germany
sodiumdihydrogenphosphate dihydrate	Merck: Darmstadt, Germany
sodiumdodecylsulphate, SDS	Serva: Heidelberg, Germany
tris	MP-Biomedical: Eschwege, Germany
tween 20	Sigma: Steinheim, Germany
uranyl acetate	Merck: Darmstadt, Germany

4.2 Animals

Collagen IX-deficient mice contain a targeted disruption of the Col9α1 gene (Fassler et al., 1994; Hagg et al., 1997). Briefly, exon 8 of α1(IX) was disrupted by inserting a phosphoglycerate kinase-1 (Pgk-1) promoter-neomycin gene cassette. This replacement vector provided 10 kb of homology at the 5' end and 1.2 kb of homology at the 3' end. To select against random integration, a viral thymidine kinase cassette was included at the 3' end of the vector. The linearized targeting vector was electroporated into the D3 embryonic stem (ES)

cell line. After double selection in G418 and gancyclovir, the enrichment of targeted clones was about 15-fold.

Biglycan-deficient mice were generated by using homologous recombination in ES cells (Xu et al., 1998). Two independent ES cell clones were used to produce chimiras capable of germlin transmission of the targeted allele.

Wild-type, biglycan- and Col9α1-deficient mice were maintained and bred under specific-pathogen-free conditions in single ventilated cages. Wild-type and transgenic mice with a similar genetic background were used for *in vivo* investigation.

4.3 Tissues

In this study bovine articular cartilage and rib cages of newborn mice were used for *in vivo* investigation. Fresh bovine articular cartilage was obtained from a local slaughterhouse (Hamm). Adult bovine articular cartilages were dissected and were either used directly or stored at -80 ^{o}C. The rib cages of newborn mice (~ 2 day old) were freed from surrounding non-cartilaginous tissue and were stored at -20 ^{o}C. Chicken embryo corneas were used for collagen VI microfibril purification. They were also dissected and stored at -20 ^{o}C after dissection.

4.4 Extraction of fibril fragments

All extraction buffer solutions contained proteinase inhibitors that listed in table 4-2.

Table 4-2. List of used proteinase inhibitors in fibril fragments extraction (according to Riordan and Vallee, 1972; Deutscher, 1990; Gegenheimer, 1990; Jazwinski, 1990).

Proteinase inhibitor	inhibited protienases
6-aminohexanoic acid	Ser-
benzamidine HCl	Ser-
EDTA	Metallo-

Table 4-2. (continue)

Proteinase inhibitor	inhibited protienases
NEM	Cystein- (Thiol-)
PMSF	Ser-

4.4.1 Extraction of fibril fragments from bovine articular cartilage

An overview of the extraction procedure and partial purification of fibril fragments from cartilage tissue is shown in Figure 4-1. Cartilage slices were homogenized with a Polytron (Kinematiga, Littau/Luzern, Switzerland) in 15 volumes of extraction buffer (PBS, pH 7.4) containing a mixture of proteinase inhibitors. The suspension was centrifuged at 27,000 ×g for 20 min (JA 25.15, Beckman) and the clear supernatant was collected. This procedure was repeated twice with fresh extraction buffer, and all supernatants were combined. The supernatants were diluted in PBS containing the mixture of proteinase inhibitors. Then the suspension was ultracentrifuged at 104,350 ×g, (45 Ti, Beckman), 4 ^{o}C for 2 h. The pellets were resuspended in PBS. These extracts were used in subsequent experiments. In some experiments, the pellets were treated with Tris buffer containing 1, 3, and 5 M guanidine hydrochloride for 3 h at 4 ^{o}C under mild stirring. After the ultracentrifugation (104,350 ×g) the pellets were washed with distilled water for 2 h at 4 ^{o}C. Then the suspension was centrifuged with the ultracentrifugation (104,350 ×g). Finally, the pellets were resuspended in PBS.

Extraction buffer solution

NaCl	0.15 M
NaH_2PO_4. $2H_2O$, pH 7.4	2 mM
6-aminohexanoic acid	0.1 M
benzamidine HCl	1 mM
EDTA	20 mM
NEM	5 mM
PMSF	0.1 mM

Tris buffer solution

NaCl	0.1 M
Tris HCl, pH 7.4	50 mM

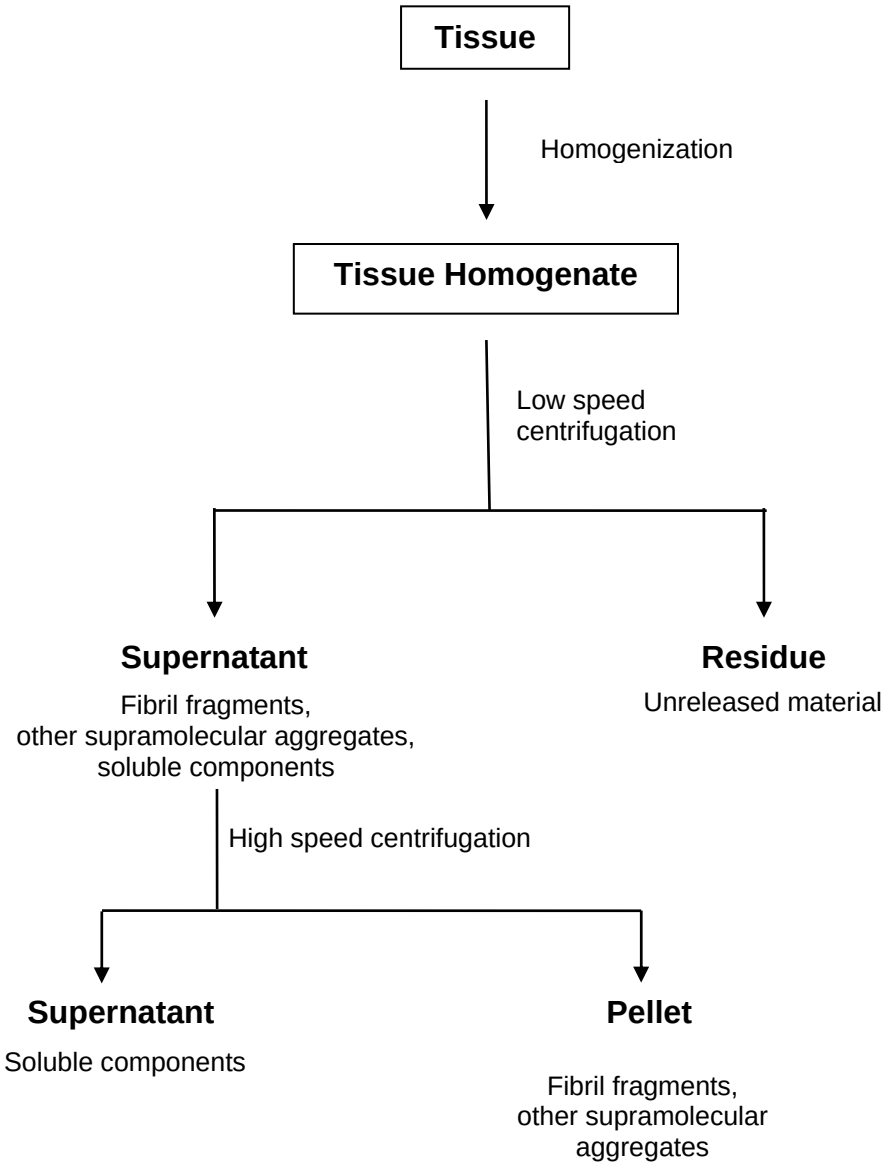

Figure 4-1: Procedure of the extraction and partial purification of fibril fragments from cartilage tissue.

4.4.2 Fibril fragment extraction from rib cages of newborn mice

Rib cages of newborn mice were homogenized with a Polytron (Kinematiga, Littau/Luzern, Switzerland) in 500 µl of the extraction buffer containing inhibitors as same as used for bovine cartilage. The suspension was centrifuged at 20,800 ×g (5417R, Eppendorf) for 3 min. The clear supernatant was collected. This procedure was repeated twice with fresh extraction buffer. All supernatant were combined and used for the immuno-gold electron microscopy.

4.5 Antibodies for immuno gold electron microscopy

Primary antibodies were polyclonal affinity-purified goat-antibodies to human and bovine collagen VI (AB782, Chemicon); monoclonal mouse antibodies to chicken collagen II with cross-reactivity to human homologue (MAB8887, Chemicon); polyclonal antibodies against bovine COMP (DiCesare *et al.*, 1994), (friendly gift from Dr. Frank Zaucke, University of Cologne); polyclonal rabbit antibodies against bovine matrilin-1 (Hauser and Paulsson, 1994), (friendly gift from Dr. Raimund Wagener, University of Cologne); polyclonal affinity-purified antibodies to the NC-4 domain of murine collagen IX (Budde *et al.*, 2005); (friendly gift from Dr. Bastian Budde, University of Muenster), rabbit antiserum against human biglycan (Hausser *et al.*, 1989; 1992), (friendly gift from Dr. Daniela G. Seidler, Philadelphia); rabbit anti-serum against human decorin (Kresse *et al.*, 2001), (friendly gift from Dr. Daniela G. Seidler, Philadelphia).

Secondary antibodies were goat anti-rabbit immunoglobulins conjugated to 12 nm gold particles (Jackson Immuno Research); goat anti-mouse immunoglobulins conjugated to 18 nm gold particles (Jackson Immuno Research).

4.6 Gold conjugate preparation

Colloidal gold 10 nm were prepared by reduction of $HAuCl_4$ by potassium carbonate (according to data sheet, Aurion) and conjugated after titration to collagen VI affinity purified polyclonal antibodies (AB782, Chemicon). The required gold conjugate was prepared using the undiluted antibody solution. After addition of the minimal amount of antibody solution to the gold sol (Aurion), the mixture was allowed to stand for 2 min. 2% bovine serum albumin was added to block the remaining free surface areas on the gold particles and to prevent aggregation of the gold conjugate. The mixture was centrifugated at 20,800 ×g (5417R, Eppendorf), 4 ^{o}C for 1 h. The fluffy parts were collected and resuspended in PBS with 1% BSA, pH 7.6.

Before using the gold conjugates for labelling, electron microscopy was used to evaluate the particles size, the size distribution and the presence of

clusters on a quantitative basis. Gold conjugates were attached to poly-L-lysine coated formvar grids. The particle density per unit surface area should be kept low in order to be able to evaluate the presence of clusters. After the quantitative analysis of the complex, 83.57% of singlet gold particles and 6.70% of triple gold particles were detected. From a rule, gold conjugates with at least 75% singlets and at the most 5% triplets of gold particles are considered to be acceptable (according to the data sheet of gold sol, Aurion). Therefore, it might be acceptable to use these gold conjugates for immuno electron microscopy.

4.7 Immuno gold electron microscopy

All steps were carried out at room temperature. Aliquots of fibril fragment extracts were spotted onto sheets of parafilm. Nickel grids (200-400 mesh) covered with formvar and coated with carbon were floated on the drops for 10 min to allow adsorption of fibril fragments. The grids were dried on filter paper (did after all steps) then subsequently washed with PBS, and treated for 30 min with 2% (w/v) dried skim milk in PBS (2% blocking solution), centrifuged twice at 20,800 ×g (5417R, Eppendorf), for 10 min prior to application. The adsorbed material was allowed to react for 2 h with antibodies against some extracellular matrix components (see 4.5) diluted in 1:50-1:100 0.2% blocking solution. After washing five times with PBS, the grids were put on drops of 0.2% blocking solution containing colloidal gold particles (12 or 18 nm) coated with goat antibodies to rabbit or chicken immunoglobulins (see 4.5, concentration 1:30). Collagen VI antibody gold conjugates (see 4.6, concentration 1:2 or undiluted) were adsorbed on the grid after the blocking step then following by the uranyl acetate staining. For double-labeling experiments, a mixture of gold particles of two different sizes was used. Finally, the grids were washed with distilled water and negatively stained with 2% uranyl acetate for 7 min. Control experiments were done with the first antibody omitted. Electron micrographs were taken at 60 kV with a transmission electron microscope (TEM) (Philips EM410). The re-usable film plates were scanned in a film scanner (Micron imaging plate scanner, Ditabis, Pforzheim, 6000 × 5000 pixel).

PBS, pH 7.4(sterile filtrated)

NaH_2PO_4. $2H_2O$	2 mM
NaCl	0.15 M

4.8 Protein Purifications

4.8.1 Collagens II, IX, and XI

The collagens II, IX, and XI were purified in native and fibrillogenesis-competent form from cultures of chick embryo sternal chondrocytes in agarose gels (Blaschke *et al.*, 2000).

4.8.1.1 Cell culture

Sternal chondrocytes from 17-day old chicken embryos were digested overnight with bacterial collagenase. Matrix free chondrocytes were collected by centrifugation (600 ×g, 7 min, S4180, Beckman) and embedded at a density of 2-3 × 10^6 cells/ml into gels containing 0.5% low melting agarose and Dulbecco's modified Eagle's medium. The medium was supplemented with 10% fetal calf serum, 1 mM pyruvate, 1 mM cysteine, 50 µg/ml sodium acorbate and was changed every 2-3 days. 60 µg/ml β-aminopropionitrile was added to the medium to prevent lysyl oxidase-derived cross-linking. After 14 days, medium was removed and the cultures were either used directly or stored at -20 ^oC.

4.8.1.2 Collagen purification

An overview of the procedures used for collagen purification is shown in Figure 4-2. After thawing, noncollagenous material was extracted from the agarose cultures at 18 °C with PBS containing proteinase inhibitors. Agarose pellets containing the collagens were recovered by centrifugation (18,542 ×g, 30 min, JLA 10.500, Beckman), and the supernatants were discarded. All of the following steps were performed at 4 °C. Collagens were solubilized subsequently with buffer containing 100 mM Tris-HCl, pH 7.4, 1 M NaCl. Collagens II, IX, and XI were precipitated by adding solid NaCl to give a final concentration of 4.5 M and were recovered by centrifugation.

For chromatographic separation, the crude collagen pellets were dissolved in 100 mM Tris-HCl, pH 7.4, containing 2 M urea and 100 mM NaCl, and were passed over a DEAE-cellulose column (3.5 × 11 cm, 100 ml, DE52; Whatman Ltd.) equilibrated with the same buffer. Most collagen IX was bound to the column, while collagens II and XI and residual collagen IX were recovered from the breakthrough fractions (fraction A in Figure 4-2). After dialysis against 10 mM Tris HCl, pH 7.4, containing 2 M urea and 50 mM NaCl, fraction A proteins were applied to a carboxymethyl-cellulose column (3.5 × 11 cm, 100 ml, CM52; Whatman, Ltd.). Collagen II was recovered from the breakthrough fractions (fraction C), and collagen XI was eluted by a stepwise increase of NaCl to 0.5 M (fraction D).

Collagen XI was further purified by dialyzing fraction D against 50 mM Tris-HCl, pH 7.4, containing 2 M urea and 5 mM NaCl, followed by chromatography on DEAE-cellulose (1 × 7 cm). Collagen XI bound to the columns under these conditions and was eluted in pure form by buffer containing 0.2 M NaCl (fraction F). Final purification of collagen II in fraction C was achieved by DEAE-cellulose chromatography in 50 mM Tris-HCl, pH 7.4, containing 2 M urea and 5 mM NaCl. The pure protein was recovered from the breakthrough fractions (fraction E). Purified proteins in fractions E and F were dialyzed extensively against 100 mM Tris-HCl, pH 7.4, containing 1 M NaCl, and then precipitated by the addition of solid NaCl to a final concentration of 4.5 M. Following centrifugation, pellets were redissolved in storage buffer at appropriate concentrations and clarified by centrifugation.

PBS, pH 7.4

NaH_2PO_4. $2H_2O$	20 mM
NaCl	0.15 M
6-aminohexanoic acid	0.1 M
benzamidine HCl	10 mM
EDTA	50 mM
NEM	10 mM
PMSF	0.1 mM

Storage buffer, pH 7.4

Tris HCl	100 mM
NaCl	0.4 M

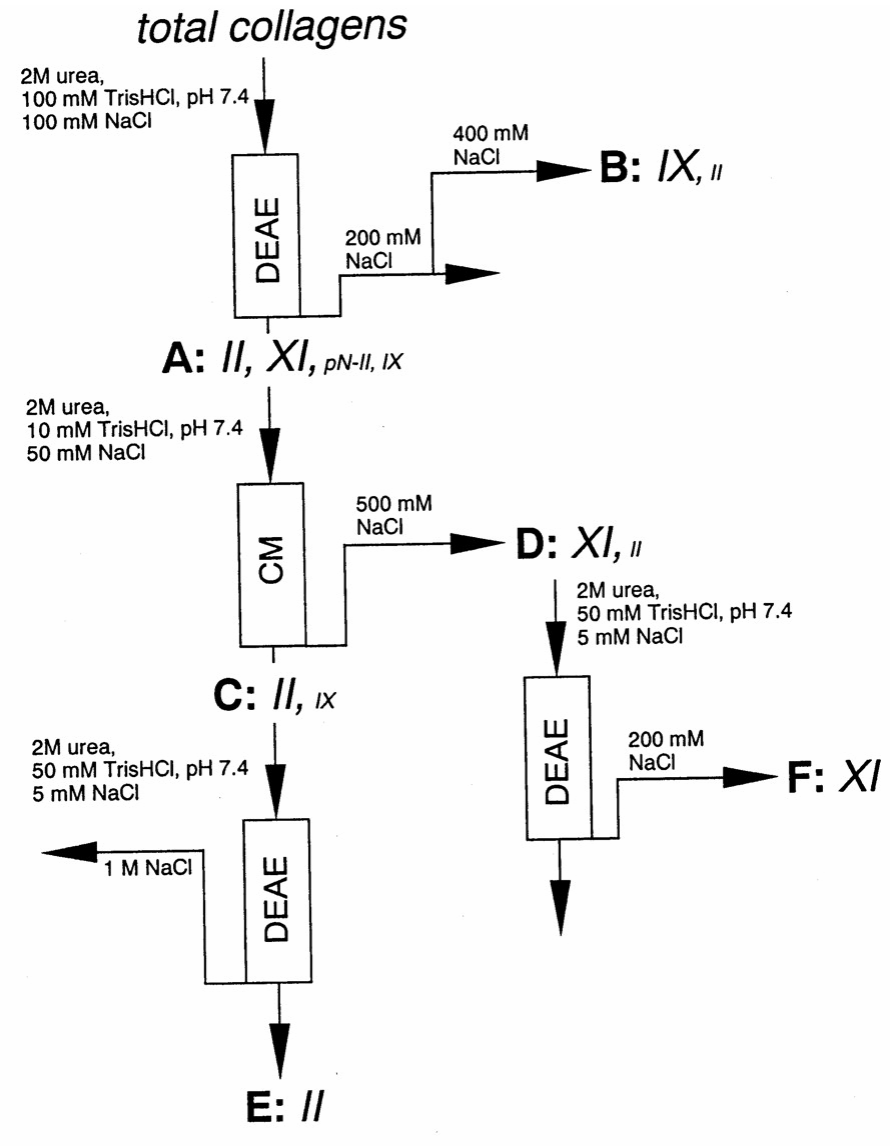

Figure 4-2. Flowchart representation of the protocol for purification of cartilage collagens from chondrocyte cultures in agarose. The mixture of total collagens corresponds to the proteins of 1 M NaCl extracts of the cultures after precipitation by 4.5 M NaCl. (according to Blaschke et al., 2000).

