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A Novel Marker of Tissue Junctions, Collagen XXII*

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Abstract

Here we describe a novel specific component of tissue junctions, collagen XXII. It was first identified by screening an EST data base and subsequently expressed as a recombinant protein and characterized as an authentic tissue component. The *COL22A1* gene on human chromosome 8q24.2 encodes a collagen that structurally belongs to the FACIT protein family (fibril-associated collagens with interrupted triple helices). Collagen XXII exhibits a striking restricted localization at tissue junctions such as the myotendinous junction in skeletal and heart muscle, the articular cartilage-synovial fluid junction, or the border between the anagen hair follicle and the dermis in the skin. It is deposited in the basement membrane zone of the myotendinous junction and the hair follicle and associated with the extracellular matrix in cartilage. *In situ* hybridization of myotendinous junctions revealed that muscle cells produce collagen XXII, and functional tests demonstrated that collagen XXII acts as a cell adhesion ligand for skin epithelial cells and fibroblasts. This novel gene product, collagen XXII, is the first specific extracellular matrix protein present only at tissue junctions.

Tissue integrity of all organs is critically dependent on suprastructural aggregates containing collagens. The family of collagens is specified in man by 42 genes encoding polypeptides assembled into at least 28 distinct, trimeric collagens. Their functions are indirectly illustrated by a multitude of human diseases resulting from mutations in collagen genes. To

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date more than 1000 different mutations are known to cause “collagen diseases” in a wide spectrum of organ systems, including the skeletal system, ligaments and other soft connective tissues, the kidney, bone marrow, skin, eye, and as shown recently, the brain (1–3).

Depending on their occurrence in supramolecular assemblies and other structural features, collagens are subdivided into different classes, such as fibrillar, network-forming, beaded filament-forming, fibril-associated, or transmembrane collagens. The major fibrillar collagens often have a wide tissue distribution in mesenchymal connective tissues, such as bone, cartilage, tendons, or dermal connective tissue (1). However, other collagens, *e.g.* the network-forming basement membrane collagens, can exhibit a very limited tissue localization lining epithelia, endothelia, or muscle cells and separating them from the surrounding extracellular matrix. Such a restricted expression pattern is believed to indicate both a highly specialized mechanical role in maintaining integrity of a tissue compartment and a role in regulation of cellular functions (4).

The FACITs,¹ fibril-associated collagens with interrupted triple helices, are quantitatively minor collagens that often copolymerize into suprastructures with the major collagens and mediate ligand interactions between the fibrils and their environment (5). Typically, these collagens contain triple helical as well as other functional protein modules, including VWA domains and fibronectin type III-like domains. VWA domains are found in a variety of proteins, *e.g.* the prototype von Willebrand factor, collagens, matrilins, and integrins (for review, see Ref. 6). The general notion about the function of VWA domains is mediation of protein-protein interactions. For example, the classical collagen binding receptors, integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$, bind to their target via VWA domains (7). Although not yet proven, it is likely the VWA domains of FACIT collagens also bind to other proteins. Two new VWA-containing proteins, collagens XX and XXI, were recently identified by screening databases (8,9). However, except for the gene structure and the predicted molecular domain structure, very little is known about these molecules, their distribution, or their functions in tissues.

Tissue junctions have critical functions in joining tissue compartments and in transmitting forces. However, because of a lack of specific markers for such junctions, their molecular and cellular composition and morphogenesis have remained elusive. The only exception may be the myotendinous junction (MTJ), which represents the link between muscles and tendons, the biology and pathology of which has been studied in more detail. To overcome the drastic forces at MTJs, the muscle increases the contact area by forming finger-like interdigitations of the basement membrane zone at the junction. Some molecules are enriched but not exclusively present at the MTJ, *e.g.* integrin $\alpha_7\beta_1$, or tenascin (10–12). Consequently, mutations in integrin $\alpha_7\beta_1$ cause muscular dystrophy (13,14). Recent developmental studies have provided evidence for the requirement of communication between muscle and tendon for morphogenesis. The morphogenetic origin of the muscle and tendon cells was shown to be the somites (12,15); in muscle cell ablation experiments tendons were not formed in a muscle-less wing (16). However, except for the initial generation of the precursors, practically nothing is known about the signaling between the tissue compartments, which will lead to formation of the MTJ. In this study we identified and characterized a novel marker, collagen XXII (Col XXII), which exhibits a unique localization at tissue junctions in the muscle, tendons, heart, articular cartilage, and skin.

¹The abbreviations used are: FACIT, fibril-associated collagen with interrupted triple helices; VWA, von Willebrand factor A-like; TSPN, N-terminal thrombospondin-like domain; Col XXII, collagen XXII; NC1 domain, corresponds to the N-terminal non collagenous region of Col XXII; MTJ, myotendinous junction; kb, kilobase(s); RACE, rapid amplification of cDNA ends; pAb, polyclonal antibody.

EXPERIMENTAL PROCEDURES

cDNA Isolation

A BLAST search (17) of the data base of expressed sequence tags (dbEST, Ref. 18) for homology with the C-terminal amino acid sequence of human collagen XII (accession number, NM_004370) yielded one clone (GenBank™ accession number AA296964) as a possible candidate for a novel collagen cDNA. The EST clone, which was about 1520 bp in length, was purchased from the American Type Culture Collection (ATCC) and sequenced in its entirety. The sequence revealed codons for 167 amino acid residues, and the 3'-untranslated region was 1.0 kb in length. From the sequence of the EST clone, nested primers were designed for 5' RACE using a human placental cDNA Marathon library (Clontech, Palo Alto, CA) as template, as previously described (19). For 5' RACE, the Long Expand PCR kit (Roche Applied Science) was used for the PCR reaction. By performing in total three RACEs with primer sequences derived from each previous RACE, overlapping segments representing the full-length 6.4-kb mRNA were obtained. To confirm the nucleotide sequence and as a control for PCR-induced nucleotide substitutions, gene-specific primers were used to reamplify the entire cDNA on human cartilage/bone cDNA. A first strand cDNA synthesis kit (BD Biosciences Clontech) was used to synthesize cDNA from total RNA using random primers following the manufacturer's protocol; PCR was used to generate overlapping clones complementary to the entire human $\alpha 1$ (XXII) collagen mRNA. Sequencing of all the PCR products obtained from the cDNA confirmed the nucleotide sequence of the human collagen XXII. The full-length cDNA of Col XXII is deposited in GenBank™ under accession number AF406780.

To generate a genetic relationship map of the VWA domain-containing proteins, the VWA of protein sequences were analyzed with the GrowTree program (GCG Sequence Analysis Software Package, Genetics Computer Group Inc.). The following sequences were used: matrilin1 (NP_002370, 35–221); matrilin2 (NP_085072, 51–237); matrilin3 (NP_002372, 77–263); matrilin4 (NP_003824, 28–224); collagen XII 1 (NP_004361, 1192–1376), collagen XII 2 (NP_004361, 2317–2501); collagen XIV 1 (CAA72402, 1–185), collagen XIV 2 (XX, 151–335); collagen XXI (NP_110447, 31–212); collagen XXII (AAN03620, 32–218). The WVA were analyzed, and the Jukes-Cantor method was chosen to correct the distances for multiple substitutions at a single site; the tree was created with the unweighted pair group method using arithmetic averages (UPGMA) algorithm.

Northern Blot Analysis

A 1368-bp PCR product (AF406780, nucleotides 529–1896) was labeled with [³³P]dCTP (PerkinElmer Life Sciences) using the *rediprime* DNA-labeling system (Amersham Biosciences). The Northern blot (Clontech) was prehybridized in 50% formamide, 5× saline/sodium phosphate/EDTA, 1× Denhardt's, 1% SDS, 10% dextran sulfate, and 0.1 mg/ml salmon sperm DNA (Invitrogen) at 42 °C for 2 h. Without further purification the probe was denatured in the same buffer plus 1:10 (v/v) human Cot-1 DNA (Roche Applied Science) and 1:10 (v/v) sheared salmon testis DNA (Invitrogen) at 94 °C for 5 min, placed on ice, added to the blots, and hybridized for 20 h. Blots were washed 3 times in 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 1% SDS at 42 °C, and 2 times in 0.1× SSC, 1% SDS at 42 °C. Blots were placed on BioMax MR film (Eastman Kodak Co.) with a BioMax TranScreen-LE intensifying screen (Kodak) for 20 h at –70 °C.

Recombinant Expression of Secreted Collagen XXII and Its Fragments

The following fragments were amplified by PCR and subcloned into an episomal expression vector: NC1 domain (nucleotides 529–1896) and full-length cDNA (nucleotides 529–5328). One μ g of total RNA from human cartilage/bone was reverse-transcribed, and PCR was

performed following the manufacturer's instructions (Herculase DNA polymerase; Stratagene). The PCR product was purified on an agarose gel (Qiagen) and subcloned (rapid DNA ligation kit; Roche Diagnostics) into a modified PCEP-4 (gift from Ernst Poeschl) expression vector. For convenience, an His₈ tag followed by a thrombin cleavage site was included adjacent to the NheI site in the vector. The ligated DNA was transformed into TOP 10 cells (Invitrogen). Plasmids were isolated from the bacteria (Qiagen) and sequenced with gene-specific primers (Thermo Sequenase cycle sequencing kit; Amersham Biosciences). 293-EBNA cells (Invitrogen) were transformed (FuGENE; Roche Diagnostics) with the expression vector and selected after 2 days with puromycin (Sigma).

Stably transfected 293-EBNA cells were pseudo-subcloned, and the highest protein-producing clones were expanded for large scale production. Two liters of supernatant from these cells were collected and supplemented with 1 mM Pefablock (Merck). After ammonium sulfate precipitation (45% saturation), the precipitate was collected by centrifugation, dialyzed against the binding buffer (200 mM NaCl, 20 mM Tris-HCl, pH 8), and applied onto a nickel-chelated Sepharose column (Amersham Biosciences). The elution was done by applying binding buffer containing increasing concentrations of imidazole (10–150 mM) to the column. In some cases, the His tag was digested with thrombin (isolated from bovine plasma; Sigma) according to the protocol from EMD Biosciences. The digested protein was again applied to a nickel-chelated Sepharose column to remove the His tag, and since the protein still weakly binds to the matrix, the fragment was again eluted with increasing imidazole concentration.

Antibody Production

The human Col XXII NC1 protein was injected intradermally into a rabbit (R34) for antibody production following standard procedures (20). The R34 antiserum was passed over a protein G column (Amersham Biosciences) and eluted with triethylamine (Sigma). The neutralized eluate was affinity-purified by applying it to a human Col XXII NC1 protein column that was prepared by coupling the protein without His tag to activated CNBr-Sepharose. Bound antibodies were eluted with triethylamine and immediately neutralized (19).

Immunodetection of Collagen XXII

The above polyclonal antibody pAb R34 was used for immunofluorescence staining of cryosections of 1-day-old mice and adult mouse tissues. The incubation with the first antibody (1:2000) was done overnight at 4 °C followed by a 1-h incubation with fluorescein isothiocyanate- or rhodamine-coupled secondary antibody at room temperature. The staining was observed by immunofluorescence microscopy. For immunoblotting the proteins were separated on SDS-PAGE with 4.5, 5.0, or 7.5% polyacrylamide under non-reduced or reduced conditions and transferred onto nitrocellulose. The affinity-purified antibodies were diluted 1:10,000 and incubated overnight followed by an incubation with horseradish peroxidase-linked anti-rabbit secondary antibody (Amersham Biosciences) for 2 h. The signals were visualized with chemiluminescence substrate Renaissance™ (PerkinElmer Life Sciences).

For immunoelectron microscopy with pAb R34, recombinant Col XXII was subjected to rotary shadowing using previously published methods (21). Native fibrils were isolated from bovine cartilage, placed on grids, immunostained with pAb R34 and colloidal gold-labeled secondary antibodies, and analyzed with transmission electron microscopy as described (22). Immunoelectron microscopy with immunogold labeling on ultra-thin sections of skin, muscle, and articular surface was carried out as described previously (23).

Tissue Extractions

For analysis of Col XXII tissue form, adult mouse tissues were dissected, homogenized in Tris-buffered saline (1 g of tissue/ml), and extracted sequentially at 4 °C with the following buffers; TBS for 30 min; 0.5 M NaCl, 50 mM Tris-HCl, pH 7.4, overnight; 2 M urea, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.4, overnight. All buffers contained 10 mM EDTA, 1 mM *N*-ethylmaleimide, and 1 mM Pefabloc (Merck) as proteinase inhibitors. Between the extractions the tissue was centrifuged at $14,000 \times g$ at 4 °C for 30 min, and 50–100 μ l of the supernatants were used for the enzyme digestions and 10–50 μ l for immunoblotting.

Collagenase Digestion

For assessment of the domain structure of Col XXII, the recombinant collagen and tissue extracts were subjected to collagenase digestion. The incubation with 40 units/ml highly purified bacterial collagenase (Advanced Biofacturers Inc., Lynbrook, NY) was carried out in 50 μ l of extraction buffer containing 5 mM CaCl₂ and 1 mM Pefablock at 37 °C for 4 h (34). The reaction was stopped by adding EDTA to a final concentration of 20 mM.