4.8.2 Collagen VI microfibrils

Native collagen VI microfibrils were purified from chicken corneas. Corneas were cut into pieces and homogenized with the Polytron in 2 volumes of Tris buffer, pH 7.4. The homogenate was digested with 2% (w/w) of bacterial

collagenase type 1 (Worthington biochemical corporation) overnight at room temperature. The suspension was centrifuged for 20 min at 48,000 ×g (JA-25.15, Beckman). After centrifugation for clarification 500 µl aliquots of the supernatant were filtrated and then fractionated on a Superose 6 (Amersham Biosciences) molecular sieve column on HPLC (P-900, Amersham Biosciences). The column was run at 0.2 ml/min, monitored at 280 nm with a UV spectrophotometer (UV-900, Amersham Biosciences), and fractions of 1 ml were collected. Purity and suprastructure of the extracted collagen VI microfibrils was proved by SDS-PAGE, immunoblotting and immuno-gold electron microscopy.

Extraction buffer

NaCl	400 mM
Tris HCl, pH 7.4	50 mM
6-aminohexanoic acid	0.1 M
benzamidine HCl	10 mM
EDTA	20 mM
NEM	10 mM
PMSF	2 mM

4.9 Recombinant of NC4-domain of collagen IX

The NC4-domain was a friendly gift from Dr. Bastian Budde, University of Muenster (Budde *et al.*, 2005). RNA was prepared by lithium chloride-urea extraction of whole rib cages of neonatal mice, proteinase K digestion, and subsequent isopropanol precipitation and reverse transcribed into cDNA using Stratascript reverse transcriptase (Stratagene, Germany). A 762-bp fragment encoding the entire NC4 domain of the α1 chain of collagen IX was generated by PCR. After PCR amplification, purification, and digestion with the corresponding restriction enzymes (New England Biolabs), the construct was ligated into the pCEP-Pu vector (Smyth et al., 2000) containing a BM40 signal peptide and a C-terminal six-His sequence, resulting in a fusion protein of α1(IX) NC4 and a His epitope. The vector construct was then transiently transfected into 293-EBNA cells (Invitrogen, Germany) using the lipofection reagent FuGENE 6 (Roche, Germany) according to the protocol of the manufacturer. Selection for successfully transfected cells was performed by culturing in selection medium: Dulbecco's modified Eagle medium (DMEM)/F12 (1:1) with final concentrations of 2 mM

glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% (v/v) fetal calf serum, 175 µg/ml Geneticin (Gibco, Germany), and 0.5 µg/ml puromycin (Sigma, Germany).

Positive clones were kept for three to four passages in selection medium followed by culturing in serum-free DMEM/F12 (1:1) medium. The cells were viable and produced large amounts (ca. 0.5 mg/liter of medium) of NC4 protein for 18 to 21 days. Media conditioned for 2 days were pooled, dialyzed against 20 mM sodium phosphate, pH 6.0, and applied to cationic-exchange chromatography (carboxymethyl cellulose CM-52; Whatman, England). The protein was eluted by a sodium chloride gradient (0.05 to 0.30 M NaCl, with a main protein peak at 0.14 M). The NC4-containing fractions were pooled and further purified by a TALON cobalt affinity resin which allows high-affinity binding of the six-His tag introduced at the C-terminus (Clontech, Germany).

4.10 SDS-PAGE

Samples were pooled and prepared for electrophoresis by three volume of cold ethanol precipitation. The precipitates were resuspended in SDS sample buffer containing 5% β-mercaptoethanol and boiled at 95 ^{o}C for 5 min. The samples were run on 4.5–15% polyacrylamide gradient gels (Laemmli, 1970). Alternatively, some samples were boiled in non-reducing SDS sample buffer. The electrophoresis was performed at room temperature with a current between 15-20 mA. A molecular weight standard, Prestained Broad Range Standard (Bio-Rad, Munich) was applied. The gel was stained with coomassie blue (see 4.11) or transferred to nitrocellulose membrane (see 4.12).

SDS-sample buffer (4×)	
Tris HCl, pH 6.8	60 mM
SDS	2% (w/v)
Glycerol	10% (v/v)
Bromphenolblue	0.001% (w/v)
Optional :	
β-Mercaptoethanol	5% (v/v)

Running buffer	
Tris HCl, pH 8.8	25 mM
SDS	0.1% (w/v)
Glycine	0.2 M

Running gel buffer (4×)	
Tris HCl, pH 8.8	1.5 M

Stacking gel buffer (4×)	
Tris HCl, pH 6.8	0.5 M

SDS	0.4% (w/v)	SDS	0.4% (w/v)

Running gel solution	
Running gel buffer	1×
Acrylamide/Bis-acrylamide (32:1)	4.5 and 15% (w/v)
TEMED	0.1%
Ammonium persulphate	100 µg/ml
Stacking gel solution	
Stacking gel buffer	1×
Acrylamide/Bis-acrylamide (32:1)	4.5% (w/v)
TEMED	0.33%
Ammonium persulphate	75 µg/ml

4.11 Coomassie staining

The gel from electrophoresis (4.10) was put in destaining solution I for 20 min, in oder to fix proteins contained in the gel. Afterwards, the gel was stained with coomassie solution for 1 h 30 min. Then the gel was destained with destaining solution I for 1 h following by destaining solution II for 1 h. The destaining solution II was replaced with fresh destain solution II and the gel was soaked overnight. Finally, the gel was washed twice with gel drying solution for 30 min and was dried between two gel drying films.

Destaining solution I		Destaining solution II	
Methanol	50% (v/v)	Methanol	10% (v/v)
Acetic acid	10% (v/v)	Acetic acid	10% (v/v)

Coomassie solution		Gel drying solution	
Coomassie blue R-250	0.1% (w/v)	Ethanol	25% (v/v)
Acetic acid	10% (v/v)	Glycerol	2% (v/v)
Isopropanol	25% (v/v)		

4.12 Immunoblotting

The separated proteins from electrophoresis (see 4.10) were transferred onto a nitrocellulose membrane (Whatman, Florham Park, NJ, USA). The membrane was placed face-to-face with the gel covered with two whatman-papers at both sides. Transfering of the proteins from gels onto nitrocellulose membrane was performed by using wet electrophoretic elution. For this purpose, the gel-

membrane sandwich was placed between carbon electrodes in a buffer tank with transfer buffer. Electrophoretic elution was run overnight at 4 ^{o}C with current at 10 kV and voltage at 30 mA. Efficiency of transfer was checked by a staining of the blot membrane with Ponceau S (Serva, Heidelberg). Afterwards, the dye was remove by repeated washing and free binding sites on nitrocellulose membrane were blocked with TBS containing 5% (w/v) of dried skim milk for 1 h. Proteins on the membrane were then allowed to react for 2 h with a monoclonal antibody against chicken VI collagen (39, Hybridoma Bank), diluted in TBST containing 2.5% (w/v) of dried skim milk. After repeated washing with TBST, membrane was incubated for 2 h with a horseradish peroxidase-coupled goat anti-mouse immunoglobulins antibody (DAKO). After repeated washing with TBST, bound antibodies were visualized with a substrate solution consisting of TBS supplemented with 0.18 mg/ml 4-chloro-1-naphthol and 0.04% (v/v) H_2O_2. Chemiluminescent detection was performed by putting a roentgen film (Hyperfilm, Amersham Bioscience, Freiburg) on the membrane. The separated protein image was developed from the roentgen film by using a film developing machine (Curix 60, Agfa).

Transfering buffer

Tris HCl	50 mM
Glycine	380 mM
SDS,	0.1% (w/v)
Methanol	20% (v/v)

4.13 *In vitro* fibrillogenesis

Collagens II, IX, and XI were purified in native and fibrillogenesis-competent form from cultures of chick embryo sternal chondrocytes in agarose gels (see 4.8.1). Mixtures of collagens II, IX, and XI and mixtures of collagens II and XI were prepared in storage buffer at molar proportions of 8:1:1 and 8:1, respectively (see Table 4-3). In the latter mixtures, storage buffer were added instead of collagen IX. Defined mixtures thereof were degassed under vacuum. 100-µl samples were transferred to microcuvettes (Multicell, light path, 1 cm, Beckman, Palo Alto, CA). *In vitro* fibrillogenesis was initiated by adding of 100 µl of distilled water. The cuvettes were sealed and placed immediately into a spectrophotometer (Beckman

UV 640, equipped with a Multicell holder, Micro Auto 12), connected to a water bath at 37 ^{o}C. After 3 h, in some experiments (Table 4-3, A and C), collagen VI microfibrils were added with final concentration 5 µg/ml. Fibrillogenesis was continued for one more hour. The kinetics of fibrillogenesis was monitored by turbidity development at 313 nm. Finally, the reconstitution products were examined by immunogold electron microscopy (see 4.7 and Hansen and Bruckner, 2003).

Table 4-3. List of collagen volume used in different experiments. The same concentrations of all collagens were used (250-300 µg/ml).

Experimen	Volume of collagens/ µl			
	II	IX	XI	VI
A	80	10	10	10
B	80	10	10	- *
C	80	- *	10	10
D	80	- *	10	- *

- *: Storage buffer were used instead of collagens.

Storage buffer

NaCl	0.4 M
Tris HCl, pH 7.4	0.1 M

4.14 Binding assay (ELISA)

Polystyrene microtiter plates (F96; Nunc, Wiesbaden, Germany) were coated overnight at 4 ^{o}C with 100 µl/well of recombinant NC4-domain of collagen IX, and the purified collagens II, IX, and XI in a concentration of 10 µg/ml in 20 mM Tris-HCl, containing 0.15 M NaCl, pH 7.4 (TBS). Plates were washed three times with TBS. After blocking with 2% BSA in 200 µl TBS for an hour, the plates were washed four times with TBS containing 0.05% Tween 20, TBST. Then 1 to 100 µg/ml bovine collagen VI (Rockland Inc.) in 100 µl TBST was added and incubated at room temperature for an hour. After washing four times with TBST, collagen VI polyclonal antibody (AB7821, Chemicon) was added at 1:20,000 in 100 µl TBST and incubated at room temperature for an hour. Then the plates were washed four

times with TBST and incubated with a horseradish peroxidase-coupled anti-rabbit immunoglobulin antibody at 1:10,000 in 100 µl TBST at room temperature for an hour. After washing four times with TBST, binding was detected using 200 µl 0.04% (w/v) *o*-phenylenediamine, 24.3 mM citric acid, 51.4 mM Na_2HPO_4, and 0.012% (v/v) H_2O_2. The reaction was stopped by adding 100 µl of 2.5 M H_2SO_4. Absorbance at 490 nm was measured using a microplate reader (Dynatech Laboratories, Sussex, United Kingdom; software, Mikro WIN V2.38, Mikrotek, Overath). The similar procedure was used for pepsin treated commercial collagen VI.

5. RESULTS

5.1. Collagen VI antibody-gold conjugates bind to the globular domains of collagen VI microfibrils

Suprastructural fragments were isolated from bovine articular cartilage (see 4.4.1). These mechanically generated fragments retain some of their authentic tissue properties. Fragments of D-periodically banded fibrils are clearly distinguishable from more amorphous-appearing extrafibrillar aggregates after negative staining with uranyl acetate. Collagen VI was identified by binding of a polyclonal antibody against collagen VI (AB782, Chemicon) directly conjugated to gold particles (see 4.6). These collagen VI antibody gold conjugates bound to microfibrils (Figure 5-1). The distances between the lateral groups of immunogold particles were measured. Such measurements revealed distances of 108 ± 11 nm (n = 284) corresponding to the distance of the globular domains of collagen VI microfibrils (Engval *et al*., 1986; Bruns *et al*., 1986; Baldock *et al.*, 2003). Therefore, the antibody against collagen VI is specific to the globular domains of collagen VI microfibrils.

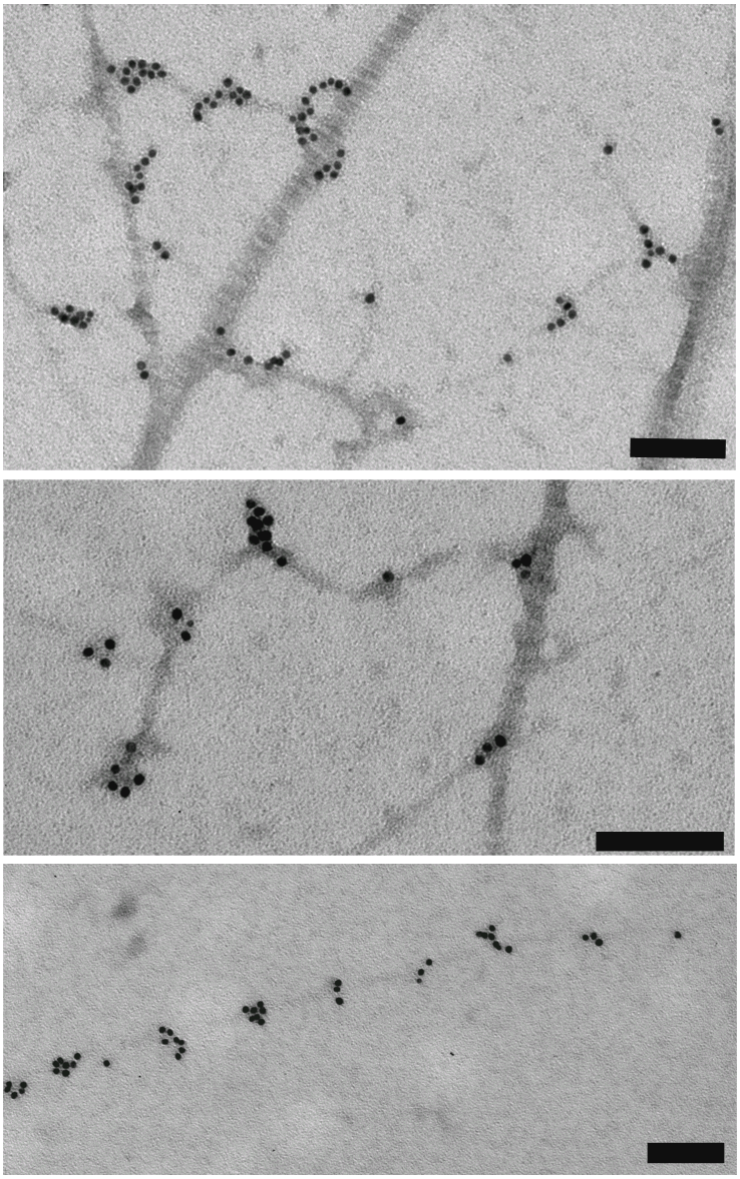

Figure 5-1. Suprastructural localization of collagen VI on fibril fragments from bovine articular cartilage by direct immunogold electron microscopy. The globular domains of cartilage microfibrils are heavily labeled with collagen VI antibody gold conjugates. Bar, 100 nm.

5.2 Localization of matrix proteins on the globular domains of collagen VI microfibrils in bovine articular cartilage

The distribution of matrix proteins in fibril fragments from bovine articular cartilage was investigated. Collagen VI microfibrils were identified by collagen VI antibody gold conjugates labelling. By using electron microscope, collagen VI

microfibrils were found in association with D-periodically banded fibrils and network-like structures (small gold particles in Figure 5-2, 5-3). After a quantification of cartilage banded fibrils, 34% (n =153) of the banded fibrils were found in association with collagen VI microfibrils. Interestingly, most of collagen VI microfibrils associating with the banded fibrils were found in the association by twisting around the banded fibrils (Figure 5-4).

On the other hand, biglycan, decorin, matrilin-1, and COMP were identified by indirect immuno-gold labelling. They were also found in association with banded fibrils and network-like structures (large gold particles, Figure 5-2, 5-3). The labelling of biglycan revealed that biglycan co-localized with the globular domains of collagen VI microfibrils in a regular binding pattern [Figure 5-2 (A,B)]. Decorin, matrilin-1, and COMP also co-localized with some of the globular domains of collagen VI microfibrils [Figure 5-2 (C,D) and Figure 5-3 (A,B); (C,D), respectively]. However, the gold particles representing COMP were found at some distance from the globular domains of collagen VI microfibrils whereas the gold particles representing biglycan, decorin, and matrilin-1 were found in close ficinity of the globular domains.

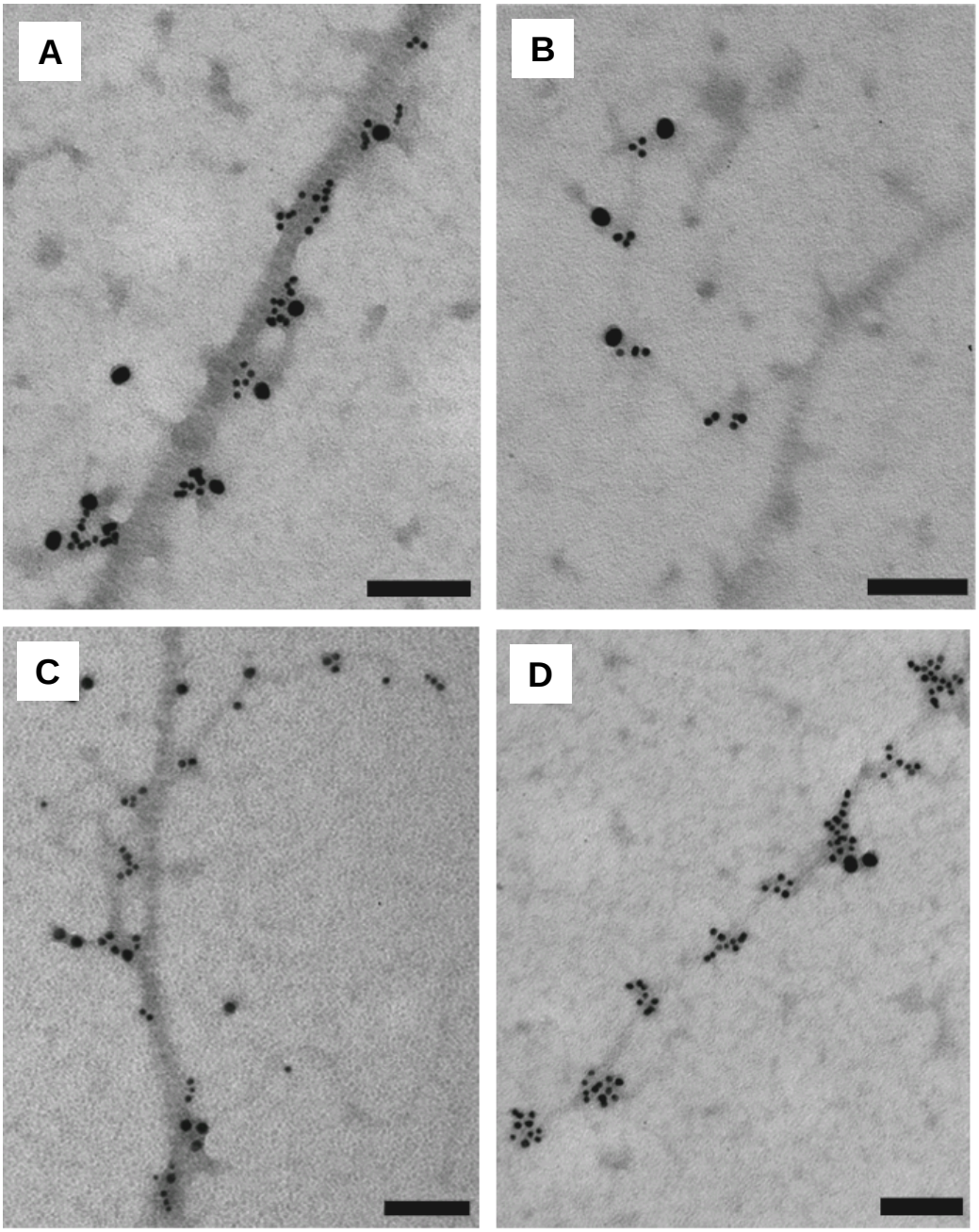

Figure 5-2. Microfibrils labelled with collagen VI antibody gold conjugates (small gold particles) were positive in indirect immuno-labelling for biglycan (A, B) and decorin (C, D). Bar, 100 nm.