Cell Adhesion Assays

Skin epithelial cells (HaCaT) and fibroblasts (WI-26) were cultivated under standard conditions. Multiwell tissue culture plates (96 wells, Costar Corp., Faust, Germany) were coated with serial dilutions of Col XXII (0–165 nM) overnight at 4 °C. After saturation with 1% bovine serum albumin (fraction V, Sigma), the plates were immediately used for short term cell adhesion assays. After 30 min the floating cells were washed away, and the number of adherent cells were counted (24). All assays were done in triplicate.

In Situ Hybridization

In situ hybridization was performed as previously described (25) using cRNA probes generated from PCR products. The T7 polymerase recognition site was added to the reverse primers. For mouse collagen COL1A1 the region between nucleotides 3653 and 4242 (BC050014) was chosen as a probe. The mouse cDNA was obtained by comparison of the human Col XXII cDNA to the mouse genome. The following primers localized within the VWA and thrombospondin N-terminal-like domain (TSPN) domain region was used to amplify the probe from embryonic mouse cDNA (forward, 5'-GCCACTTCAACTCTCGCGAGGAGG-3'; reverse, 5'-CACAAAGCCGACGCCTCAGCTTGC-3'). Images shown here were processed using Adobe Photoshop (Releases 7.0); no enhancements other than contrast and brightness have been made to these images.

RESULTS

Identification and Cloning of Collagen XXII

Several novel partial cDNA sequences were identified in a human dbEST sequence data base search for clones containing Gly-X-Y triplets. One of these sequences was extended using rapid PCR amplification of cDNA ends. After three rounds of amplification, the full-length cDNA for Col XXII was obtained. It contains a predicted open reading frame of 1626 amino acids, including a putative signal peptide (27 amino acids) (Fig. 1; Ref. 26). The predicted protein is a secreted molecule with an N-terminal domain similar to a VWA-like domain followed by a TSPN and a long collagenous domain with several interruptions of the Gly-X-Y repeats. The TSPN sequence contains two consensus *N*-glycosylation sites, and a short linker sequence containing a polyproline stretch joins the globular and the collagenous region. The collagenous domain contains six small imperfections in which the third amino acid of Gly-X-Y triplet is missing and larger interruptions of 4–38 amino acids throughout

the collagenous sequence. The last 105-amino acid collagenous stretch at the C-terminal end contains the imperfections and amino acid organization common to FACIT proteins, *i.e.* two interruptions of the Gly-X-Y triplets followed by two Cys residues (Fig. 1). Thus, the overall structure of Col XXII is similar to the FACIT or FACIT-related proteins.

As judged by their domain organizations, the closest relative of Col XXII is human collagen XXI; it contains the same non-collagenous domain structure (9). The collagenous domain, however, is shorter in collagen XXI, in which the first collagenous stretch is missing (Fig. 2). Both molecules contain two cysteines on either side of the collagenous domain. The Col XXII VWA domain is 43% identical to the VWA domains of human collagen XXI, 37% in collagen XII and 36% in Matrilin-1 (Fig. 3). The TSPN domain, typically located between the non-collagenous domain and the collagenous domain, is a common feature of proteins of the FACIT subfamily. Comparing the TSPN domain of Col XXII to those in other collagens indicated a close relation to collagen XII and XXI. However, the overall homologies between the different TSPN domains were too low for the construction of a statistically relevant phylogenetic tree.

The Collagen XXII Gene COL22A1

Comparison of the *COL22A1* gene encoding collagen XXII with sequences in the genome databases revealed an orthologue for collagen XXII in mouse, zebra fish, and puffer fish. *COL22A1* is present on human chromosome 8q24.2 and spans 326 kb (NT_028251). The mouse *col22a1* gene is localized on chromosome 15 D2 to D3. According to the NCBI evidence viewer, the coding region of *COL22A1* contains 66 exons, separated by 65 introns, and the largest introns, intron 1 and 3, are more than 30 kb in size. The exon-intron boundaries follow the classical splice donor and acceptor consensus sequences (not shown). The signal peptide coding sequence is localized in the second exon. The VWA domain is encoded by exon 3, whereas the TSPN sequence is distributed over exons 4–9. Most exons encoding the collagenous sequences are rather small, and the majority of them encode for only 5–6 Gly-X-Y repeats.

Production and Characterization of an Antibody to Collagen XXII

The N-terminal globular region of Col XXII (NC1 domain), including a His tag and a thrombin cleavage site, was produced in 293-EBNA cells using an episomal expression vector. After purification with a nickel-containing column and removal of the His tag by cleavage with thrombin, the protein was used for immunization. The affinity-purified rabbit antibody pAb R34 efficiently recognized the NC1 domain of Col XXII in immunoblots. The calculated molecular mass of this domain is 53 kDa; however, the recombinant protein migrates with an electrophoretic mobility corresponding to an apparent mass of 70 kDa (Fig. 4A, lane 4). Removal of the His tag had no effect on the ability of the antibodies to recognize Col XXII (Fig. 4A, lane 4). Importantly, despite structural similarities between this domain in collagens XXI and XXII, no cross-reactivity with collagen XXI was seen (Fig. 4A, lane 3).

Recombinant Full-length Col XXII

Recombinant human Col XXII was expressed in 293-EBNA cells and affinity-purified using the His tag (Fig. 4B, lanes 1 and 3). Under non-reducing conditions, monomeric, dimeric, and trimeric molecules were detected, indicating partial inter-chain disulfide bonding (Fig. 4B, lane 2). The monomeric polypeptide chains of full-length Col XXII had an electrophoretic mobility consistent with a mass of about 200 kDa (Fig. 4B); in some cases a smaller product of about 70 kDa, corresponding to the NC1 domain, was observed. The full-length molecule was collagenase-sensitive, and digestion yielded the collagenase-resistant NC1 domain (Fig. 4B, lane 4).

Visualization of recombinant Col XXII by transmission electron microscopy after rotary shadowing indicated that the recombinant molecule formed structures resembling other FACIT collagens (Fig. 5, A–C). Full-length Col XXII has a thin rod-like structure that corresponds to the collagenous domain, with a contour length of about 301 ± 15 nm. The rod contains several kinks representing the interruptions of the collagenous domain. At the N-terminal region the globules correspond to the VWA and TSPN domains (Fig. 5, A and D). From these images the flexible structure of Col XXII becomes evident.

Col XXII in Tissue Extracts

Col XXII was easily extracted with a high salt buffer from muscle and, less efficiently, from skin (Fig. 6A, lanes 1 and 2). Very little additional protein was released by subsequent urea extraction of either tissue (Fig. 6A, lanes 4 and 5). In addition to the 200-kDa Col XXII band, smaller bands of 110–120 and 70 kDa were visible in an immunoblot. Indeed, Col XXII proved to be very sensitive to proteolysis. Despite rapid protein isolation methods and use of potent proteinase inhibitor cocktails during extraction, a single band was not seen in tissue extracts. Similarly, if the recombinant full-length protein was stored for some time at 4 °C, a comparable fragmentation pattern was obtained (not shown). Collagenase digestion of tissue extracts abolished the 200-kDa and the 160-kDa bands, and immunoblotting with pAb34 demonstrated that the 70-kDa band corresponded to the NC1 domain (Fig. 6A, lane 3). These observations confirmed that in tissue extracts, partial proteolytic degradation of the collagenous stretch of the molecule had taken place.

Tissue Distribution of Col XXII mRNA

Northern blot analysis of human tissues showed Col XXII mRNA to be highly expressed in skeletal muscle and heart (Fig. 6B). A single band of about 6.4 kb was detected, indicating no alternative splice variants. The size of the *COL22A1* mRNA corresponds well to the length of the cloned cDNA. Semiquantitative RT-PCR on mouse tissues revealed additional signals in cartilage, skin, and keratinocytes and in the eye (not shown). In contrast, almost no signal was obtained from neuronal tissues or other organs such as bone, liver, kidney, or lung.

Col XXII mRNA Is Expressed in Muscle Cells, Not in Fibroblasts

By *in situ* hybridization, *COL22A1* mRNA was detected exclusively in muscle cells at the muscle attachment sites to tendon elements and ribs (Fig. 7, A and B). Dense alkaline phosphatase reaction products were observed only in the muscle cells closest to the rib or aponeurosis. No signal was detected in muscle fibers at any other location in the muscle. For comparison, a collagen I probe was used to identify fibroblasts at the tendinous sheet zone. As shown in Fig. 7, C and D, Col XXII mRNA is absent from the region positive for collagen I message. Controls with antisense probes remained negative (data not shown).

Tissue Distribution of Col XXII Protein

Tissue distribution of Col XXII was determined by indirect immunofluorescence in postnatal mouse tissues. A striking observation was that Col XXII is expressed only at sites of tissue junctions in muscle, cartilage, heart, and skin (Fig. 8). In skeletal muscle Col XXII was found juxtaposed to tendon insertion sites, *i.e.* in tendinous sheets, so called aponeurosis, which separate muscles (Fig. 8A), the myotendinous junction (Fig. 8B), and the muscle attachment site to the rib (Fig. 8C). In the heart Col XXII was present at the insertion points of the *chordae tendineae* (Fig. 8D) into the atrial muscle. Immunoelectron microscopy indicated close proximity of Col XXII to the basement membrane outlining the finger-like myotendinous junction (Fig. 9, A and B).

In articular cartilage a small narrow band of Col XXII was detected at the cartilage surface facing the synovial fluid (Fig. 8E). Similarly, ultrathin sections surface-labeled for Col XXII exhibited a narrow positive zone of gold particles close to the articular surface (Fig. 9D). Because Col XXII belongs to the FACITs, its association with collagen-containing fibrils was examined by immunoelectron microscopy of fragments of native cartilage fibrils isolated from tissue homogenates (27). Gold particles representing Col XXII were not localized to the large collagen-containing fibrils but were found in the filamentous extrafibrillar material surrounding the fibrils, such as fibrillin (Fig. 9, E and F).

In the skin, Col XXII was found in a striking layer surrounding the lower third of anagen hair follicles (Fig. 8G). In cross-sections, for example of the mouse tail, the staining was reminiscent of thin ring-like structures embedded in the dermal matrix (Fig. 8H). No signal was detected in the interfollicular epidermis or in the uppermost region of the hair follicle. However, a positive staining was consistently observed in the sebaceous glands associated with the hair follicles. At the lower region of the hair follicle, the staining coincided with the presence of myofibroblast (Fig. 8H). Immunoelectron microscopy showed the gold particles to be in and around the lamina densa of the follicular basement membrane, which was strongly invaginated with cellular protrusions and closely interconnected with thin cross-banded fibrils in parallel orientation with the follicle wall (Fig. 9C).

Cell Attachment to Col XXII

Because collagens are well known ligands for cell surface receptors, the cell binding properties of Col XXII were assessed. Integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are the classic “collagen receptors”; therefore, two cell types expressing these integrins, HACAT keratinocytes and WI-26 lung fibroblasts, were tested for binding to Col XXII. Both cells bound to Col XXII in a concentration-dependent manner, and saturation was reached already at the coating concentration of 10 $\mu\text{g/ml}$ (Fig. 10). Binding efficiency of the two cell types was clearly different; WI26, which expresses both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins, bound more efficiently than HACAT cells, which express only $\alpha_2\beta_1$ integrins.

DISCUSSION

Most members of the collagen protein family are unique to vertebrates, and their genes are closely linked to the evolution of bones, tendons, vasculature, and organs. Because not all collagens are deposited in tissues in great abundance, some family members have remained undiscovered. Recently, with the support of the human genome sequencing project and the expressed single sequence tag data base, several new collagens have been traced (28,29). Here we identified a short cDNA clone of Col XXII, a novel collagen, from the dbEST database. After definition of the full-length cDNA by RACE amplification, interpretation of the translated cDNA revealed the existence of a novel FACIT collagen. In addition to human, putative EST clones for mouse, zebra fish, chick, and rat Col XXII were found in different databases.

The novel gene, *COL22A1*, was also predicted with the help of a gene prediction program (6), which also identified other additional, structurally closely related, VWA-containing collagen genes in the human genome. The *COL22A1* gene was found in almost all ongoing vertebrate genomic sequencing projects. However, surprisingly, the *COL21A1* gene, which is its closest related gene, could not be discerned in mouse, zebra fish, or rat genomes.

The closest structural relative of human Col XXII is human collagen XXI, Col XXI. Both proteins share the typical structure of FACIT collagens, an N-terminal globular domain followed by a short C-terminal collagenous stretch. The domain organization of the two molecules is very similar except that Col XXI lacks a large segment of the collagenous

domain (Fig. 2; Ref. 9). The C-terminal collagenous domains of FACITs are believed to interact directly or indirectly with collagen-containing fibrils in tissues (30). However, our electron microscopic studies indicate that Col XXII is not directly associated with collagen-containing fibrils. Rather, based on the observations on native fibril extracts from articular cartilage, it seems to interact with components of microfibrils, such as fibrillins or collagens VI. A similar situation prevails for collagen XVI, another FACIT protein, which was recently shown to be associated with fibrillin (22).