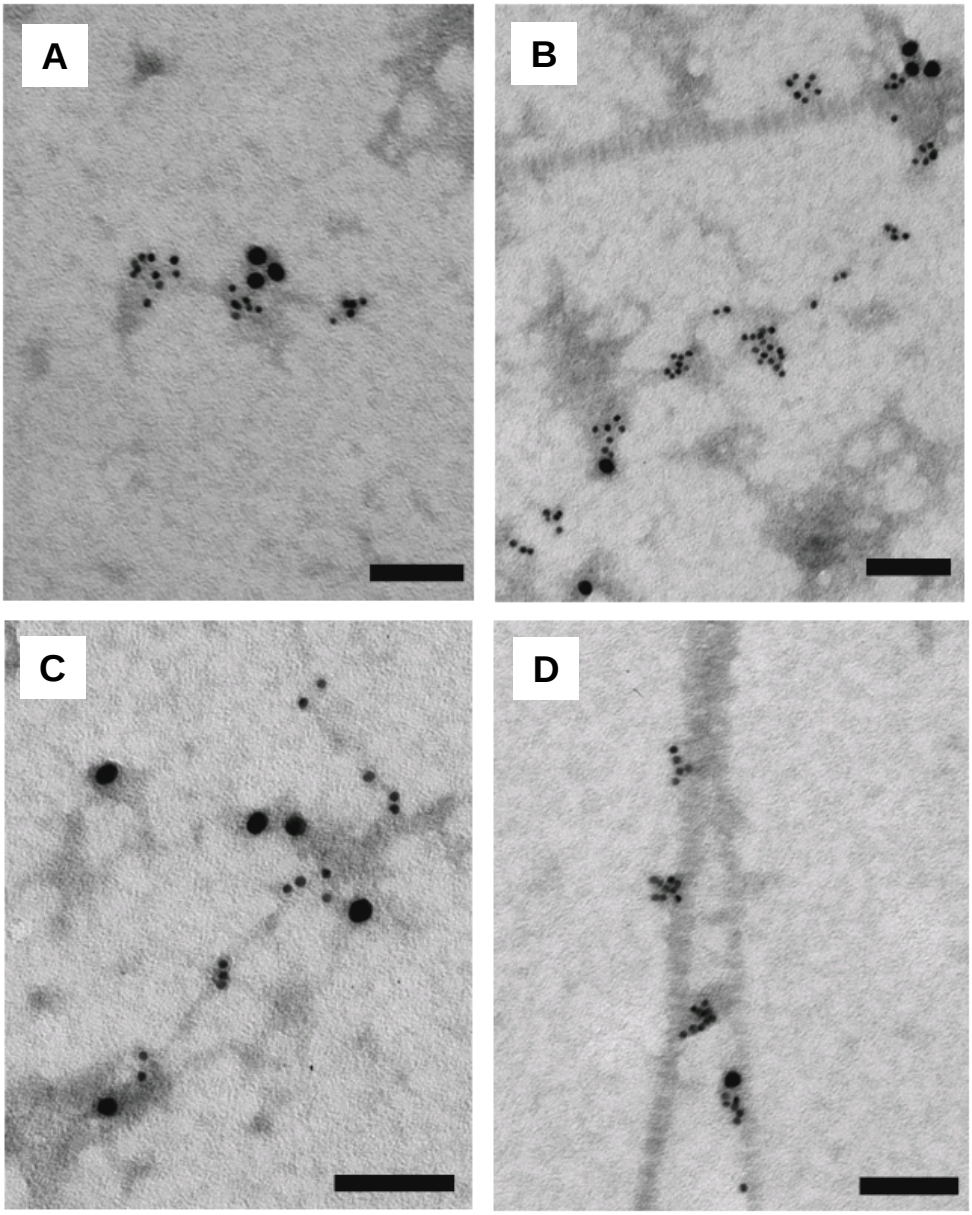

Figure 5-3. Microfibrils labelled with collagen VI antibody gold conjugates (small gold particles) were positive in indirect immuno-labelling for matrilin-1 (A, B) and COMP (C, D). Bar, 100 nm.

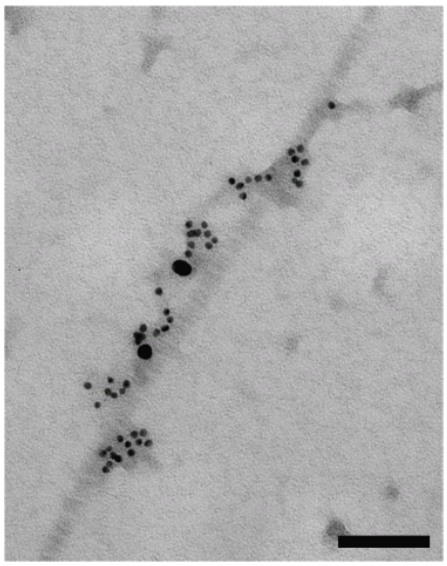

Figure 5-4. Suprastructural localization of collagen VI and biglycan on fibril fragments from bovine articular cartilage by immunogold electron microscopy. Microfibrils are heavily labelled with antibodies against collagen VI (small gold particles) in colocallization with biglycan (large gold particles). Collagen VI microfibrils are firmly associated by twisting around thin banded fibrils (b). Bar, 100 nm.

5.3 Collagen VI microfibrils interact with thin banded fibrils

From section 5.2, the population of collagen VI microfibrils twisting around the banded fibrils was taken into consideration. Interestingly, collagen VI microfibrils were found preferentially twisted around the thin banded fibrils but not the large banded fibrils (Figure 5-5). The cartilage thin banded fibrils contain collagens II, XI and, optionally, collagen IX (Hagg *et al*., 1998; Mendler *et al*., 1989). In order to assure that the banded fibrils are cartilage banded fibrils, collagens II and IX were identified by indirect immunogold labelling in combination with the collagen VI labelled with collagen VI antibody gold conjugates. Collagen XI-labelling was not performed because collagen XI located in the core of the cartilage banded fibrils was immunochemically masked (Poole *et al*., 1987). Electron microscopy showed that thin banded fibrils interacting with collagen VI microfibrils displayed a labelling with antibodies against collagens II and IX (Figure 5-6A and 5-6B). Therefore, collagen VI microfibrils interact with cartilage banded fibrils containing collagens II, IX, and XI.

The interaction between collagen VI microfibrils and cartilage banded fibrils could result from physical entanglement without molecular interaction. The extracted fibril fragments were treated with 1, 3, and 5 M guanidine hydrochloride (GuHCl) and the stripped banded fibrils were re-investigated by immuno-gold electron microscopy for the presence of collagen VI microfibrils and biglycan (see 4.4.1 and 4.7). Electron microscopy revealed that the banded fibrils were somewhat disrupted after the treatment with GuHCl. Interestingly, after the treatment with GuHCl biglycan still binds to the globular domains of collagen VI microfibrils associated with banded fibrils (Figure 5-7). The gold particles labelling biglycan on microfibrils twisting around banded collagen fibrils were counted. Some of gold particles were bound directly on the banded fibrils while others bound on the globular domains of collagen VI microfibrils interacting with the banded fibrils. The fractions of the gold particles binding to the globular domains of collagen VI microfibrils are increased after the treatment with GuHCl (Figure 5-8). This implies that biglycan which did not bind to the globular domain of collagen VI microfibrils was removed from the banded fibrils.

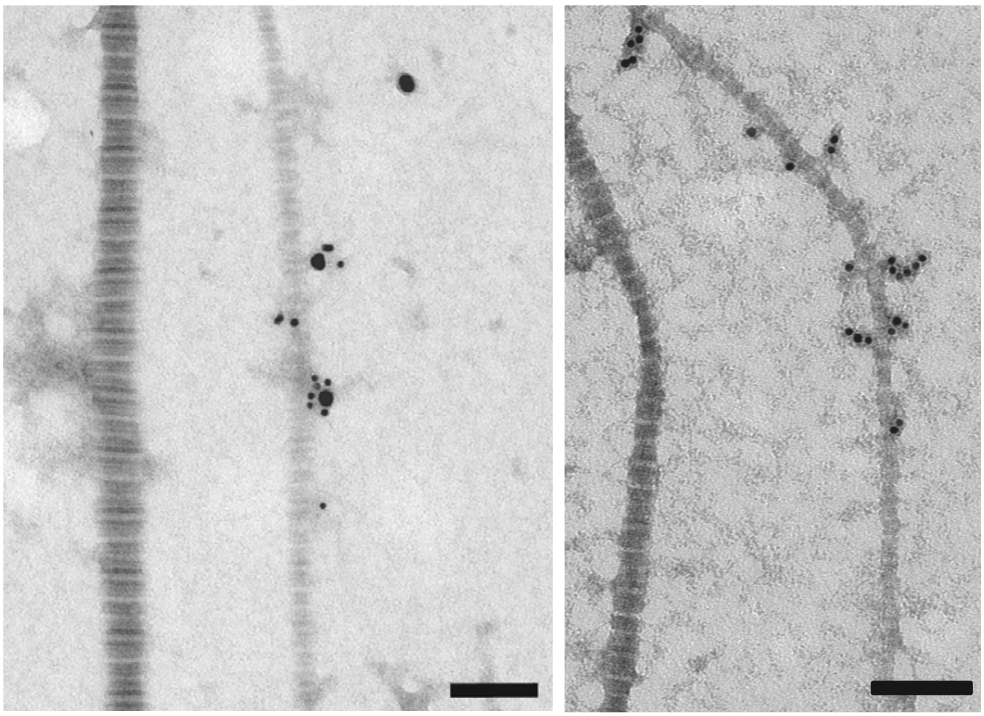

Figure 5-5. Immunoelectron micrographs of ultrastructural localization of collagen VI (small gold particles) and biglycan are on thin fibrils. Bound antibodies were visualized by direct immunogold lablling to collagen VI but by indirect immuno staining with gold-conjugated antibodies for biglycan. Bar, 100 nm.

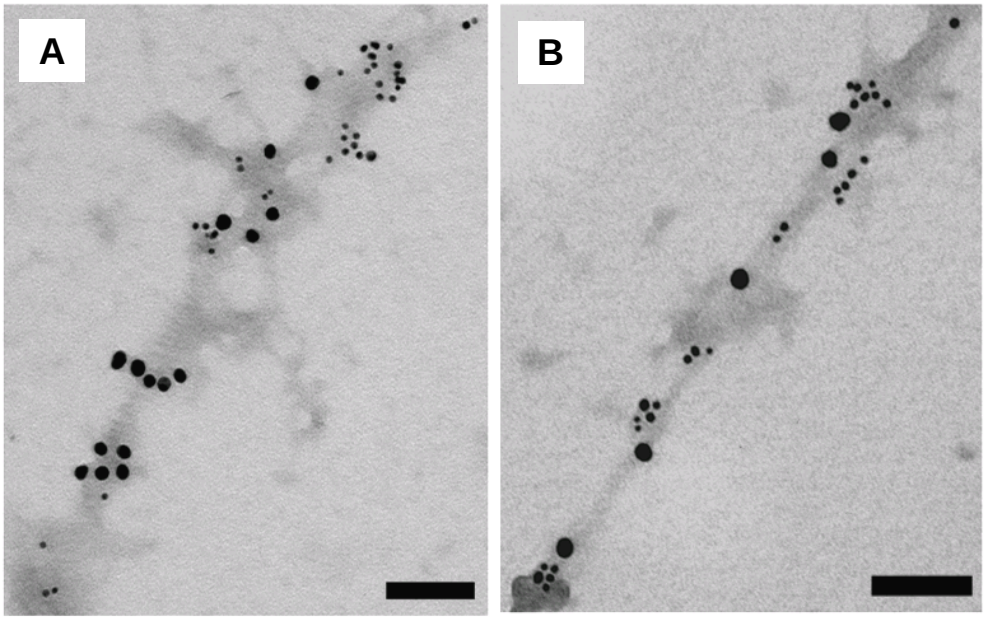

Figure 5-6. Immunoelectron micrographs of ultrastructural localization of collagen VI (small gold particles) interacts with the banded fibrils containing collagens II and IX (A and B, respectively). Bound antibodies were visualized by direct immunogold lablling to collagen VI but by indirect immuno staining with gold-conjugated antibodies for collagens II and IX. Bar, 100 nm.

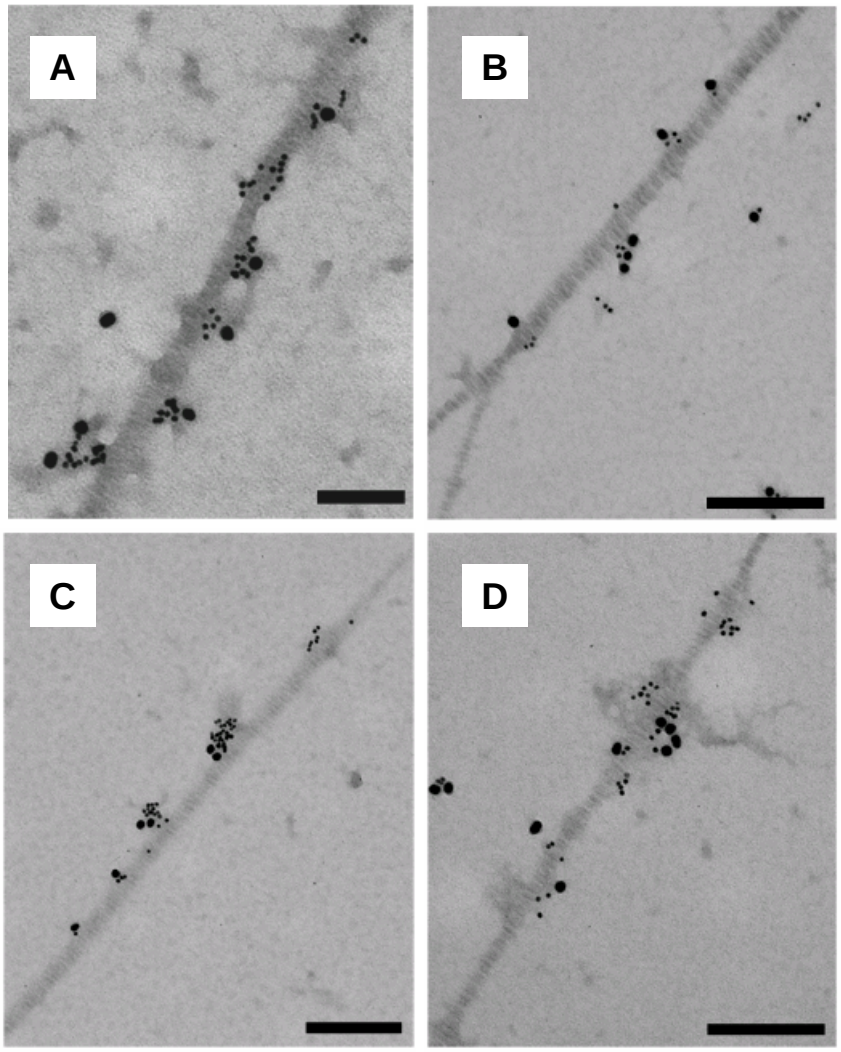

Figure 5-7. Electron micrographs of ultrastructural localization of collagen VI (small gold particles) and biglycan (large gold particles) on the fibrils after the treatment with 0, 1, 3, and 5 M guanidine hydrochloride are shown in A, B, C, and D, respectively. Bar, 100 nm for A, 200 nm for B, C, and D.

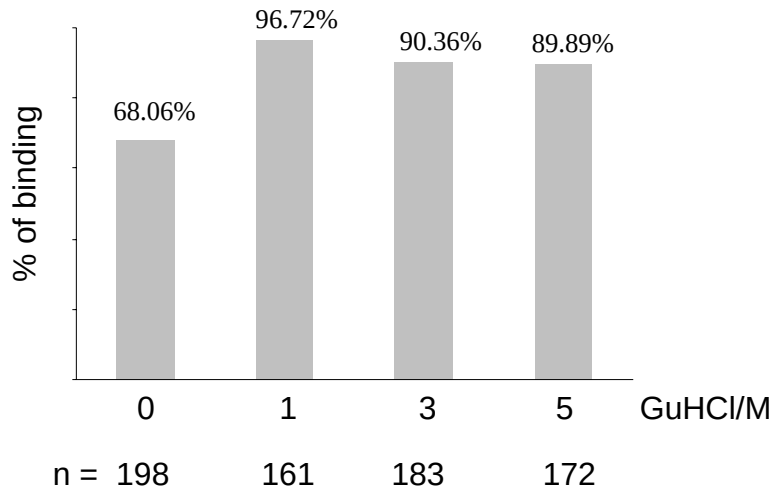

Figure 5-8. The percentage of gold particles representing biglycan that bind to collagen VI twisting around banded fibrils, compared with the total gold particles representing biglycan on the same banded fibrils before and after treatment with guanidine hydrochloride (GuHCl). Data are from two repeated experiments.

5.4 The interaction of collagen VI microfibrils with cartilage banded collagen fibrils is disrupted in collagen IX-deficient mice

The investigation of fibril fragments from articular bovine cartilage revealed that biglycan interacted to the globular domains of collagen VI microfibrils in a regular binding pattern (see 5.2). Furthermore, collagen VI microfibrils were preferentially twisted around the thin baded fibrils containing collagen II, IX, and XI (see 5.3). But the large banded fibrils lacking of collagen IX molecule did not interact the microfibrils. Therefore, biglycan and collagen IX presumably are important for the interaction of collagen VI microfibrils with the banded collagen fibrils.

In order to substantiate the notion that biglycan and collagen IX in tethering collagen VI microfibrils to the cartilage banded fibrils, the composition of fibril fragments from costal cartilage of wild-type, biglycan-, and collagen IX-deficient newborn mice were investigated (see 4.4.2).

Fibril fragments from wild-type cartilage displayed a labelling with the collagen VI antibody gold conjugates. Collagen VI microfibrils bind regularly along the thin banded fibrils of wild-type and biglycan-knockout mice (Figure 5-9A and B,

respectively). The distances between the lateral groups of gold particles representing the globular domains of collagen VI microfibrils were measured. The measurement revealed that the lengths between the lateral globular domains of the microfibrils were the same in wild-type (89 ± 12 nm) and biglycan-knockout mice (91 ± 11nm). The collagen VI antibody gold conjugates were also specific to the fibril fragments from collagen IX-deficient mice. However, the gold particles were not regularly associated with banded fibrils but were a bit further away from the surface of the banded fibrils (55%; n = 232, Figure 5-9C). We concluded that the interaction of collagen VI microfibrils with the banded fibrils depended on collagen IX.

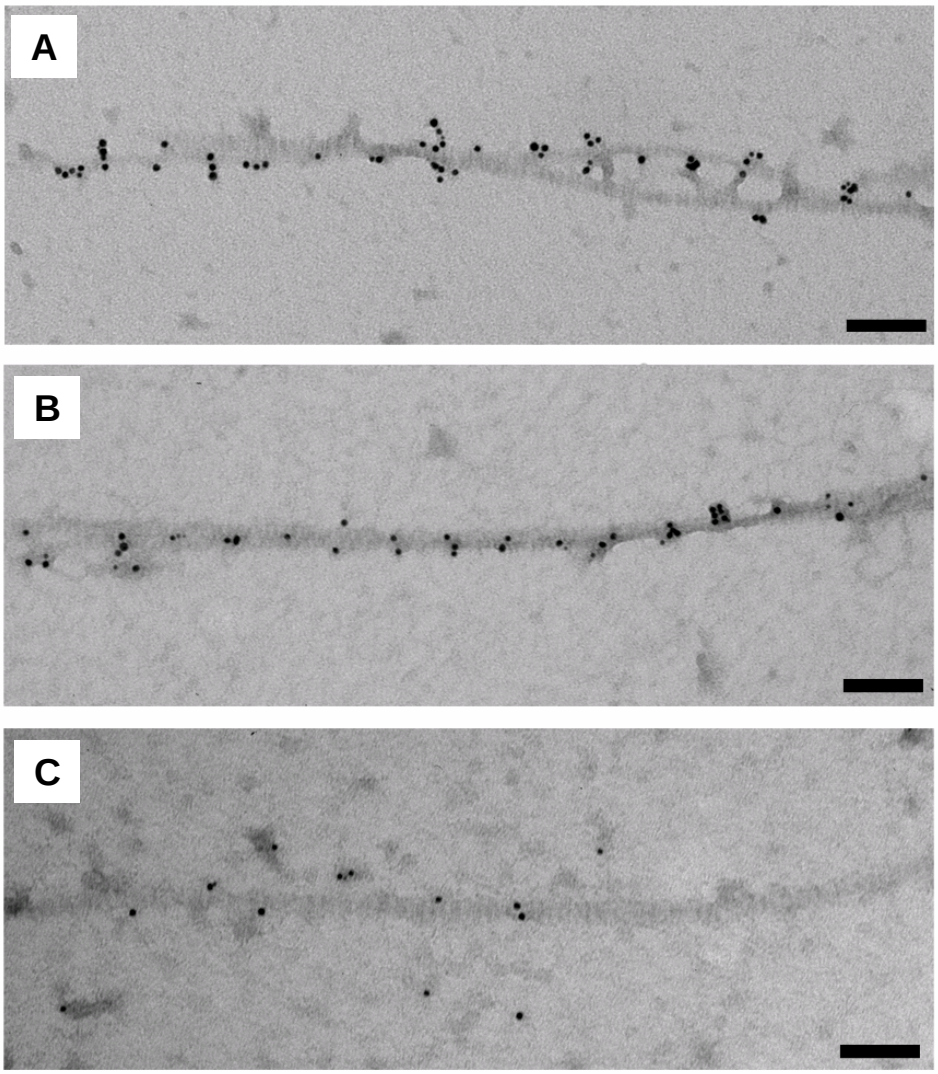

Figure 5-9. Ultrastructural localization of collagen VI on fibril fragments from newborn mice ribcages by direct immunogold electron microscopy. Collagen VI microfibrils has a regular binding pattern in wild type (A) as well as in biglycan knockout mice (B). This binding pattern was disrupted in collagen IX-knockout mice (C). Bar, 100 nm.

5.5 Isolation of collagen VI microfibrils from chicken cornea

Collagen VI microfibrils from chicken cornea were isolated after bacterial collagenase digestion. This enzyme digests collagens in banded fibrils while leaving intact collagen VI-containing microfibrils. The extracts were chromatographed on Superose 6 column (see 4.8.2) and the fractions pooled as indicated (Figure 5-10). The profiles demonstrated a clear separation of the high molecular weight aggregates (arrow) from small molecules. Purity of the isolated

collagen VI microfibrils were pure as judged by SDS-Polyacrylamide gradient gels of 4.5-15% stained with coomassie blue. All fractions without the reducing agent (β-mercaptoethanol) on coomassie blue stained gels are shown in Figure 5-11. The high molecular weight aggregates in fractions 6 to 8 failed to penetrate the gels (Figure 5-11). The small molecules were eluted from the column in fractions 14 to 20 (Figure 5-11). Figure 5-12 shows the coomassie blue stained gels of all fractions after treatment with reducing agent. The high molecular weight aggregates were reduced to different peptides (fractions 6 and 7 in Figure 5-11). There are five prominent bands between 75 to 140 kDa and a diffuse band at 25 kDa.

Immuno blotting experiments using monoclonal antibodies specific for collagen VI confirmed the presence of collagen VI in chicken cornea. Unreduced as well as reduced samples showed a positive immuno reaction (Figure 5-12 and 5-13, respectively) in the fraction of the high molecular weight aggregates. The purified collagen VI microfibrils consisted of disulfide bonded multimers which did not enter the gel in the absence of reducing agents and were visible as strong bands on the top of the gels (fractions 6 to 8, Figure 5-11). Upon reduction the multimers were dissociated into prominent five bands occurring between 75-140 kDa (Figure 5-11). Immunoblotting with the antibody showed only three bands corresponding to an apparent molecular weight of 140, 100, and 75 kDa (Figure 5-12). The 140 kDa-polypeptide corresponds to intact α1(VI) and α2(VI) chains (Engvall *et al*., 1986). The 100 kDa-polypeptide is consistent with an α1(VI)-fragment (Trüeb and Bornstein, 1984). The 75 kDa-polypeptide corresponds to a pepsin treated α1(VI)-fragment (Colombatti *et al*., 1987). Therefore, the monoclonal antibody is considered to specific for the α1(VI) chain. Furthermore, indirect immuno-gold electron microscopy analysis demonstrated collagen VI microfibrils as shown in Figure 5-14. The staining revealed globules representing the globular domains of collagen VI microfibrils. They were labeled with the monoclonal antibody against collagen VI. The faint lines connecting the globular domains represent the triple helical part of collagen VI microfibrils. The lengths between globular domains was 103 ± 10 nm (n = 137), consistent with the periodicity of collagen VI-containing microfibrils.

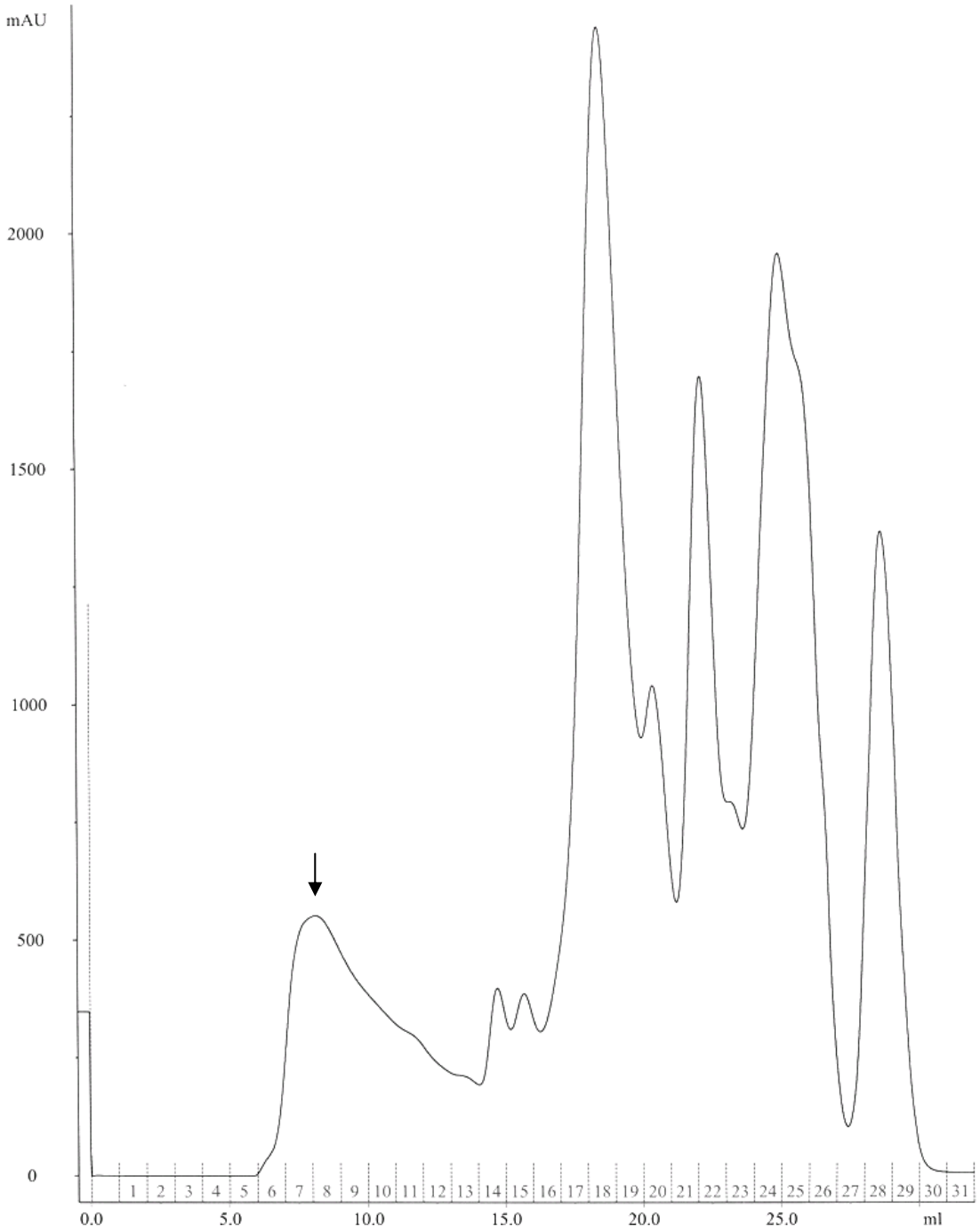

Figure 5-10. Chromatograph of fractions from the Superose 6 (Amersham Biosciences) molecular sieve column. The column was run at 0.2 ml/min, monitored at 280 nm with a UV spectrophotometer and fractions of 1 ml were collected.

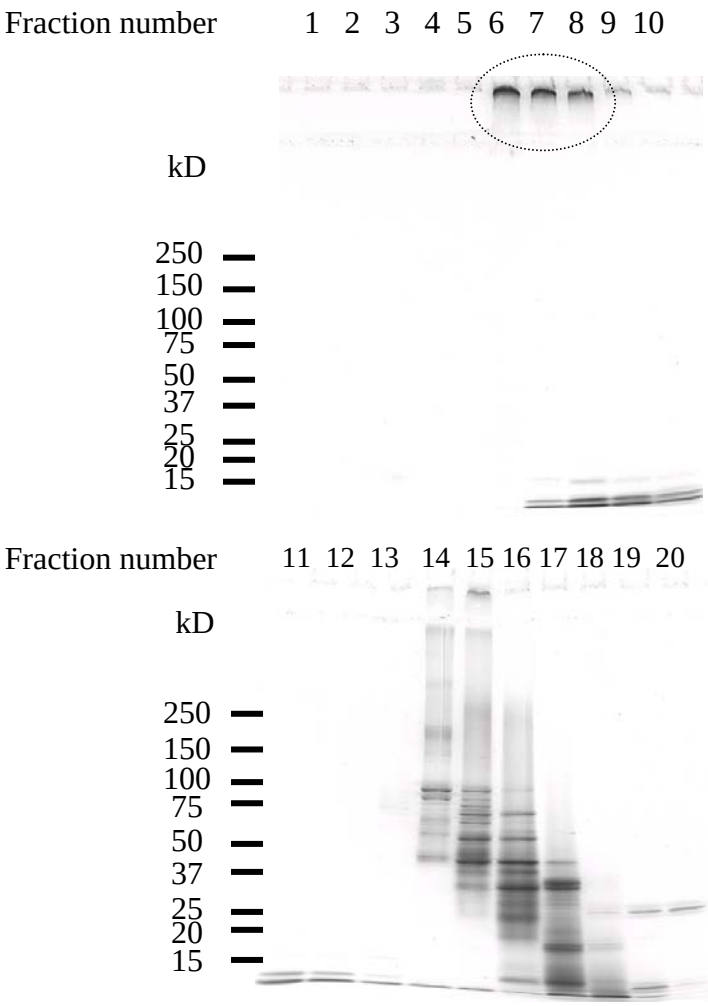

Figure 5-11. SDS-PAGE, 4.5-15%, fraction number 1-30 under non-reducing condition. High molecular aggregates failed to penetrate the gel (in the cycle).

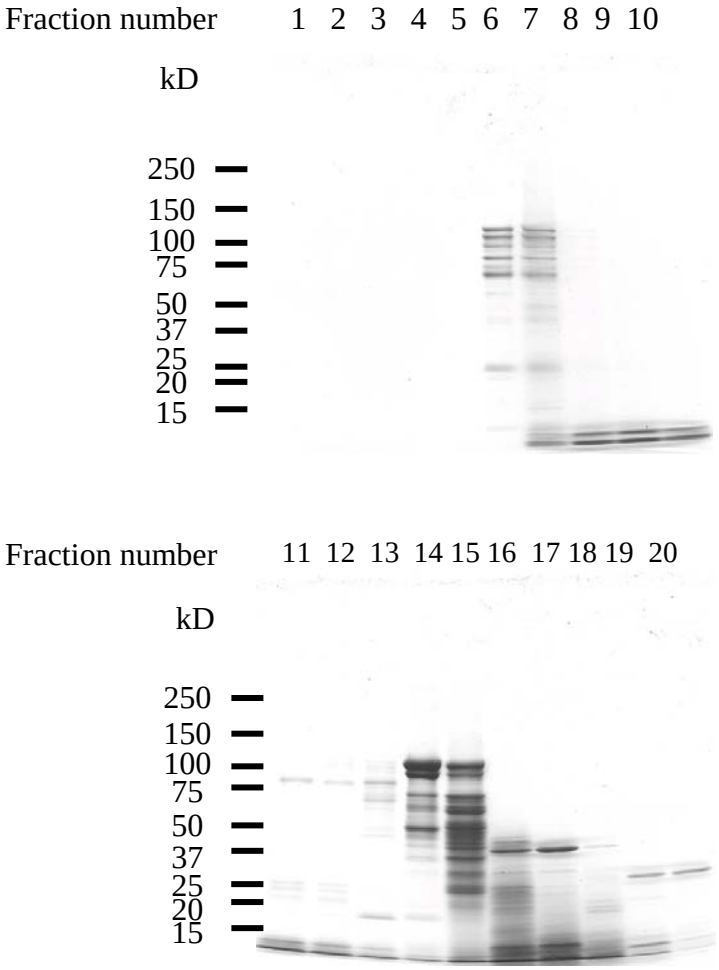

Figure 5-10. SDS-PAGE, 4.5-15%, fraction number 1-30 under reducing condition.

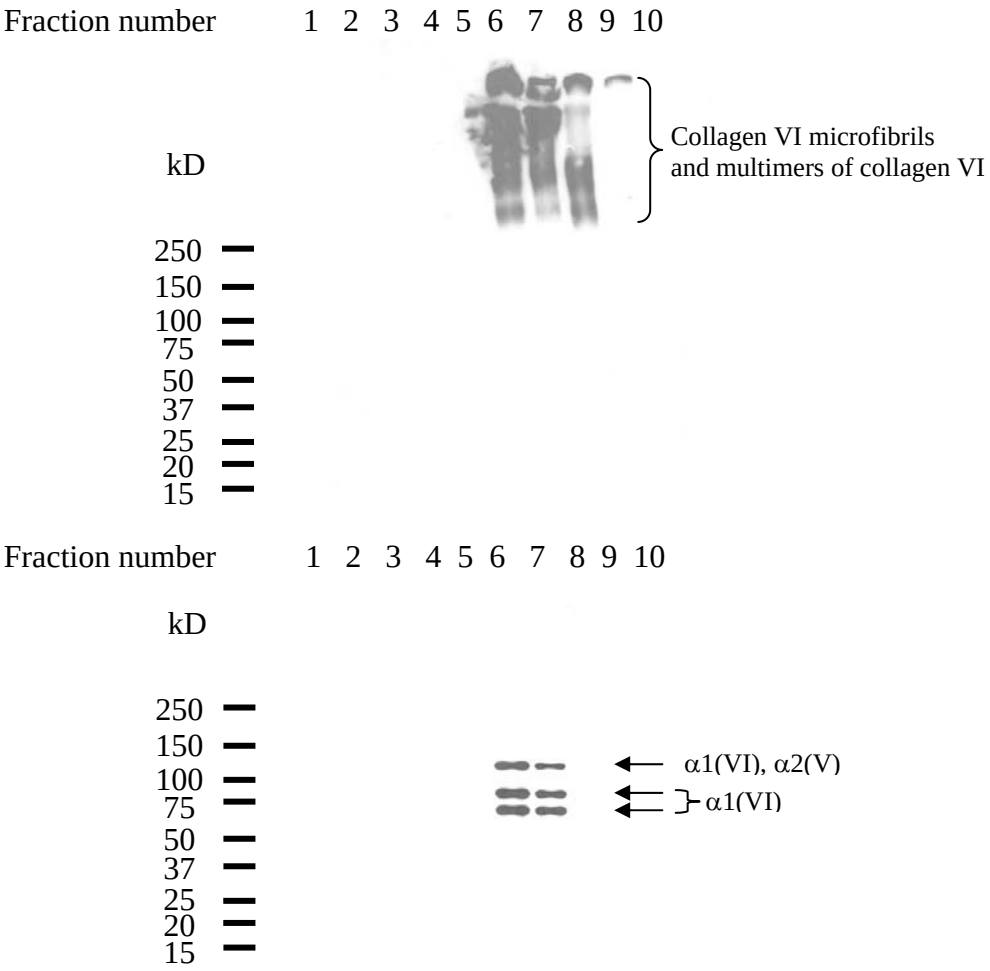

Figure 5-11. Immunoblots analysis of fraction number 1-10 under non-reducing (top) and reducing condition (below).

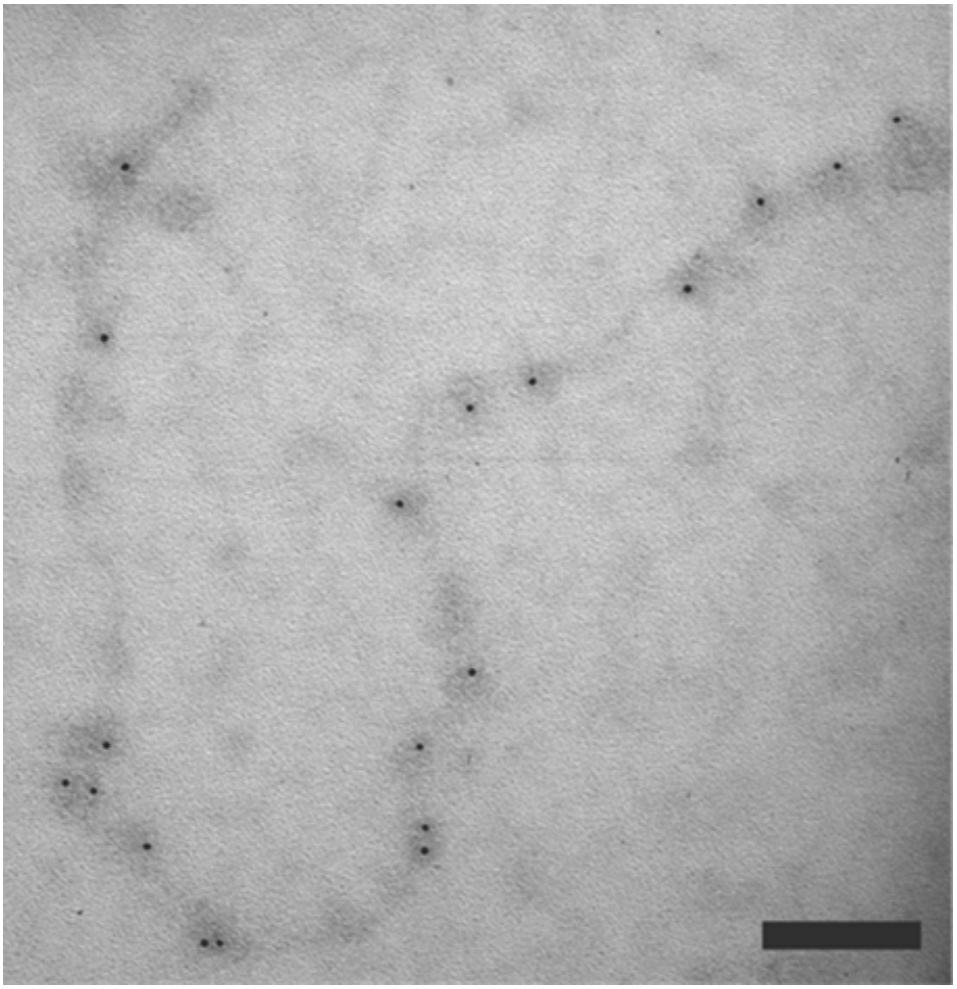

Figure 5-12. Electron micrograph of purified collagen VI microfibrils from chicken cornea. The gold particles represent the globular domain of collagen VI microfibrils. Bar, 200 nm

5.6 Collagen VI microfibrils bind to collagens II, IX and NC4-domain of collagen IX in binding assay (ELISA)

In order to study the monomolecular interaction between solubilized collagen VI and the purified chicken collagens II, IX, and XI (see 4.8.1) each representing molecular constituents of banded fibrils as well as the recombinant NC4-domain of collagen IX (see 4.9), binding experiments were performed. Constant amounts of the soluble collagens II, IX and XI in their monomeric state and the recombinant NC4-domain of collagen IX were immobilized on microtiter

plates. Pepsin treated and untreated commercial collagen VI microfibrils (Rockland Inc.) were used as ligand at concentrations ranging from 1-100 µg/ml (see 4.12).