The structure of the recombinantly expressed Col XXII, as seen in the electron microscope after rotary shadowing (Fig. 5), fits well with the calculated length of the molecule. With the total number of 1045 amino acid residues in the collagenous domains and the 0.289-nm rise per residue in a triple-helical conformation, the predicted length of the triple-helical rod is 302 nm. This is in excellent agreement with the observed 304 ± 15 -nm tail. The sequence of Col XXII contains four major and several minor interruptions of the collagenous domain, a fact that explains the flexibility of this molecule.

The tissue form of Col XXII was identified by immunoblotting of mouse tissues with an antibody against the recombinant NC-1 domain of human Col XXII (antibody pAb R34). After Northern blot and RT-PCR analysis of different tissues and cells revealed strong expression of *COL22A1* in muscle and heart (and weaker in cartilage, skin, and eye), muscle and skin extracts were analyzed first. In immunoblots of both tissues, pAb R34 recognized a 200-kDa band that corresponds to the authentic full-length Col XXII molecule. In muscle extracts, additional bands were seen. These most likely represent proteolytic cleavage products generated during extraction and protein chemical analysis. The non-collagenous NC-1 domain was stable, and the cleavage occurred within the collagenous regions. This is a rather common phenomenon for large secreted proteins, such as collagens and laminins (31,32). However, the fact that Col XXII and Col XXI are highly homologous led us to exclude cross-reactivity carefully. First, immunoblots overloaded with the recombinant NC-1 domain of human Col XXI did not exhibit a signal with the antibody pAb R34. Second, an intensive search for the *COL21A1* gene in the mouse genome databases did not yield evidence for the existence of murine collagen XXI. Thus, the tissue form of monomeric collagen XXII corresponds to that of the recombinant molecule visualized by rotary shadowing electron microscopy. However, the functional suprastructure form of Col XXII *in situ* remains unknown at present.

The tissue distribution pattern of Col XXII is unique. By immunofluorescence staining, the protein was localized to specific tissue junctions. In the skin, a sheet-like structure surrounding the hair follicle contains Col XXII. This sheet is Col XXII-positive in areas of the lower follicle, where myofibroblasts line the outer surface of the hair follicle along the junction between the follicle and the dermis (33). In the joints, the surface of the articular cartilage, *i.e.* the junction between cartilage and synovial fluid, was labeled with Col XXII antibodies within a very thin, confined band. At the ultrastructural level, this region contains highly organized thin collagen-containing fibrils and other suprastructural elements. Our experiments with extraction of native fibrils from articular cartilage demonstrated that Col XXII is not associated with the classic cross-banded “collagen fibrils” but, rather, with microfibrils. In arthritic human joints, Col XXII is still detectable with immunofluorescence staining, but the staining pattern is broadened and fuzzier.² Future studies will show how Col XXII is integrated into functional suprastructures of the articular surface and around the hair follicle and which the cellular origin of this protein is.

²M. Chiquet, S. Wagner, and M. Koch, unpublished observation.

In muscle, heart, and ciliary body, Col XXII was localized at the insertion sites of tendons or zonula fibrils into the muscle. The scaffolds, which are necessary for the integrity of tissues and the transmission of locomotive forces, are formed by polymeric protein structures, in which collagens play an integral role (34). Of all the sites in which Col XXII was found, the MTJ is best studied.

MTJ are crucial elements in the transmission of mechanical force from the muscle via tendons to the skeletal elements. Several molecules have been identified at this site. Tenascin, an oligomeric extracellular matrix protein, was one of the first markers identified (11), but its function at this site still remains unknown. Other components, such as $\alpha_7\beta_1$ integrin, laminin 2, and the dystrophin glycoprotein complex are pivotal, since mutations in their genes lead to pathologic changes of the MTJ both in human diseases and in mouse models (13,35,36). The present study demonstrates that muscle cells close to the MTJ synthesize collagen XXII and deposit it into the interdigitated basement membrane zone structures of this junction (37). Because molecules containing VWA domains have been implicated in protein-protein interactions (38), it is feasible that Col XXII binds to components of the basement membrane, such as collagens, proteoglycans, laminins, or the integrin collagen receptors.

The morphogenesis of MTJ during the development of tendons and muscles is an intriguing process that is not well known. Because the formation of MTJ requires both tendon and muscle cells, it is evident that also both soluble and stationary signaling molecules must be involved. Fibroblast growth factor (FGF) 4 and FGF-8 have been detected at this site by *in situ* hybridization (39,40), and epidermal growth factor-like molecules or wnt signaling molecules are further candidates for regulation of MTJ formation and, potentially, also of Col XXII expression. Col XXII, through its limited expression pattern is a suitable marker to study the signals involved in the cross-talk between the tendon and the muscle.

Taken together, collagen XXII is a novel molecule present in specialized basement membrane zones and certain other tissue junctions in different organs. Through its restricted expression pattern, it will be an excellent marker to study tissue junction formation during development and regeneration and to discern pathologic processes involving two or more tissues.