The commercial collagen VI (Rockland Inc.) is not completely digested with pepsin. The coomassie blue stained gel showed the high molecular weight aggregates of commercial collagen VI (lane A, Figure 5-15). The three α chains of collagen VI were separated after reduction with β-mercaptoethanol (lane C, Figure 5-15). Lane B in Figure 5-15 shows the peptide fragments of collagen VI after the reduction followed by a second digestion with pepsin.

As expected, collagen XI does not bind to collagen VI microfibrils and collagen II is fast saturated at a concentration about 10 µg/ml of collagen VI microfibrils. Collagen IX and NC4-domain of collagen IX are also interact to collagen VI microfibrils (Figure 5-15). The NC4-domain of collagen IX has the highest intensity of the binding. Nevertheless, the association constants (K_d) of the binding of collagen VI microfibrils with collagens II, IX, and NC4-domain of collagen IX are 1.5×10^{-7}, 19.15×10^{-6}, and 3.0×10^{-7} M, respectively. Collagen II and the NC4 domain of collagen IX have a comparable binding affinity to collagen VI microfibrils. Therefore, collagen IX may interact with collagen VI microfibrils via NC4-domain.

The binding of pepsin treated collagen VI was obliterated with all substrates (Figure 5-17). Because pepsin treated collagen VI lost the globular domains the globular domains of collagen VI microfibrils are important for the interaction with the banded fibrils.

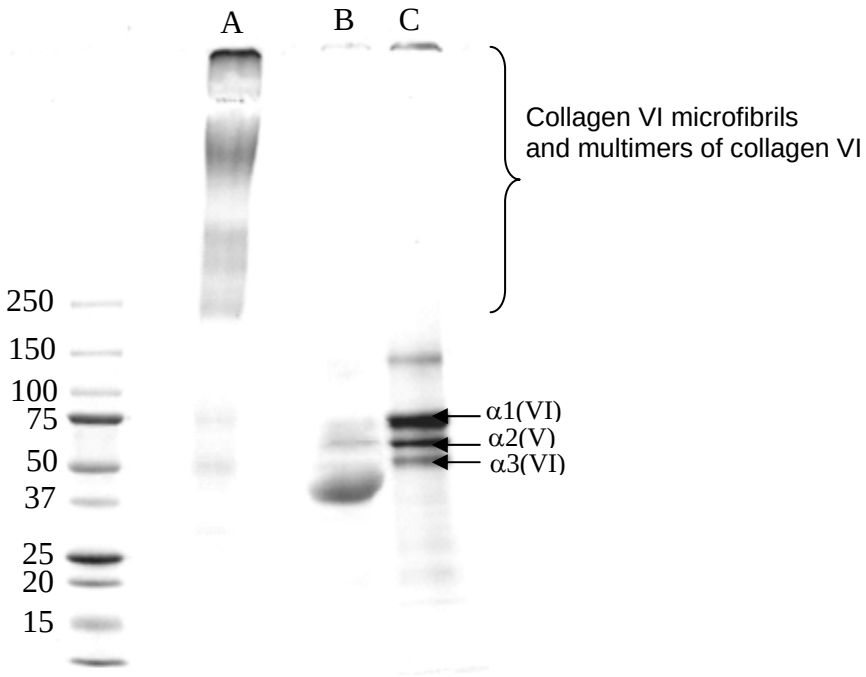

Figure 5-15. SDS-PAGE, 4.5-15% gel of commercial collagen VI (Rockland Inc), untreated (A), after a reduction (C), and after a reduction followed by a pepsin digestion representing three band of collagen VI (B) (Trüeb et al. 1987).

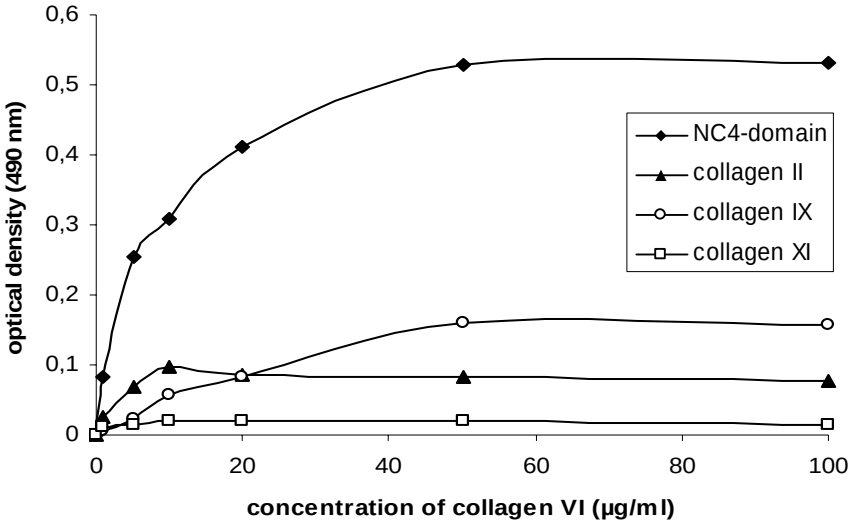

Figure 5-16. Binding of collagen VI to NC4-domain of collagen IX, collagens II, IX, and XI were determined by using a binding assay. The NC4-domain and the collagens were coated at a concentration of 10 µg/ml in storage buffer and incubated with in concentrations 0-100 µg/ml of collagen VI for 1 h. Data are the means of triplicate determinations.

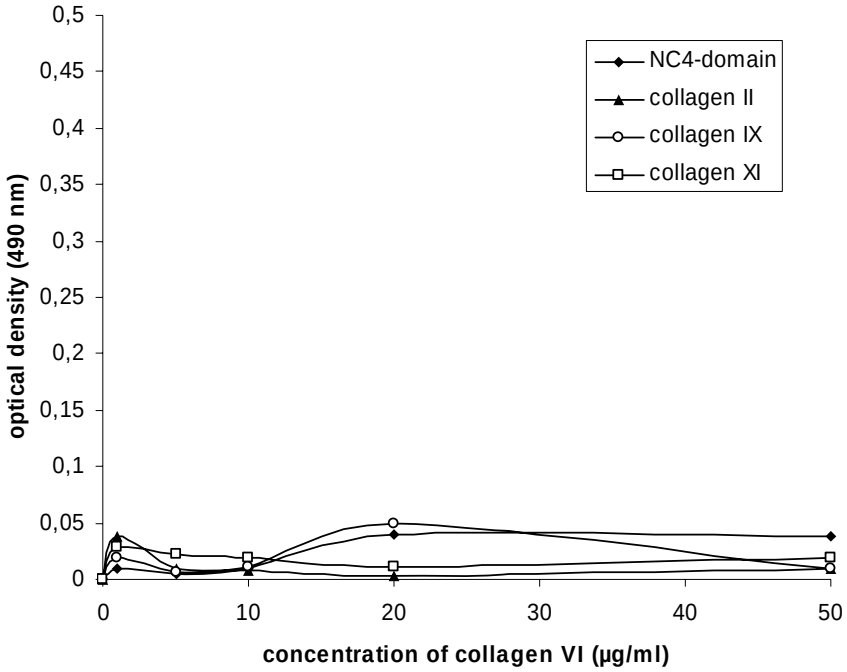

Figure 5-17. Binding of pepsin treated collagen VI to NC4-domain of collagen IX, collagens II, IX, and XI were determined by using a binding assay. The NC4-domain and the collagens were coated at a concentration of 10 µg/ml in storage buffer and incubated at concentration ranging from 0-50 µg/ml of collagen VI for 1 h. Data are the means of triplicate determinations.

5.7 Collagen VI microfibrils interact with the reconstituted fibrils containing collagens II, IX, and XI

In order to substantiate the notion that collagen IX is necessary for the binding of collagen VI microfibrils to banded fibrils, *in vitro* reconstitution experiments were preformed (see 4.11). Fibrils were reconstituted from soluble chicken collagens II, IX, and XI (see 4.8.1) for 3 h at 37 ^{o}C. Then commercial and isolated (see 4.8.2) collagen VI microfibrils were added. The development of turbidity kinetic, hyperbolic turbidity curves, of the fibrillogenesis is shown in Figure 5-18. The reconstitution products of collagens II, IX, and XI (A and B) showed higher turbidity than reconstitution products of collagens II and XI (C and D). The addition of commercial (data not shown) and isolated chicken collagen VI microfibrils slightly enhanced the turbidity (A and C), whereas the addition of buffer, only slightly decreased the turbidity (B and D). Added collagen VI

microfibrils were visualized in the EM as beaded filaments by labelling with an antibody against the globular domains of collagen VI. As expected, the analysis of the reconstituting products revealed that both collagen VI microfibrils, commercial (Rockland, Inc.) and isolated chicken collagen VI, interact only with banded fibrils containing collagens II, IX, and XI (Figure 5-19A and C). Microfibrils do not interact with reconstituted fibrils without collagen IX (Figure 5-19B and D). Furthermore, doubly-labelled collagen fibrils containing collagens II, IX, and XI revealed a clear co-localisation between the globular domains of collagen VI and the NC4-domain of collagen IX (Figure 5-19A).

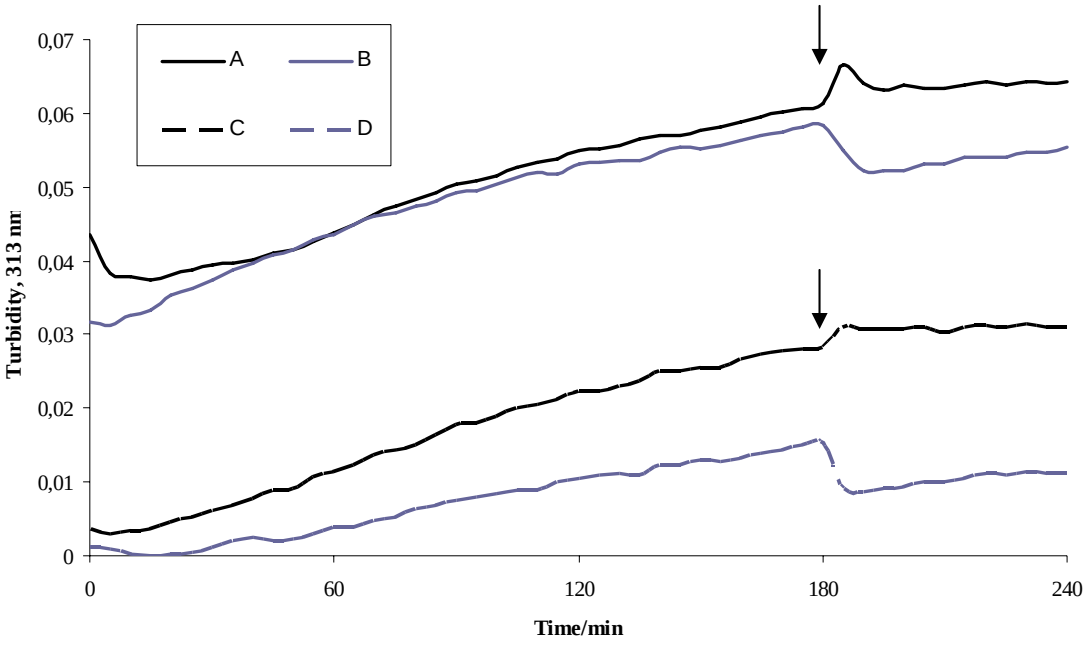

Figure 5-18. Fibrillogenesis of mixtures of collagens II, IX and XI (A and B) and collagens II and XI (C and D) monitored by development of turbidity at 313 nm. Chicken collagen VI microfibrils were added after 3 h (arrow), (A and C).

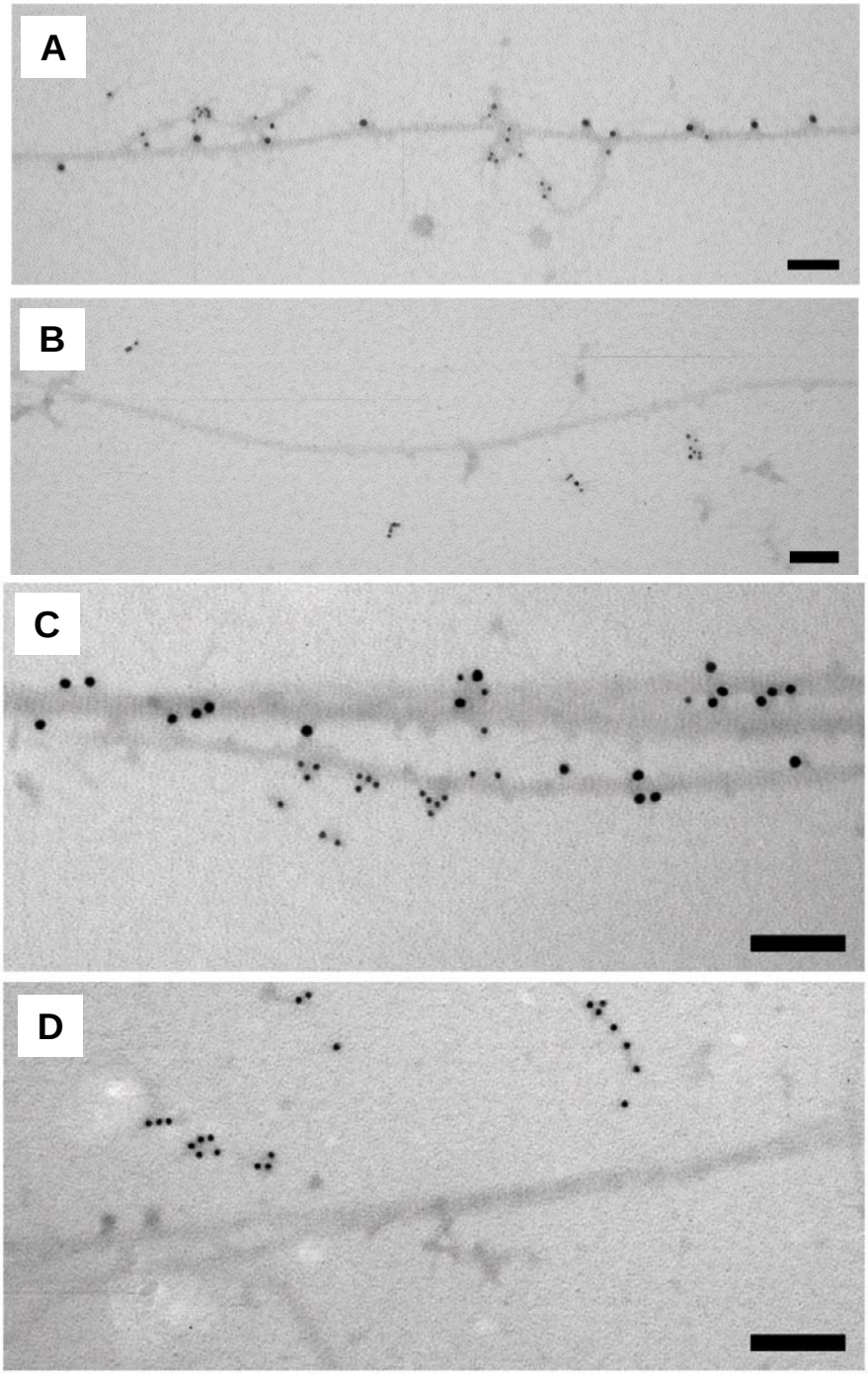

Figure 5-19. Immunoelectron micrographs of reconstituted fibrils from mixtures of collagens II, IX and XI (A and C) and collagens II and XI (B and D). The concentrations of collagens are 300 µg/ml. After 3 h of fibrillogenesis, commercial collagen VI was added to 5 µg/ml at the end. Then the fibrillogenesis was allowed to proceed for 1 h. A and B represent to the addition of commercial collagen VI microfibrils (Rockland Inc.) whereas C and D represent the adding of isolated chicken collagen VI microfibrils. Small gold particles represent collagen VI while large gold particles represent collagen IX. Bar, 100 nm.

6. DISCUSSION

The important biochemical properties of the cartilage are the result of the composite structure of the extracellular matrix containing a dense network of collagen fibrils. The fibril network is embedded in a high concentration of proteoglycans. There are many distinct proteins and glycoproteins occurring in mutual associations and their molecular assemblies. A major challenge of extracellular matrix biology is to understand the mechanisms that serve to control and organize the three-dimensional fibril network. Therefore, it is necessary to identify the molecular assembly components and to examine the nature of their interactive capabilities. In the present work, the issue has been addressed by two complementary approaches:

(i) the examination of fragments of authentic fibrils mechanically released from the tissue.

(ii) *in vitro* binding studies with the soluble components of interest that were discussed in ELISA and fibrillogenesis analysis of the present work.

These strategies were used for investigation of the tissues from bovine cartilage, chicken cornea and rib cages of newborn mice. Surprisingly, the first method has been applied in relatively few studies, although its potential stands out clearly. The authentic fibrils were obtained by mechanical tissue disruption. The fibril fragments could be examined by immuno-gold electron microscopy. After negative staining the fibril structure was shown in more detail. In addition, it is possible to carry out biochemical analysis of these macromolecular aggregates.

6.1 The molecular components of collagen VI-containing suprastructure

As discussed in 2.2.1.2, collagen VI interacts with a large number of extracellular matrix molecules. Supramolecular aggregates containing collagen VI are composite structures with other integrated molecules modulating the structural and functional properties of the collagen VI-containing suprastructure. In the present study, biglycan, decorin, and matrilin-1 were shown to interact to the

globular domains of collagen VI microfibrils which is in agreement with the studies of Wiberg and her co-workers (Wiberg *et al.*, 2001; 2002; 2003). Matrilin-1, -3, and -4 bind to the globular domains of collagen VI microfibrils purified from chondrosarcoma tissues via biglycan or decorin (Wiberg *et al.*, 2003). Furthermore, biglycan and decorin bind to the same triple helical site near the N-terminus. This interaction is mediated by the core protein and the presence of the glycoaminoglycans chains had no effect on binding (Wiberg *et al.*, 2001). Biglycan interacts with the tetramer induce formation of hexagonal lattices rather than beaded microfibrils. This was dependent on the presence of the glycoaminoglycan chains. Decorin which bind to the same site, was less effective in inducing hexagonal lattice formation presumably because of one glycosaminoglycan chain (Wiberg *et al.*, 2002). These binding of the proteoglycans via their core proteins and their glycosaminoglycan chains subsequently serve to keep collagen VI molecules separated while assembly is initiated. Therefore, the proteoglycans appear to have a central role in the organization of collagen VI into a network-like structure.