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1	MAGLRGNAVA	GLLWMLLLWS	GGGGCQAQRA	GCKSVHYDLV	FLDTSSSVG	50
51	KEDFEKVRQW	VANLVDTFEV	GPDRTVRGVV	RYSDRPTTAF	ELGLFGSQEE	100
101	VKAAARRLAY	HGGNTNTGDA	LRYITARSFS	PHAGGRPRDR	AYKQVAILLT	150
151	DGRSQDLVLD	AAAAAHRAGI	<u>RIFAVGVGEA</u>	<u>LKEELEETAS</u>	<u>EPKSAHFVHV</u>	200
201	SDFNAIDKIR	GKLRRLCEN	VLCPSVRVEG	DRFKHTNGGT	KEITGFDLMD	250
251	LFSVKEILGK	RENGAQSSYV	RMGSFPVVQS	TEDVFPQGLP	DEYAFVTTFR	300
301	FRKTSRKEDW	YIWQVIDQYG	IPQVSIRLDG	ENKAVEYNAV	GAMKDAVRVV	350
351	FRGSRVNDLF	DRDWHKMALS	IQACNVSLHI	DCALVQTLPI	EERENIDIOG	400
401	KTVIGKRLYD	SVPIDFDLQR	IVIYCDSRHA	ELETCCDIPS	<u>GPCOVTVVTE</u>	450
451	PPPPPPPPORP	PTPGSEQIGF	LKTINCS	CPA	GEKGEMGVAG	500
501	IGAIGPVGAP	GPKGEKGDVG	IGPFQGEKG	EKGLGLPGP	PGRDGSKGM	550
551	GEPGELGEPG	LPGEVGMGRP	QPPGLPGPP	GRVGAPLQG	ERGEKGTGE	600
601	KGERGLDGFP	GKPGDTGQQG	RPGPSGVAGP	QGEKGDVGA	GPPGVGSAV	650
651	QEEGLKGEQG	APGPRGHQGA	PGPPGARGPI	GPEGRDPPP	LQGLRGKKGD	700
701	MGPPGIPGLL	GLQPPGPPG	VPGPPGPGGS	PGLPGEIGFP	GKPGPPGPTG	750
751	PPGKDGPNP	PGPPGKGE	GERGEDGLP	KPGLRGEIGE	QGLAGRPGEK	800
801	GEAGLPGAPG	FPGVRGEKGD	QGEKGLGLP	GLKGRGEKG	EAGPAGPPGL	850
851	PGTISLFTPH	FRMPGEQPK	GEKGDPLPG	EPGLQGRPGE	LGPGQPTGPP	900
901	GAKGQEGAHG	APGAAGNPGA	PGHVAPGPS	GPPGSVAGP	LRGTPGKDGE	950
951	RGEKGAAGEE	GSPGPVGP	DPGAPGLPG	PKGKDGEPG	LRGSPGLPGP	1000
1001	LGTKAACGKV	RGSENALGG	QCVKDRGAP	GIPGPSGRG	DHGIGVAGPP	1050
1051	GPSGPPGDKG	SPGSRGLPGF	PGPQGPAGRD	GAPGNPGERG	PPGKPLSL	1100
1101	LSPGDINLLA	KDVCNDCP	PPGLPGLPGF	KGDKGVPGK	GREGTEGKKG	1150
1151	EAGPPGLPGP	PGIAGPQGSQ	GERGADGEVG	QKGDQGHV	PGFMGPPGNP	1200
1201	GPPGADGIAG	AAGPPGIQGS	PGKEGPPGPQ	GPSGLPGIPG	EEGKEGRDGK	1250
1251	PGPPGEPGKA	GEPGLPPEG	ARGPPGFKGH	TGDSGAPGR	GESGAMGLPG	1300
1301	QEGLPGKDG	TGPTGPQGPQ	GPRGPPGKNG	SPGSPGEPG	SGTPGQKGSK	1350
1351	GENGSPGLPG	FLGPRGPPGE	PGEKGVPGKE	GVPKPGEPG	FKGERGDPGI	1400
1401	KGDKGPPGK	GQPGDPGIPG	HKHTGLMGP	QGLPGENGPV	GPPGPPGQPG	1450
1451	FPGLRGES	PSMETLRLIQE	ELGKQLETRL	AYLLAQMPPA	YMKSSQGRPG	1500
1501	PPGPPGKDG	PGRAGPMGEP	GRPGQGLEG	PSGPIGKGE	RGAKGDPGAP	1550
1551	GVGLRGEMGP	PGIPGQPEP	GYAKDGLPGI	PGPOGETGPA	GHPGLPGPPG	1600
1601	PPGQCDPSQC	AYFASLAARP	GNVKGP			1626

FIG. 1. The complete amino acid sequence of human Col XXII as predicted from the corresponding cDNA sequence

A 28-amino acid signal peptide sequence precedes the N terminus (*underlined*). The different domains are outlined; a VWA domain is followed by the TSPN domain. In the TSPN two potential *N*-glycosylation sites are *circled*. Between the globular domains and the collagenous domain a linker region with a polyproline stretch is present. The collagenous region is interrupted by several larger amino acid stretches and six smaller imperfections (*circled*) of the Gly-X-Y repeats.

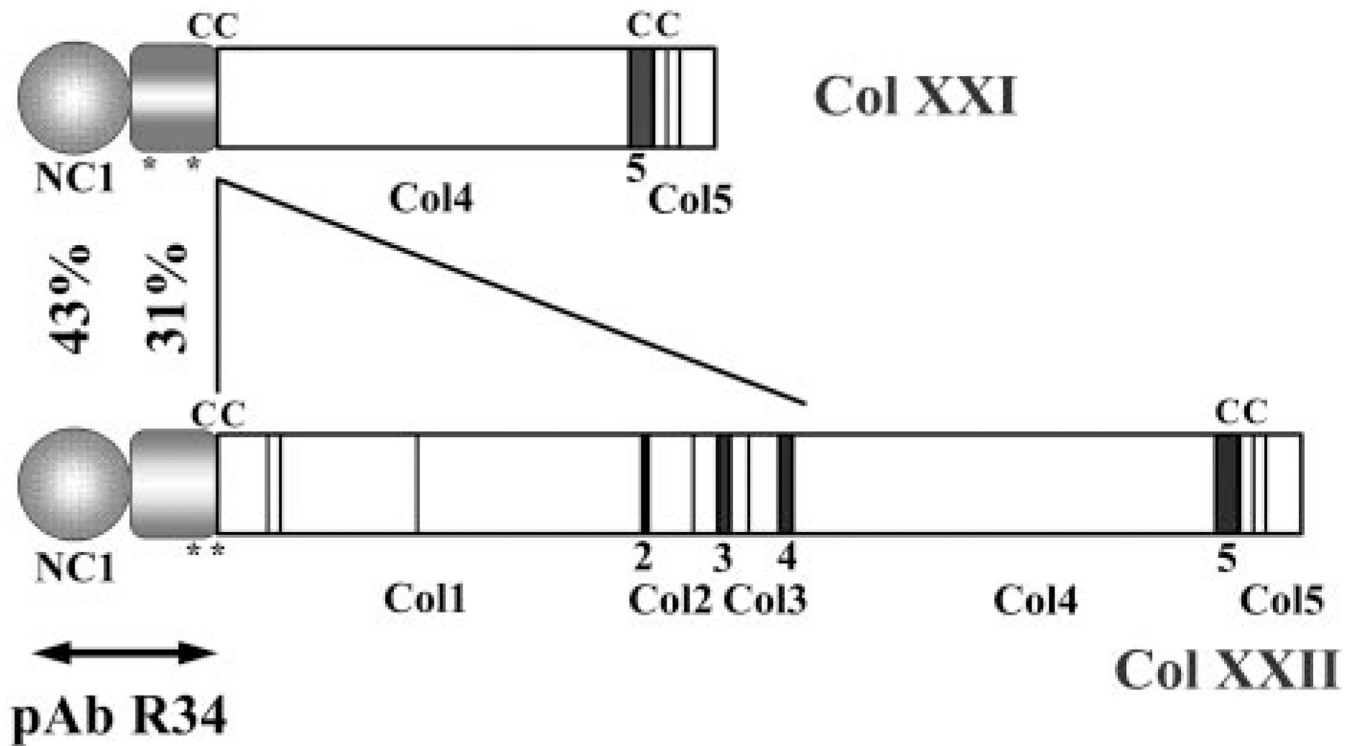


FIG. 2. Schematic presentation of the domain structure of collagen XXI and XXII

At the N terminus, the VWA domain (*circle, gray*) and a TSPN domain (*square, gray*) are the building blocks of the NC1 domain. The Gly-X-Y triplets Col1-Col5 (*open squares*) are interrupted several times (*black squares*) by small NC2-NC5 linker domains. Smaller imperfections of the collagen triplets are indicated by *thin lines*. The putative N-linked glycosylation sites are marked by *asterisks*. The cysteines (C), which are presumably involved in the inter-chain stabilization, are marked *above* the schematic drawings. The *double arrow* indicates the region that was used to generate the polyclonal antibody R34. Collagens XXI and XXII are quite similar in their domain structure. However, the region spanning the collagenous domains Col1–Col3 and the non-collagenous domains NC1–NC4 of collagen XXII is missing in collagen XXI (connecting lines between the two drawings).