Moreover, in the present work, COMP binds close to the globular domains of collagen VI microfibrils. COMP has not previously been observed in collagen VI-containing suprastructure. However, the gold particles representing COMP were found at some distance from the globular domain of collagen VI microfibrils whereas the biglycan, decorin, and matrilin-1 were found in close proximity to the globular domains. Moreover, COMP was identified as a prominent binding partner for matrilin-1, -3, and -4 (Mann *et al.*, 2004). Therefore, it is possible that COMP indirectly is attached to globular domains of collagen VI microfibrils via matrilin-1, -3 and/or -4.

6.2 The isolation of collagen VI microfibrils from chicken corneas

Cornea contains relatively large amounts of collagen VI (Zimmermann *et al.*, 1986). Therefore, mostly intact collagen VI microfibrils were isolated from chicken by bacterial collagenase digestion following a procedure by Spissinger and Engel (1994). Chicken corneas were digested with bacterial collagenase and

were purified on a Superose 6 column. The purified collagen VI microfibrils consisted of disulfide bonded multimers which failed to penetrate the gel in the absence of reducing agents. Furthermore, the microfibrils could be detected and identified by immuno-gold electron microscopy. Upon reduction, the multimers were dissociated into five prominent bands occurring between 75-140 kDa representing the α-chains of collagen VI (Engvall *et al*., 1986; Trüeb and Bornstein, 1984; Colombatti *et al*., 1987).

6.3 The interaction of collagen VI microfibrils with banded fibrils containing collagens II, IX, and XI

A major novel observation of the present work was that collagen VI microfibrils undergo tight interactions with thin banded collagen fibrils in cartilage. Collagens II, IX, and NC4-domain of collagen IX, but not collagen XI, interacted with collagen VI microfibrils in ligand binding assay. Immobilized collagen II has the highest binding affinity to collagen VI microfibrils. This result is consistent to the result obtained by Bidanset and her co-workers (1992). However, the binding of collagen VI to collagen II monomers differs from binding to fibril-bound collagen II. The present work shows that collagen VI microfibrils do not bind to large banded fibrils which, however, do contain collagen II as the most abundant molecular component.

On the other hand, the binding of collagen VI microfibrils to the NC4-domain of collagen IX is a relatively weak binding ($K_d = 3\times10^{-7}$ M). However, the repetitive projections of COL3- and NC4-domain of collagen IX (Vaughan *et al*., 1988) present a substrate with multiple binding sites and, hence, an extremely high avidity for collagen VI microfibril binding.

Interestingly, the pepsin treated collagen VI microfibrils which lacks the globular domains could not interact with the ligands. Therefore, the globular domains of collagen VI microfibrils are important for the interaction with the banded fibrils.

Moreover, the investigation took advantage of the possibility that *in vitro* fibrillogenesis could confirm the conclusions from the investigations into authentic

suprastructures. Collagen VI microfibrils interact with the reconstituted fibrils containing collagens II, IX, and XI but do not interact with the reconstituted fibrils containing collagens II and XI. This result implies that collagen IX is important for the interaction and supports the results from the binding assays and costal cartilage of collagen IX-deficient mice.

Biglycan was found at the globular domains of collagen VI microfibrils in a regular pattern that twisted around banded fibrils. This result is consistent to the results from Wiberg and her coworkers (2001, 2002, 2003). First, biglycan was considered to play an important role as an adaptor between the banded fibrils and collagen VI microfibrils (Wiberg *et al*., 2003). However, after the treatment with 5 M guanidine hydrochloride, biglycan still binds to the globular domains of collagen VI microfibrils associated with banded fibrils. On the other hand, biglycan which did not bind to the globular domain of collagen VI microfibrils was removed. From these data it implies that biglycan does not play as an adaptor for the interaction but collagen VI microfibrils directly bind on the banded fibrils or via other matrix proteins (see Figure 6-1). This hypothesis is supported by the result from the investigation of biglycan-deficient costal cartilage of newborn mice. Collagen VI microfibrils have a regular binding on the banded fibrils in the knock-out mice as well as in the wild type mice.

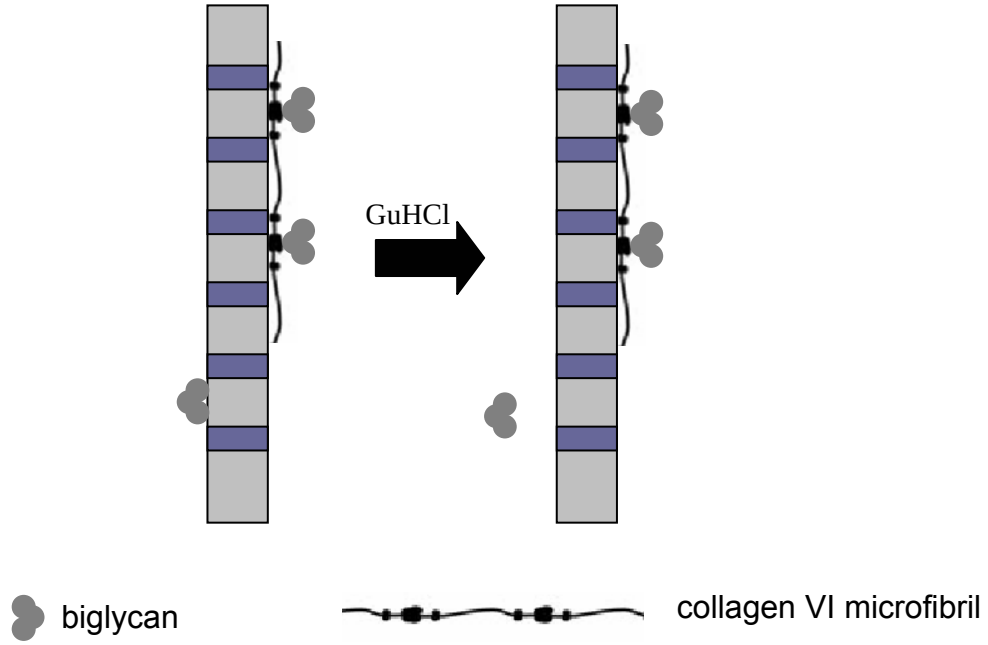

Figure 6-1. Model for the binding of collagen VI microfibrils on the banded fibrils in the extracellular matrix of articular cartilage. Biglycan directly bound to the banded fibrils was removed after treated with 5 guanidine hydrochloride. Because collagen VI still bind to the banded fibrils after the treatment, therefore, biglycan do not play as an adaptor for the binding but be a component of the supramolecular assembly of collagen VI microfibrils.

6.4 Collagen VI-containing suprastructure assembly

Budde and his co-workers (2005) have proposed two possible models of collagen VI microfibril binding to cartilage banded fibrils: Both models imply to binding of matrilin-3 which binds to the globular domains of collagen VI microfibrils via biglycan or decorin (Wiberg *et al*., 2003). Further:

(i) matrilin-3 binds to COL3-domain of collagen IX which contained in the banded fibrils (Budde *et al*., 2005). This domain projects from the banded fibril surface into perifibrillar space (Vaughan *et al*., 1988).

(ii) (ii) matrilin-3 binds to COMP (Mann *et al*., 2004) which interacts to collagen IX molecules.

However, from the results presented here, other conclutions may be drawn (Figure 6-2). In collagen VI-containing suprastructures, the globular domains of collagen VI microfibrils interact with NC4-domains of collagen IX, a component of

banded collagen fibrils projecting from the surface of the banded fibrils. Further, biglycan and decorin connect collagen VI microfibrils to matrilin-1, -3 and -4 (Wiberg *et al.*, 2003). These three matrilins interact with COMP (Mann *et al*., 2004). Moreover, matrilin-1 interacts with aggrecan (Paulsson and Heinegard, 1979; Hauser *et al*., 1996). It is presumably that collagen VI-containing suprastructures interact with aggrecan, the major component in network-like structure. Therefore, the collagen VI-containing suprastructure interactions suggest a possible role as a bridging suprastructure in cartilage matrix.

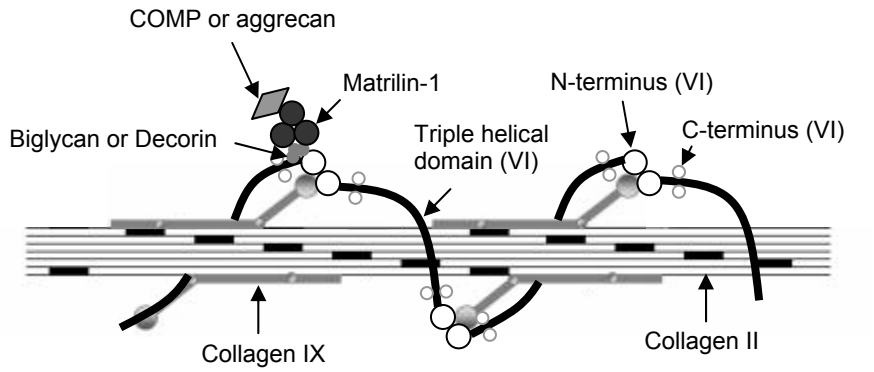

Figure 6-2. Model for supramolecular assembly of collagen VI microfibrils and cartilage banded fibrils. The globular domains of collagen VI microfibrils bind directly to the NC4-domain of collagen IX. Biglycan and decorin play as adaptor molecules to connect collagen VI microfibrils to matrilin-1. COMP contacts to collagen VI microfibrils are mediated by matrilin-1.

7. CONCLUSIONS

Hyaline articular cartilage plays important roles in the mechanics of the body. The tissue transmits weight-bearing forces between long bones and facilitates joint articulation. Therefore, the functionally essential component of the tissue is its abundant extracellular matrix. The few cells embedded into this matrix are responsible for the turnover and for the repair of the tissue. The extracellular matrix of hyaline cartilages consists of two major suprastructural elements, an extensive network of collagen II-rich fibrils embedding a highly hydrated complex of aggregating proteoglycan and hyaluronanic acid called network-like structures. Analogously into the mechanics of concrete strengthened with steel rods, the roles of the extrafibrillar matrix and the fibrils are to resist compression and tensile forces, respectively.

Collagen VI-containing aggregates are prominent examples of the suprastructural plasticity of extracellular matrix aggregates, depending on the exact composition. In order to gain more insight into the organization and molecular composition of matrix suprastructures the present work was performed by *in vivo* and in vitro studies. Collagen VI microfibrils are found in both of two suprastructural elements, banded collagen fibrils and network-like structures, of cartilage extracellular matrix. First group, tigh collagen VI-binding through regularly presented NC4-domains of collagen IX binding with extremely high avidity to regularly spaced globular domains of collagen VI microfibrils. The twisted is necessary to account for 100 nm versus 64 nm D-periodicity. Another group interacts to the network-like structures. Both collagen VI-containing suprastructures contains matrilin-1, decorin, and biglycan. These results are consistent to the previous reports of Wiberg and her co-workers (2001, 2002, and 2003). In addition, cartilage oligomeric matrix protein (COMP) is a novel component of the collagen VI-containing suprastructures.

In collagen VI-containing suprastructures, the globular domains of collagen VI microfibrils interact with NC4-domain of collagen IX, a component of banded collagen fibrils. Further, biglycan and decorin connect collagen VI microfibrils to matrilin-1, -3 and -4 (Wiberg *et al.*, 2003). In fact, matrilin-1 interacts with aggrecan

and COMP (Paulsson and Heinegard, 1979; Hauser et al., 1996; Mann *et al*., 2004). It is presumably that collagen VI-containing suprastructures interact with aggrecan, the major component in network like structure. Therefore, the collagen VI-containing suprastructure interactions suggest a possible role as a bridging suprastructure in cartilage matrix.

8. REFERENCES

Andres, J.L., K. Stanley, S. Cheifetz, and J. Massague. 1989. Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-beta. J. Cell Biol. 109:3137-3145.

Arey, L.B. 1974. Developmental Anatomy. 7th ed, WB Saunders, Philadelphia.

Baldock, C., M.J. Sherrat, C.A. Shuttleworth, and C.M. Kielty. 2003. The supramolecular organization of collagen VI microfibrils. J. Mol. Biol. 330:297-307.

Baldwin, C.T., A.M. Reginato, and D.J. Prockop. 1989. A new epidermal growth factor-like domain in the human core protein for the large cartilage specific proteoglycan. J. Biol. Chem. 264:15747-15750.

Banyard, J., L. Bao, and B.R. Zetter. 2003. Type XXIII collagen, a new transmembrane collagen identified in metastatic tumor cells. J. Biol. Chem. 278:20989-20994.

Belluoccio, D. and B. Trueb. 1997. Matrilin-3 from chicken cartilage. FEBS Lett. 415:212-216.

Bidanset, D.J., C. Guidry, L.C. Rosenberg, H.U. Choi, R. Timpl, and M. Hook. 1992. Binding of the proteoglycan decorin to collagen type VI. J. Biol. Chem. 267:5250-5256.

Birk, D.E., J.M. Fitch, J.P. Babiarz, and T.F. Linsenmayer. 1988. Collagen type I and V are present in the same fibril in the avian corneal stroma. J. Cell Biol. 106:999-1008.

Birk, D.E. and P. Bruckner. 2005. Collagen Suprastructures. Top Curr. Chem. 247:185-205.

Blaschke, U.K., E.F. Eikenberry, D.J.S. Hulmes, H.-J. Galla, and P. Bruckner. 2000. Collagen XI nucleates self-assembly and limits lateral growth of cartilage fibrils. J. Biol. Chem. 275:10370-10378.

Boot-Handford, R.P., D.S. Tuckwell, D.A. Plumb, C.F. Rock, and R. Poulsom. 2003. A novel and highly conserved collagen (pro(alpha)1(XXVII)) with a unique expression pattern and unusual molecular characteristics establishes a new clade within the vertebrate fibrillar collagen family. J. Biol. Chem. 278:31067-77.

Brewton, R., D.W. Wright, and R. Mayne. 1991. Structural and functional comparison of type IX collagen-proteoglycan from chicken cartilage and vitreous humor. J. Biol. Chem. 266:4752-4757.

Briggs, M.D., S.M. Hoffman, L.M. King, A.S. Olsen, H. Mohrenweiser, J.G. Leroy, G.R. Mortier, R.S. Lachman, E.S. Gaines, et al. 1995. Pseudoachondroplasia and multiple epiphyseal dysplasia due to mutations in the cartilage oligomeric matrix protein gene. Nat. Genet. 10:330-336.

Briggs, M.D., G.R. Mortier, W.G. Cole, L.M. King, S.S. Golik, J. Bonaventure, L. Nuytinck, A. De Paepe, J.G. Leroy, et al. 1998. Diverse mutations in the gene for cartilage oligomeric matrix protein in the pseudoachondroplasia-multiple epiphyseal dysplasia disease spectrum. Am J. Hum. Genet. 62:311-319.

Bruckner-Tuderman, L., B. Hopfner, and N. Hammami-Hauasli. 1999. Biology of anchoring fibrils: lessons from dystrophic epidermolysis bullosa. Matrix Biol. 18:43-54.

Bruns, R.R., W. Press, E. Engvall, R. Timpl, and J. Gross. 1986. Type VI collagen in extracellular, 100-nm periodic filaments and fibrils: identification by immunoelectron microscopy. J. Cell Biol. 103:393-404.

Budde, B., K. Blumbach, J. Ylöstalo, F. Zaucke, H.W.A. Ehlen, R. Wagener, L. Ala-Kokko, M. Paulsson, P. Bruckner, and S. Grässel. 2005. Altered integration of matrilin-3 into cartilage extracellular matrix in the absence of collagen IX. Mol. Cell Biol. 25:10465-10478.

Camacho, V.O., E. Bertini, R.Z. Zhang, S. Petrini, C. Minosse, P. Sabatelli, B. Giusti, M.L. Chu, and G. Pepe. 2001. Ullrich scleroatonic muscular dystrophy is caused by recessive mutations in collagen type VI. Proc Natl. Acad. Sci. USA. 98:7516-7521.

Cameron, D.A., and R.A. Robinson. 1958. Electron microscopy of epiphyseal and articular cartilage matrix in the femur of the newborn infant. J. Bone Joint Surg. (Am). 40:163-170.
Chapman, J.A., M. Tzaphlidou, K.M. Meek, and K.E. Kadler. 1990. The collagen fibril: a model system for studying the staining and fixation of a protein. Electron Microsc. Rev. 3:143-182.

Chen, Z., D. Heinegard, and Y. Sommarin. 1994. Distribution and expression of cartilage oligomeric matrix protein and bone matrix protein shows marked changes during rat femoral head development. Matrix Biol. 14:773-778.

Chen, Q., D.M. Johnson, D.R. Haudenschild, M.M. Tondravi, and Goetinck, P.F. 1995. Cartilage matrix protein forms a type II collagen-independent filamentous network: Analysis in primary cell cultures with a retrovirus expression system. Mol. Biol. Cell. 6:1743-1753.

Cheng, F., D. Heinegard, A. Malmstrom, A. Schmidtchen, K. Yoshida, and L.A. Fransson. 1994. Patterns of uronosyl epimerization and 4-/6-O-sulphation in chondroitin/dermatan sulphate from decorin and biglycan of various bovine tissues. Glycobiology. 4:685-696.

Chu, M., Mann K., R. Deutzmann, D.P. Conway, C. Hsu-Chen, M.P. Bernard, and R. Timpl. 1987. Characterization of three constituent chains of collagen type VI by peptide sequences and cDNA clones. Eur. J. Biochem. 168:309-317.

Chu, M., R.Z. Zhang, T.C. Pan, D. Stokes, D. Conway, H.J. Kuo, R. Glanville, U. Mayer, K. Mann, and R. Deutzmann. 1990. EMBO J. 9:385-393.

Cohn, D.H. 2001. Defects in extracellular matrix structural proteins in the osteochondro-dysplasias. Novartis Found. Symp. 232:195-210.

Colombatti, A., P. Bonaldo, K. Ainger, G.M. Bressen, and D. Volpin. 1987. Biosynthesis of chick type VI collagen: I: Intracellular assembly and molecular structure. J. Biol. Chem. 262:14454-14460.

Cremer, M.A., E.F. Rosloniec, and A.H. Kang. 1998. The cartilage collagens: a review of their structure, organization, and role in the pathogenesis of experimental arthritis in animals and in human rheumatic disease. J. Mol. Med. 76:275-288.

David, G., V. Lories, B. Decock, P. Marynen, J.J. Cassiman, and H. van den Berghe. 1990. Molecular cloning of a phosphatidylinositol-anchored membrane heparan sulphate proteoglycan from human lung fibroblasts. J. Cell Biol. 111:3165-3176.

Deak, F., D. Piecha, C. Bachrati, M. Paulsson, and I. Kiss. 1997. Primary structure and expression of matrilin-2, the closest relative of cartilage matrix protein within the von Willebrand factor type A module superfamily. J. Biol. Chem. 272:9268-9274.

Deak, F., R. Wagener, I. Kiss, and M. Paulsson. 1999. The matrilins: a novel family of oligomeric extracellular matrix proteins. Matrix Biol. 18:55-64.

Delot, E., S.G. Brodie, L.M. King, W.R. Wilcox, and D.H. Cohn. 1998. Physiological and pathological secretion of cartilage oligomeric matrix protein by cells in culture. J. Biol. Chem. 273:26692-26697.