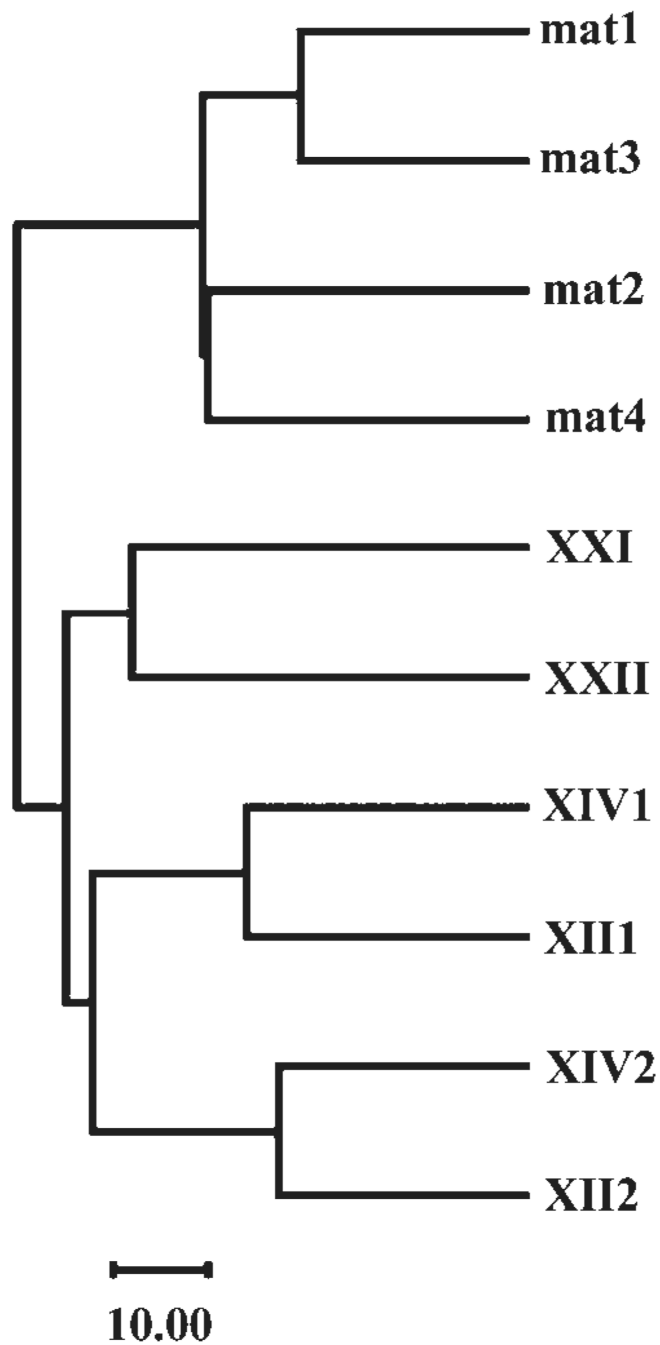


FIG. 3. Genetic relationship of the VWA domains of matrilins and collagens

The VWA domains of matrilins form one subgroup and can be further separated into two branches. FACIT collagens constitute the second group. Collagens XII and XIV are closely related and form one subgroup. Similarly, collagens XXI and XXII are closely related and form another subgroup. This tree was calculated with two different methods; both calculations yielded the same prediction.