Deutscher, M. P. 1990. Maintaining protein stability. *Methods Enzymol.* 182:83-89.

Diab, M., J.J. Wu, and D.R. Eyre. 1996. Collagen type IX from human cartilage. A structural profile of intermolecular cross-linking sites. Biochem. J. 314:327-332.

DiCesare, P.E., N. Hauser, D. Lehman, S. Pasumarti, and M. Paulsson. 1994. Cartilage oligomeric matrix protein (COMP) is an abundant component of tendon. FEBS Lett. 354:237-240.

DiCesare, P.E., M. Mörgelin, C.S. Carlson, S. Pasumarti, and M. Paulsson. 1995. Cartilage oligomeric matrix protein: isolation and charactrization from human articular cartilage. J. Orthop. Res. 13:422-428.

Doege, K., M. Sasaki, E. Horigan, J.R. Hassel, and Y. Yamada. 1987. Complete primary structure of the rat cartilage proteoglycan core protein deduced from cDNA clones. J. Biol. Chem. 262:17757-17767.

Doege, K., M. Sasaki, T. Kimura, and Y. Yamada. 1991. Complete coding sequence and deduced primary structure of the human cartilage large aggregating proteoglycan, aggrecan. J. Biol. Chem. 266:894-902.

Efimov, V.P., A. Lustig, and J. Engel. 1994. The thrombospondin-like chains of cartilage oligomeric matrix protein are assembled by a five-stranded alpha-helical bundle between residues 20 and 83. FEBS Lett. 341:54-58.

Eggli, P.S., E.B. Hunziker, and R.K. Schenk. 1988. Quantitation of structural features characterizing weight- and less-weight-bearing regions in articular cartilage: A stereological analysis of medial femoral condyles in young adult rabbits. Anat. Rec. 222:217-227

Ekman, S., F.P. Reinholt, K. Hultenby, and D. Heinegard. 1997. Ultrastructural immunolocalization of cartilage oligomeric matrix protein (COMP) in porcine growth cartilage. Calcif. Tissue Int. 60:547-553.

Engel, J. and D.J. Prockop. 1991. The zipper-like folding of collagen triple helices and the effects of mutations that disrupt the zipper. Ann. Rev. Biophys. Chem. 20:137-152.

Engvall, E., H. Hessle, and G. Klier. 1986. Molecular assembly, secretion, and matrix deposition of type VI collagne. J. Cell Biol. 102:703-710.

Eyre, D.R., S. Apon, J.J. Wu, L.H. Ericsson, and K.A. Walsh. 1987. Collagen type IX: evidence for covalent cross-linkages to type II collagen in cartilage. FEBS Lett. 220:337-341.

Eyre, D.R. 2002. Collagen of articular cartilage. Arth. Res. 4:30-35.

Eyre, D.R., T. Pietka, M.A. Weis, and J.J. Wu. 2004. Covalent cross-linking of the NC1 domain of collagen type IX to collagen type II in cartilage. J. Biol. Chem. 279:2568-2574.

Fassler, R., P.N. Schnegelsberg, J. Dausman, T. Shinya, Y. Muragaki, M.T. McCarthy, B.R. Olsen, and R. Jaenisch. 1994. Mice lacking alpha 1 (IX) collagen develop noninflammatory degenerative joint disease. Proc. Natl. Acad. Sci. USA. 91:5070-5074.

Fisher, L.F., J.D. Termine, and M.F. Young. 1989. Deduced-protein sequence of bone small proteoglycan (biglycan) shows homology with protoglycan II (decorin) and several nonconnective tissue proteins in a variety of species. J. Biol. Chem. 264:4571-4576.

Fisher, L.W., A.M. Heegaard, U. Vetter, W. Vogel, W. Just, J.D. Termine, and M.F. Young. 1991. Human biglycan gene. Putative promoter, intron-exon junctions, and chromosomal localization. J. Biol. Chem. 266:14371-14377.

Fitzgerald, J. and J.F. Bateman. 2001. A new FACIT of the collagen family: COL21A1. FEBS Lett. 505:275-280.

Fleischmajer, R., L.W. Fisher, E.D. McDonald, L.Jr. Jacobs, J.S. Perlish, and J.D. Termine. 1991. Decorin interacs with fibrillar collagen of embryonic and adult human skin. J. Struct. Biol. 106:82-90.

Fleischmajer, R., B.R. Olsen, and K. Kühn. 1990. Structure, molecular biology, and pathology of collagen. Ann. NY Acad. Sci. 580:1-592.

Font, B., E. Aubert-Foucher, D. Goldschmidt, D. Eichenberger, and M. van der Rest. 1993. Binding of collagen XIV with the dermatan sulphate side chain of decorin. J. Biol. Chem. 268:25015-25018.

Font, B., D. Eichenberger, L.M. Rosenberg, and M. van der Rest. 1996. Characterization of the interactions of type XII collagen with two small proteoglycans from fetal bovine tendon, decorin and fibromodulin. Matrix Biol. 15:341-348.

Furthmayr, H., H. Wiedemann, R. Timpl, E. Odermatt, and J. Engel. 1983. Electron-microscopical approach to a structural model of intima collagen. Biochem. J. 211:303-311.

Gegenheimer, P. 1990. Preparation of extracts from plants. *Methods Enzymol.* 182:174-193.

Gelse, K., E. Pöschl, and T. Aigner. 2003. Collagens: structure, function, and biosynthesis. Adv. Drug Del. Rev. 55:1531-1546.

Hagg, R., E. Hedbom, U. Möllers, A. Aszodi, R. Fässler, and P. Bruckner. 1997. Absence of the α1(IX) chain leads to a functional knock-out of the entire collagen IX protein in mice. J. Bio. Chem. 272:20650-20654.

Hagg, R., P. Bruckner, and E. Hedbom. 1998. Cartilage fibrils of mammals are biochemically heterogeneous: differential distribution of decorin and collagen IX. *J. Cell Biol*. 142:285-294.

Hansen, U., and P. Bruckner. 2003. Macromolecular specificity of collagen fibrillogenesis: fibrils of collagens I and XI contain a heterotypic alloyed core and a collagen I sheath. J. Biol. Chem. 278:37352-37359.

Hashimoto, T., T. Wakabayashi, A. Watanabe, H. Kowa, R. Hosoda, A. Nakamura, I. Kanazawa, T. Arai, K. Takio, D.M. Mann, and T. Iwatsubo. 2002. CLAC: a novel Alzheimer anyloid plaque component derived from a transmembrane precursor, CLAC-P/collagen type XXV. EMBO J. 21:1524-1534.

Hauser, N. and M. Paulsson. 1994. Native cartilage matrix protein (CMP). A compact trimer of subunits assembled via a coiled-coil alpha-helix. J. Biol. Chem. 269:25747-25753.

Hauser, N., M. Paulsson, D. Heinegard, and M. Mörgelin. 1996. Interaction of cartilage matrix protein (CMP) with aggrecan. Increased covalent crosslinking with maturation. J. Biol. Chem. 271:32247-32252.

Hausser, H., W. Hoppe, U. Rauch, and H. Kresse. 1989. Endocytosis of a small dermatan sulphate proteoglycan. Identification of binding proteins. Biochem. J. 263:137-142.

Hausser, H., B. Ober, E. Quentin-Hoffmann, B. Schmidt, and H. Kresse. 1992. Endocytosis of different members of the small chondroitin/dermatan sulfate proteoglycan family. J. Biol. Chem. 267:11559-11564.

Hedbom, E., P. Antonsson, A. Hjerpe, D. Aeschlimann, M. Paulsson, E. Rosa-Pimentel, Y. Sommarin, M. Wendel, A. Oldberg, and D. Heinegard. 1992. Cartilage matrix proteins. An acidic oligomeric matrix protein (COMP) detected only in cartilage. J. Biol. Chem. 267:6132-6136.

Hedbom, E. and D. Heinegard. 1993. Binding of fibromodulin and decorin to separate sites on fibrillar collagens. J. Biol. Chem. 268:27307-27312.

Hildebrand, A., M. Romaris, L.M. Rasmussen, D. Heinegard, D.R. Twardzik, W.A. Border, and E. Ruoslhti. 1994. Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. Biochem. J. 302:527-534.

Hocking, A.M., R.A. Strugnell, P. Ramamurthy, and D.J. McQuillan. 1996. Eukaryotic expression of recombinant biglycan. Post-translational processing and the importance of secondary structure for biological activity. J. Biol. Chem. 271:19571-19577.

Hollander, A.P., T.F. Heathfield, C. Webber, Y. Iwata, C. Rorabeck, R. Bourne, *et al*. 1994. Increased damage to type II collagen in osteoarthritic cartilage detected by a new immunoassay. J. Clin. Invest. 93:1722–1732.

Horton, W.A. 1993. Morphology of connective tissue: cartilage. In Connective Tissue and its Heritable Disorders. P.M. Royce, and B. Steinmann, eds. Wiley-Liss Inc., New York. 73-84.

Huber, S., K.H. Winterhalter, and L. Vaughan. 1988. Isolation and sequence analysis of the glycosaminoglycan attachment site of type IX collagen. J. Biol. Chem. 263:752-756.

Huizinga, E.G., R. Matijn van der Plas, J. Kroon, J.J. Sixma, and P. Gros. 1997. Crystal structure of the A3 domain of human von Willebrand factor: implications for collagen binding. Structure. 5:1147-1156.

Hulmes, D.J.S. 1992. The collagen superfamily-diverse structures and assemblies. Essays Biochem. 27:49-67.

Hunziker, E.B. and W. Herrmann. 1990. Ultrastructure of cartilage: Inultrastructure of skeletal tissues. E. Bonucci and P.M. Motta, editors. Kluwer Academic Publisher, Norwell, MA. pp 79-109.

Ichimura, S., J.J. Wu, and D.R. Eyre. 2000. Two-dimensional peptide-mapping of cross-linked type IX collagen in human cartilage. Arch. Biochem. Biophys. 378:33-39.

Iozzo, R.V. 1998. Matrix proteoglycans: from molecular design to cellular function. Annu. Rev. Biochem. 67:609-652.

Iozzo, R.V., D.K. Moscatello, D.J. McQuillan, and I. Eichstetter. 1999. Decorin is a biological ligand for the epidermal growth factor receptor. J Biol Chem, 274:4489-4492.

Jarvelainen, H.T., M.G. Kinsella, T.N. Wight, and L.J. Sandell. 1991. Differential expression of small chondroitin/dermatan sulphate proteoglycans, PG-I/biglycan and PG-II/decorin, by vascular smooth muscle and endothelial cells in culture. J. Biol. Chem. 266:23274-23281.

Jazwinski, S. M. 1990. Preparation of extracts from yeast. *Methods Enzymol.* 182: 154-174.

Kadler, K.E. 1995. Extracellular matrix 1: fibril-forming collagens. Protein Profile. 2:491-619.

Kassner, A., U. Hansen, N. Miosqe, D.P. Reinhardt, T. Aigner, L. Bruckner-Tuderman. P. Bruckner, and S. Grassel. 2003. Discrete integration of collagen XVI into tissue-specific collagen fibrils or beaded microfibrils. Matrix Biol. 22:131-143.

Kielty, C.M., I. Hopkinson, and M.E. Grant. 1993. Molecular, genetic, and medical aspects. In Connective Tissue and its Heritable Disorders. ed. P.M. Royce, B. Steinmann. Wiley-Liss, New York. 103-147.

Kielty, C.M., and M.E. Grant. 2002. The collagen family: structure, assembly, and organization in the extracellular matrix. In Connective Tissue and its Heritable Disorders: Molecular, Genetic, and Medical Aspects, 2nd ed, P.M. Royce, and B. Steinmann, eds. Wiley-Liss Inc., New York. 159-221.

Kivirikko, K.I., and D.J. Prockop. 1995. Collagens: molecular biology, diseases, and potentials for therapy. Annu. Rev. Biochem. 64:403-443.

Klatt, A.R., D.P. Nitsche, B. Kobbe, M. Mörgelin, M. Paulsson, and R. Wagener. 2000. Molecular structure and tissue distribution of matrilin-3, a filament-forming extracellular matrix protein expressed during skeletal development. J. Biol. Chem. 275:3999-4006.

Klatt, A.R., D.P. Nitsche, B. Kobbe, M. Macht, M. Paulsson, and R. Wagener. 2001. Molecular structure, processing, and tissue distribution of matrilin-4. J. Biol. Chem. 276:17267-17275.

Knott, L., C.C. Whitehead, R.H. Fleming, and A.J. Bailey. 1995. Biochemical changes in the collagenous matrix of osteoporotic avian bone. Biochem. J. 310:1045-1051.

Knupp, C. and J.M. Squire. 2001. A new twist in the collagen story: the type VI segmented supercoil. EMBO J. 20: 372-376.

Koch, M., J.F. Foley, R. Hahn, P. Zhou, R.E. Burgeson, D.R. Gerecke, and M.K. Gordon. 2001. Alpha 1 (XX) collagen, a new member of the collagen subfamily, fibril-associated collagens with interrupted triple helices. J. Biol. Chem. 276:23120-23126.

Koch, M., F. Laub, P. Zhou, R.A. Hahn, S. Tanaka, R.E. Burgeson, D.R. Gerecke, F. Ramirez, and M.K. Gordon. 2003. Collagen XXIV, a vertebrate fibrillar collagen with structural features of invertebrate collagens: selective expression in developing cornea and bone. J. Biol. Chem. 278:43236-43244.

Krantz, D.D., R. Zidovetzki, B.L. Kagan, and S.L. Zipursky. 1991. Amphipathic beta structure of a leucine-rich repeat peptide. J. Biol. Chem. 266:16801-16807.

Kresse, H., D.G. Seidler, M . Müller, E . Breuer, H . Hausser, P.J. Roughley, and E. Schönherr. 2001. Different usage of the glycosaminoglycan attachment sites of biglycan. J. Biol. Chem. 276:13411–13416.

Krumdieck, R., M. Hook, L.C. Rosenberg, and J.E. Volanakis. 1992. The proteoglycan decorin binds C1q and inhibits the activity of the C1 complex. J. Immunol. 149:3695-3701.

Krusius, T., and E. Ruoslahti. 1986. Primary structure of an extracellular matrix proteoglycan core protein deduced from cloned cDNA. Proc. Natl. Acad. Sci. USA. 83:7683-7687.

Krusius, T., K.R. Gehlsen, and E. Ruoslahti. 1987. A fibroblast chondroitin sulphate proteoglycan core protein contains lectin-like and growth factor-like sequences. J. Biol. Chem. 262:13120-13125.

Kuivaniemi, H., G. Tromp, and D.J. Prockop. 1991. Mutations in collagen genes: causes of rare and some common diseases in humans. FASEB J. 5:2052-2060.

Kwan, A.P., I.R. Dickson, A.J. Freemont, and M.E. Grant. 1989. Comparative studies of type X collagen expression in normal and rachitic chicken epiphyseal cartilage. J. Cell. Biol. 109:1849-1856.

Kwan, A.P., C.E. Cummings, J.A. Chapman, and M.E. Grant. 1991. Macromolecular organization of chicken type X collagen *in vitro*. J. Cell. Biol. 114:597-640.

Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680-685.

Laitinen, O. 1976. Relation to osteoporosis of age- and hormone-induced changes in the metabolism of collagen and bone. Isr. J. Med. Sci. 12:620-637.

Lane, J.M. and C. Weiss. 1975. Review of articular cartilage collagen research. Arthritis Rheum. 18:553-562.

Lui, V., R. Kong, J. Nicholls, A. Cheung, and K. Cheah. 1995. The mRNAs for three chains of human collagen type XI are widely distributed but not necessarily co-expressed: implications of for homotrimeric, heterotrimeric and hetrotypic collagen molecules. Biochem. J. 311:511-516.

Maddox, B.K., D.R. Keene, L.Y. Sakai, N.L. Charbonneau, N.P. Morris, C.C. Ridgway, B.A. Boswell, M.D. Sussman, W.A. Horton, H.P. Bächinger, and J.T. Hecht. 1997. The fate of cartilage oligomeric matrix protein is determined by the cell type in the care of a novel mutation in pseudoachondroplasia. J. Biol. Chem. 272:30993-30997.

Mann, H.H., S. Özbek, J. Engel, M. Paulsson, and R. Wagener. 2004. Interactions between the cartilage oligomeric matrix protein and matrilins. J Biol Chem, 279:25294-25298.

Marynen, P.J., J. Zhang, J.J. Cassiman, H. van den Berghe, and G. David. 1989. Partial primary structure of the 48- and 90-kilodalton proteins of cell surface-associated heparan sulphate proteoglycans. J. Biol Chem. 264:7017-7024.

Maynard, J.A., R.R. Cooper, and I.V. Ponseti. 1972. A unique rough surfaced endoplasmic reticulum inclusion in pseudoachondroplasia. Lab. Investig. 26:40-44.

Mayne, R. 1989. Cartilage collagens: what is their function, and are they involved in articular disease? Arthritis Rheum. 32:241-246.

Mayne R., R. Brewton, P. Mayne, and J. Baker. 1993. Isolation and characterization of the chains of type V/type XI collagen present in bovine vitreous. J. Biol. Chem. 268:9381-9386.

McBurney, M.W., L.C. Sutherland, C.N. Andra, B. Leclair, M.A. Rudnicki, and K. Jardine. 1991. The mouse Pgk-1 gene promoter contains an upstream activator sequence. Nucleic Acids Res. 20:5755-5761.

Mendler, M., S.G. Eich-Bender, L. Vaughan, K.H. Winterhalter, and P. Bruckner. 1989. Cartilage contains mixed fibrils of collagen type II, IX, and XI. J. Cell Biol. 108:191-197.

Miller M.D. 1996. Miller's Review of Orthopaedics 2nd ed W.B. Saunders.

Mörgelin, M., D. Heinegard, J. Engel, and M. Paulsson. 1992. Electron microscopy of native cartilage oligomeric matrix protein purified from the Swarm rat chondrosarcoma reveals a five-armed structure. J. Biol. Chem. 267:6137-6141.

Morris, N.P., D.R. Keene, and W.A. Horton. 2002. Morphology and chemical composition of connective tissue: cartilage. In Connective Tissue and its Heritable Disorders. P.M. Royce, and B. Steinmann, editor. 2nd ed. Wiley-Liss Inc., New York. pp 41-65

Müller, G., A. Michel, and E. Altenburg. 1998. COMP (cartilage oligomeric matrix protein) is synthesized in ligament, tendon, meniscus, and articular cartilage. Connect. Tissue Res. 39:233-244.

Muller-Glauser, W., B. Humbel, M. Glatt, P. Strauli, K.H. Winterhalter, and P. Bruckner. 1986. On the role of type IX collagen in the extracellular matrix of cartilage: type IX collagen is localized to intersections of collagen fibrils. J. Cell Biol., 102:1931-1939.

Myllyharju, J., and K.I. Kivirikko. 2001. Collagen and collagen-related diseases. Ann. Med. 33:7-21.

Myllyharju, J., and K.I. Kivirikko. 2004. Collagens, modifying ezymes and their mutations in humans, flies and worms. TRENDS Gen. 20:33-43.

Neame, P.J., H.U. Choi, and L.C. Rosenberg. 1989. The primary structure of the core protein of the small leucine-rich proteoglycan (PG I) from bovine articular cartilage. J. Biol. Chem. 264:8653-8611.

Newton, G., S. Weremowicz, C.C. Morton, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, and J. Lawler. 1994. Characterization of human and mouse cartilage oligomeric matrix protein. Genomics. 24:435-439.

Niyibizi C. and D.R. Eyre. 1994. Structural characteristics of cross-linking sites in type V collagen of bone: Chain specificities and heterotypic links to type I collagen. Eur. J. Biochem. 224:943-950.

Noonan, D.M., A. Fulle, P. Valente, S. Cai, E. Horigan, M. Sasaki, Y. Yamada, and J.R. Hassel. 1991. The complete sequence of perlecan, a basement membrane heparan sulphate proteoglycan, reveals extensive similarity with laminin A chain, low density lipoprotein-receptor, and the neural cell adhesion molecule. J. Biol. Chem. 266:22939-22947.

Nuytinck, L., M. Freund, L. Lagae, G.E. Pierard, T. Hermanns-Le, and A. De Paepe. 2000. Classical Ehlers-Danlos syndrome caused by a mutation in type I collagen. Am. J. Hum. Genet. 66:1398-1402.

Oldberg, A., P. Antonsson, K. Lindblom, and D. Heinegard. 1989. A collagen-binding 59 kD protein (fibromodulin) is a structurally related to the small interstitial proteoglycans PG-S1 and PG-S2 (decorin). EMBO J. 8:2601-2604.

Oldberg, A., P. Antonsson, K. Lindblom, and D. Heinegard. 1992. COMP (cartilage oligomeric matrix protein) is structurally related to the thrombospondins. J. Biol. Chem. 267:22346-22350.

Pace, J.M., M. Corrado, C. Missero, and P.H. Byers. 2003. Identification, characterization and expression analysis of a new fibrillar collagen gene, COL27A1. Matrix Biol. 22:3-14.

Paulsson, M. and D. Heinegard. 1979. Matrix proteins bound to associatively prepared proteoglycans from bovine cartilage. Biochem. J. 183:539-545.

Paulsson, M. and D. Heinegard. 1981. Purification and structural characterization of a cartilage matrix protein. Biochem. J. 197:367-375.

Paulsson, M. and D. Heinegard. 1982. Radioimmunoassay of the 148-kilodalton cartilage protein. Biochem. J. 207:213.

Peltonen, S., M. Hentula, P. Hagg, H. Yla-Outinen, J. Tuukkanen, J. Lakkakorpi, M. Rehn, T. Pihlajaniemi, and J. Peltonen. 1999. A novel component of epidermal cell-matrix and cell-cell contacts: transmembrane protein type XIII collagen. J. Invest. Dermatol. 113:635-642.

Piecha, D., S. Mulatoglu, M. Mörgelin, N. Hauser, D. Studer, I. Kiss, M. Paulsson, and F. Deak. 1999. Matrilin-2, a large, oligomeric matrix protein, is expressed by a great variety of cells and forms fibrillar networks. J. Biol. Chem. 274:13353-13361.

Piecha, D., C. Wiberg, M. Mörgelin, D.P. Reinhardt, F. Deak, P. Maurer, and M. Paulsson. 2002. Matrilin-2 interacts with itself and with other extracellular matrix proteins. Biochem. J. 367:715-721.

Poole, A.R., I. Pidoux , A. Reiner, and L. Rosenberg. 1982. An immunoelectron microscope study of the organization of proteoglycan monomer, link protein, and collagen in the matrix of articular cartilage. J. Cell. Biol. 93:921-937

Poole, C.A., M.H. Flint, and B.W. Beaumont. 1984. Morphological and functional interrelationships of articular cartilage matrices. J. Anat. 138:113-138

Poole, C.A., M.H. Flint, and B.W. Beaumont. 1987. Chondrons in cartilage: Ultrastructural analysis of the pericellular microenvironment in adult human articular cartilages. J. Orthop. Res. 5:509-522

Poole, C.A. 1992. Chondrons- the chondrocyte and its pericellular microenviroment. In Kuettner K.E., R. Schleyerbach, J.G. Peyron, and V.C. Hascall (eds). Articular Cartilage and Osteoarthritis, Raven Press, New York, 210.

Price, L.K.H., H.U. Choi, L. Rosenberg, and E.R. Stanley. 1992. The predominant form of secreted colony-stimulating factor-1 is a proteoglycan. J. Biol. Chem. 267:2190-2199.

Pringle, G.A. and C.M. Dodd. 1990. Immunoelectron microscopic localization of the core protein of decorin near the d and e bands of tendon collagen fibrils by use of monoclonal antibodies. J. Histochem. Cytochem. 38:1405-1411.

Ramachandran, G.N. and C. Ramakrishnan. 1976. In Biochemistry of Collagen. Ramachandran, G.N. & Reddi, A. H. eds., Plenum, New York, pp. 45-85.

Ratcliffe, A., and V.C. Mow. 1996. Articular cartilage. In Extracellular Matrix. W.D. Comper, editor. OPA, Amsterdam, The Netherlands. pp 234-302.

Ricard-Blum, S., F. Ruggiero, and M. van der Rest. 2005. The Collagen Superfamily. Top Curr. Chem. 247:35-84.

Riordan, J. F. und Vallee, B. L. 1972. Reactions with *N*-Ethylmaleimide and *p*-Mercuribenzoate. *Methods in Enzymology* 25:449-456.

Roughley, P.J., F. Rauch, and F.H. Glorieux. 2003. Osteogenesis imperfecta-clinical and molecular diversity. Eur. Cell Mater. 5:41-47.

Ruoslahti, E. 1988. Structure and biology of proteoglycans. A Rev. Cell Biol. 4:229-255.

Sampaio, L. de O., M.T. Bayliss, T.E. Hardingham, and H. Muir. 1988. Dermatan sulphate proteoglycan from human articular cartilage. Variation in its content with age and its structural comparison with a small chondroitin sulphate proteoglycan from pig laryngeal cartilage. Biochem. J. 254:757-764.

Sato, K., K. Yomogida, T. Wada, T. Yorihuzi, Y. Nishimune, N. Hosokawa, and K. Nagata. 2002. Type XXVI collagen, a new member of the collagen family, is specifically expressed in the testis and ovary. J. Biol. Chem. 277:37678-37684.

Saunders, S., M. Jalkanen, S. O'Farrell, and M. Bernfield. 1989. Molecular cloning of syndecan, an integral membrane proteoglycan. J. Cell Biol. 18:1547-1556.

Scacheri, P.C., E.M. Gillanders, S.H. Subramony, V. Vedanarayanan, C.A. Crowe, N. Thakore, M. Bingler, and E.P. Hoffman. 2002. Novel mutations in collagen VI genes: expansion of the Bethlem myopathy phenotype. Neurology. 58:593-602

Schäcke, H., H. Schumann, N. Hammami-Hauasli, M. Raghunath, and L. Bruckner-Tuderman. 1998. Two forms of collagen XVII in Keratinocytes: a full-length transmembrane protein and a soluble ectodomain. J. Biol. Chem. 273:25937-25943.

Schenk, R.K., P.S. Eggli, and E.B. Hunziker. 1986. Articular cartilage morphology. In Articular Cartilage Biochemistry. K.E. Kuettner, R. Schleyerbach, and V.C. Hascall, editor. Raven Press, New York. pp 3-22.

Schmid, T. and T. Linsenmayer. 1985. Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. J. Cell. Biol. 100:498-505.

Schmidt, G., H. Robenek, B. Harrach, J. Glossl, V. Nolte, H. Hormann, H. Richter, and H. Kresse. 1987. Interaction of smalldermatan sulphate proteoglycan from fibroblasts with fibronectin. J. Cell Biol. 104:1683-1691.

Scott, P.G., C.M. Dodd, E.M. Bergmann, J.K. Sheehan, and P.N. Bishop. 2006. Crystal structure of the biglycan dimer and evidence that dimerization is essential for folding and stability of class I small leucine-rich repeat proteoglycans. J. Biol. Chem. 281:13324-13332.

Seyer, J. and A. Kang. 1990. Covalent structure of type V collagen. Amino acid sequence of residues 66-267 of the human α1(V) collagen chain. Ann. NY Acad. Sci. 580:427-429.

Shen, Z., D. Heinegard, and Y. Sommarin. 1995. Distribution and expression of cartilage oligomeric matix protein and bone sialoprotein showmarked changes during rat femoral head development. Matrix Biol. 14:773-781.

Smith, R.K., L. Zunino, P.M. Webbon, and D. Heinegard. 1997. The distribution of cartilage oligomeric matrix protein (COMP) in tendon and its variation with tendon site, age and load. Matrix Biol. 16:255-271.

Smith, R.K., M. Gerard, B. Dowling, A.J. Dart, H.L. Birch, and A.E. Goodship. 2002. Correlation of cartilage oligomeric matrix protein (COMP) levels in equine tendon with mechanical properties: a proposed role for COMP in determining function-specific mechanical characteristics of locomotor tendons. Equine Vet. J. Suppl. 34:241-244.

Smyth, N., U. Odenthal, B. Merkl, and M. Paulsson. 2000. Eukaryotic expression and purification of recombinant extracellular matrix proteins carrying the Strep II tag. Methods Mol. Biol. 139:49-57.

Spissinger, T. and J. Engel. 1994. Type VI collagen beaded microfibrils from bovine cornea depolymerise at acidic pH, and depolymerization are not in fluenced by hyaluronan. Matrix Biol. 14:499-505.

Stockwell, R.A. 1979. Biology of cartilage cells. Cambridge University press, Cambridge.

Svensson, L., D. Heinegard, and A. Oldberg. 1995. Decorin-binding sites for collagen type I are mainly located in leucine-rich repeats 4-5. J. Biol. Chem. 270:20712-20716.

Thom J. and N. Morris. 1991. Biosynthesis and proteolytic processing of type IX collagen in embryonic chick sterna. J. Biol.Chem. 266:7262-7269.

Thur, J., K. Rosenberg, D.P. Nitche, T. Pihlajamma, L. Ala-Kokko, D. Heinegard, M. Paulsson, and P. Maurer. 2001. Mutations in cartilage oligomeric matrix protein causing pseudoachondroplasia and multiple epiphyseal dysplasia affect binding of calcium and collagen I, II, and IX. J. Biol. Chem. 276:6083-6092.

Trüeb, B., and P. Bornstein. 1984. Characterization of the precursor form of type VI collagen. J. Biol. Chem. 259:8597-8604.

van der Rest, R. and R. Mayne. 1987. Regulation of matrix accumulation, in: R. Mayne, R. Burgeson (Eds). Structure and Function of Collagen Types. Academic Press. Orlando.

van der Rest, R. and R. Mayne. 1988. Type IX collagen proteoglycan from cartilage is covalently cross-liked to type II collagen. J. Biol. Chem. 263:1615-1618.

van der Rest, M. and R. Garrone. 1991. Collagen family of proteins. FASEB J. 5:2814-2823.

Vaughan, L., M. Mendler, S. Huber, P. Bruckner, K.H. Winterhalter, M.I. Irwin, and R. Mayne. 1988. D-periodic distribution of collagen type IX along cartilage fibrils. J. Cell Biol. 106:991-997.

Veit, G., B. Kobbe, D.R. Keene, M. Paulsson, M. Koch, and R. Wagener. 2006. Collagen XXVIII, a novel von willebrand factor A domain-containing protein with many imperfections in the collagenous domain. J. Biol. Chem. 281:3494-3504.

Vikkula, M., P. Ritvaniemi, A.F. Vuorio, I. Kaitila, L. Ala-Kokko, and L. 1993. Peltonen. Amutation in the amino-terminal end of the triple helix of type II collagen causing severe osteochondrodysplasia. Genomics. 16:282-285.

Vikkula, M., E.C. Mariman, V.C. Lui, N.I. Zhidkova, G.E. Tiller, M.B. Goldring, S.E. van Beersum, M.C. de Waal Malefijt, F.H. van den Hoogen, H.H. Ropers, et al. 1995. Autosomal dominant and recessive osteochondrodysplasias associated with the COL11A2 locus. Cell. 80:431-437.

Vogel, K.G., M. Paulsson, and D. Heinegard. 1984. Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon. Biochem. J. 223:587-597.

Vogel, K.G. and J.A. Trotter. 1987. The effect of proteoglycans on the morphology of collagen fibrils formed in vitro. Coll. Relat. Res. 7:105-114.

von der Mark, H., M. Aumailley, G. Wick, R. Fleischmajer, and R. Timpl. 1984. Immunochemistry, genuine size and tissue localization of collagen VI. Eur. J. Biochem. 142:493-502.

von der Mark, K. 1999. Structure, biosynthesis and gene regulation of collagens in cartilage and bone, Dynamic of Bone and Cartilage Metabolism, Academic Press, Orlando. pp. 3-29.

Wagener, R., B. Knobbe, and M. Paulsson. 1997. Primary structure of matrilin-3, a new member of a family of extracellular matrix proteins related to cartilage matrix protein (matrilin-1) and von Willebrand factor. FEBS Lett. 413:129-134.

Wagener, R., B. Knobbe, and M. Paulsson. 1998. Matrilin-4, a new member of the matrilin family of extracellular matrix proteins. FEBS Lett. 436:123-127.

Wagener, R., H.W.A. Ehlen, Y. Ko, B. Kobbe, H.H. Mann, G. Sengle, and M. Paulsson. 2005. The matrilins-adaptor proteins in the extracellular matrix. FEBS Lett. 579:3323-3329.

Weiss, C., L. Rosenberg, and A.J. Helfet. 1968. An ultrastructural study of normal young adult human articular cartilage. J. Bone Joint Surg. (Am). 50:663-674

Whinna, H.C., H.U. Choi, L.C. Rosenberg, and F.C. Church. 1993. Interaction of heparin cofactor II with biglycan and decorin. J. Biol. Chem. 268:3920-3924.

Whittaker, C.A. and R.O. Hynes. 2002. Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. Mol. Biol. Cell. 13:3369-3387.

Wiberg, C., E. Hedbom, A. Khairullina, S.R. Lamande, A. Oldberg, R. Timpl, M. Mörgelin, and D. Heinegard. 2001. Biglycan and decorin bind close to the N-terminal region of the collagen VI triple helix. J. Biol. Chem. 276:18947-18952.

Wiberg, C., D. Heinegard, C. Wenglen, R. Timpl, and M. Mörgelin. 2002. Biglycan organizes collagen VI into hexagonal-like networks resembling tissue structures. J. Biol. Chem. 277:49120-49126.

Wiberg, C., A.R. Klatt, R. Wagener, M. Paulsson, J.F. Bateman, D. Heinegard, and M. Mörgelin. 2003. Complexes of matrilin-1 and biglycan or decorin connect collagen VI microfibrils to both collagen II and aggrecan. J. Biol. Chem. 278:37698-37704.

Winnemoller, M., P. Schon, P. Vischer, and H. 1992. Kresse. Interactions between thrombospondin and the small proteoglycan decorin interference with cell attachment. Eur. J. Cell Biol. 59:47-55.

Winterbottom, N., M.M. Tondravi, T.L. Harrington, F.G. Klier, B.M. Vertel, and P.F. Goetinck. 1992. Cartilage matrix protein is a component of the collagen fibril of cartilage. Dev. Dyn. 193:266-276.

Wu, J.J., P.E. Woods, and D.R. Eyre. 1992. Identification of cross-link sites in bovine cartilage type IX collagen reveals an antiparallel type II-type IX molecular relationship and type IX to type IX bonding. J. Biol. Chem. 267:23007-23014.

Wu, J.J. and D.R. Eyre. 1995. Structural analysis of cross-linking domains in cartilage type XI collagen: Insights on polymeric assembly. J. Biol. Chem. 1995. 270:18865-18870.

Xu, T., P. Bianco, L.W. Fisher, G. Longenecker, E. Smith, S. Goldstein, *et al*. 1998. Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice. Nat. Genet. 20:78-82.

Yada, T., S. Suzuki, K. Kobayashi, M. Kobayashi, T. Hoshino, K. Horie, and K. Kimata. 1990. Occurrence in chick embryo vitreous humor of a type IX collagen proteoglycan with an extraordinarily large chondroitin sulphate chain and short alpha 1 polypeptide. J. Biol. Chem. 265:6992-6999.

Yang, V.W., S.R. LaBrenz, L.C. Rosenberg, D. McQuillan, and M. Hook. 1999. Decorin is a Zn^{2+} metalloprotein. J. Biol. Chem. 274:12454-12460.

Zimmermann, D.R., B. Trüeb, K.H. Winterhalter, R. Witmer, and R.W. Fischer. 1986. Type VI collagen is a major component of the human cornea. FEBS. 197:55-58.

Zimmermann, D.R. and E. Ruoslahti. 1989. Multiple domains of the large fibroblast proteoglycan, versican. EMBO J. 8:2975-2981.

Lebenslauf

Name:	Wittaya Pimtong
Geburtsdatum:	14.07.1976
Geburtsort:	Yasothon
Staatsangehörigkeit:	Thai
Eltern:	Pong Pimtong Pa Pimtong, geb. Kokfai

Schulausbildung

1983-1989	Ban Lhao Fai-Grundschule
1989-1995	Kham Khuen Kaew Chanoopathum Gymnasium
28.03.1995	Allgemeine Hochschulreife, Yasothon, Thailand

Studium

1995-1999	Studium der Chemie an der Khon Kaen Universität
26.04.1999	Bachelor-Abschluss in der Naturwissenschaft (Chemie), Khon Kaen, Thailand
1999-2002	Master in Physikalische Chemie: „The Study of Amphiphilic Complex Formation“, an der Mahidol Universität Betreuer: Prof. Dr. Orapin Rangsiman, Institut für Chemie
19.09.2002	Masterprüfung, Bangkok, Thailand

Promotionsstudium

01.04.2003	Beginn der Dissertation am Institut für Physiologische Chemie und Pathobiochemie der Westfälischen Wilhelms-Universität Münster
	Betreuer: Prof. Dr. Hans-Joachim Galla, Institut für Biochemie. Mitbetreuer: Prof. Dr. Peter Bruckner, Institut für Physiologische Chemie und Pathobiochemie und Prof. Dr. Karl-Heinz Klempnauer, Institut für Biochemie.

Berufstätigkeit

11/2002-03/2004	wissenschaftliche Hilfskraft am Institut für Physiologische Chemie und Pathobiochemie der Universitätsklinikum Münster
ab 04/2004	wissenschaftlicher Mitarbeiter am Institut für Physiologische Chemie und Pathobiochemie der Universitätsklinikum Münster

Curriculum Vitae

Name:	Wittaya Pimtong
Date of Birth:	14.07.1976
Place of Birth:	Yasothon
Nationality:	Thai
Name of Parents:	Pong Pimtong
	Pa Pimtong

Education

1983-1989	Ban Lhao Fai Primary School, Yasothon, Thailand
1989-1995	Kham Khuen Kaew Chanoopathum Secondary School
28.03.1995	Higher Education Entrance Qualification, Yasothon, Thailand

Higher Education

1995-1999	Undergraduated Studies in Chemistry at Khon Kaen University
26.04.1999	Undergraduate Examination, Khon Kaen, Thailand
1999-2002	Graduated Studies in Physical Chemistry: “The Study of Amphiphilic Complex Formation”, at Mahidol University Supervisor: Prof. Dr. Orapin Rangsiman, Dept. of Chemistry
19.09.2002	Graduation, Bangkok, Thailand

Ph. D. Studies

01.04.2003	Begining of Ph. D. Studies at Dept. of Physiological Chemistry and Pathobiochemistry Supervisor: Prof. Dr. Hans-Joachim Galla, Dept. of Biochemistry. Co-supervisors: Prof. Dr. Peter Bruckner, Dept. of Physiological Chemistry and Pathobiochemistry and Prof. Dr. Karl-Heinz Klempnauer, Dept. of Biochemistry.

Professional Occupation

11/2002-03/2004	research assistant in Dept. of Physiological Chemistry and Pathobiochemistry at University Hospital Muenster
ab 04/2004	research associate in Dept. of Physiological Chemistry and Pathobiochemistry at University Hospital Muenster