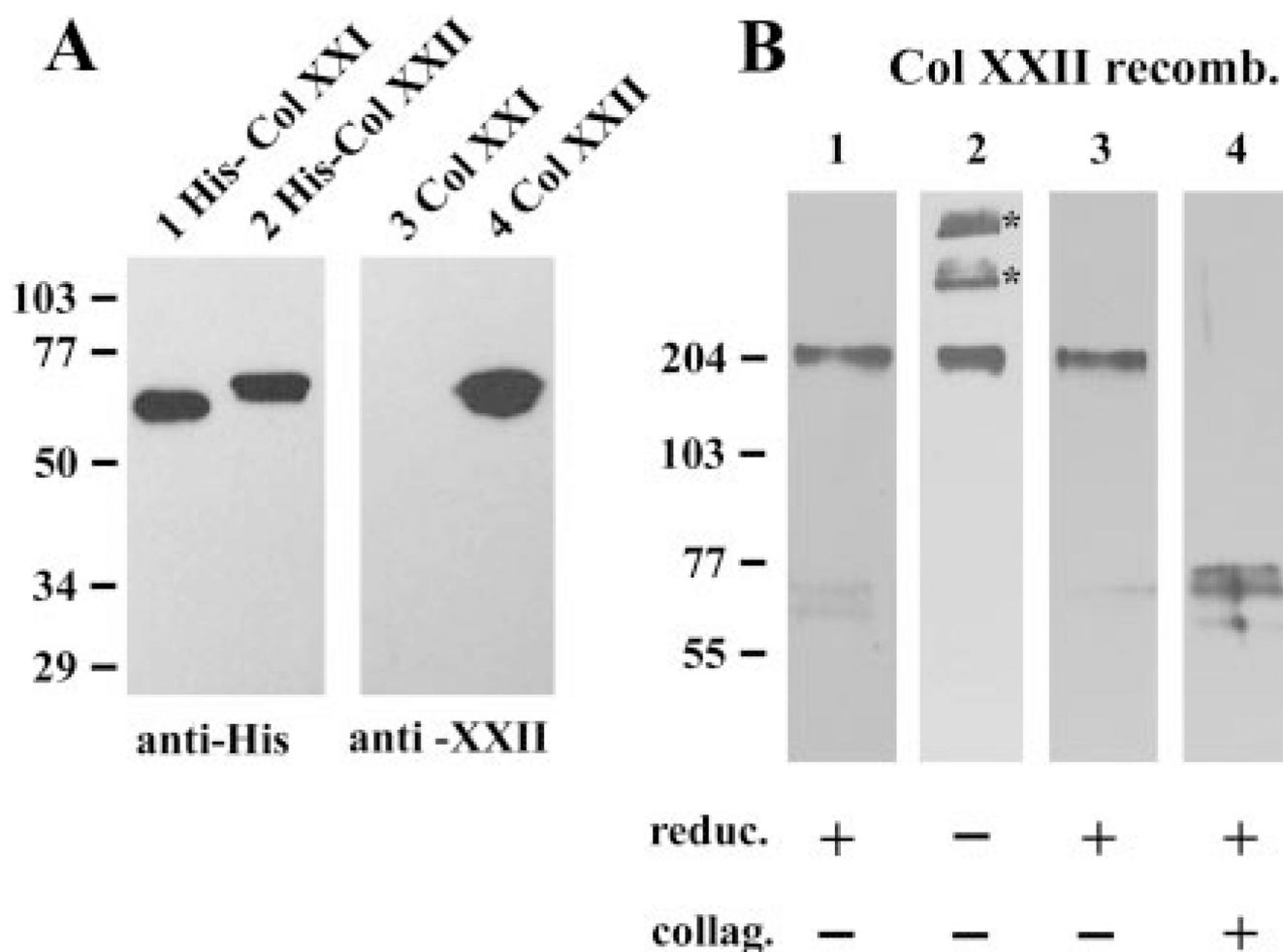


FIG. 4. Immunoblot analysis of recombinant Col XXII

A, aliquots of culture media from 293-EBNA cells expressing the NC1 globular domain of collagens XXI and XXII were electrophoretically separated, and the histidine-tagged proteins were detected using an anti-His antibody (1,2) and the affinity-purified antibody pAbR34 (3,4). pAbR34 recognizes the NC1 protein of Col XXII but not of Col XXI. B, full-length Col XXII was expressed in 293-EBNA cells, and the medium was immunoblotted (pAbR34). A single band of about 200 kDa was seen under reducing conditions (*lane 1*). Under non-reducing conditions, also dimeric and trimeric forms were present (*lane 2*, *asterisk*). Treatment of recombinant Col XXII with collagenase abolished the full-length form and yielded the collagenase-resistant NC1 domain (*lane 4*). *Lane 3*, control incubation without collagenase. Molecular mass standards in kDa are shown on the *left*.

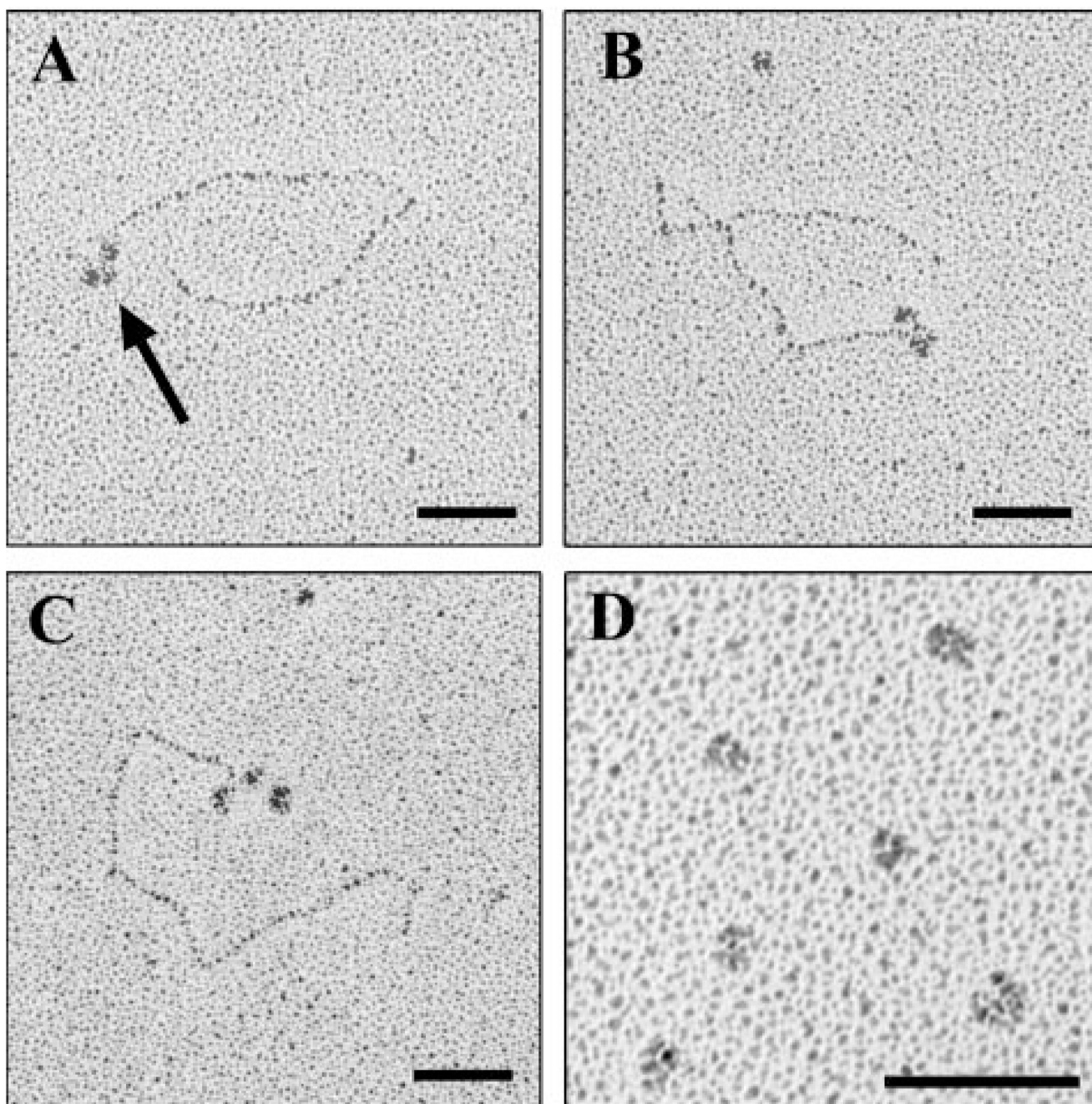


FIG. 5. Rotary shadowing images of purified recombinant Col XXII

A–C, three examples of a full-length molecule are shown. The thin rod-like structure represents the collagenous region, and the kinks represent the non-collagenous interruptions. The N terminus contains three globular domains (*arrow*). D, recombinant NC1 domain forms single globules. Bars, 50 nm.

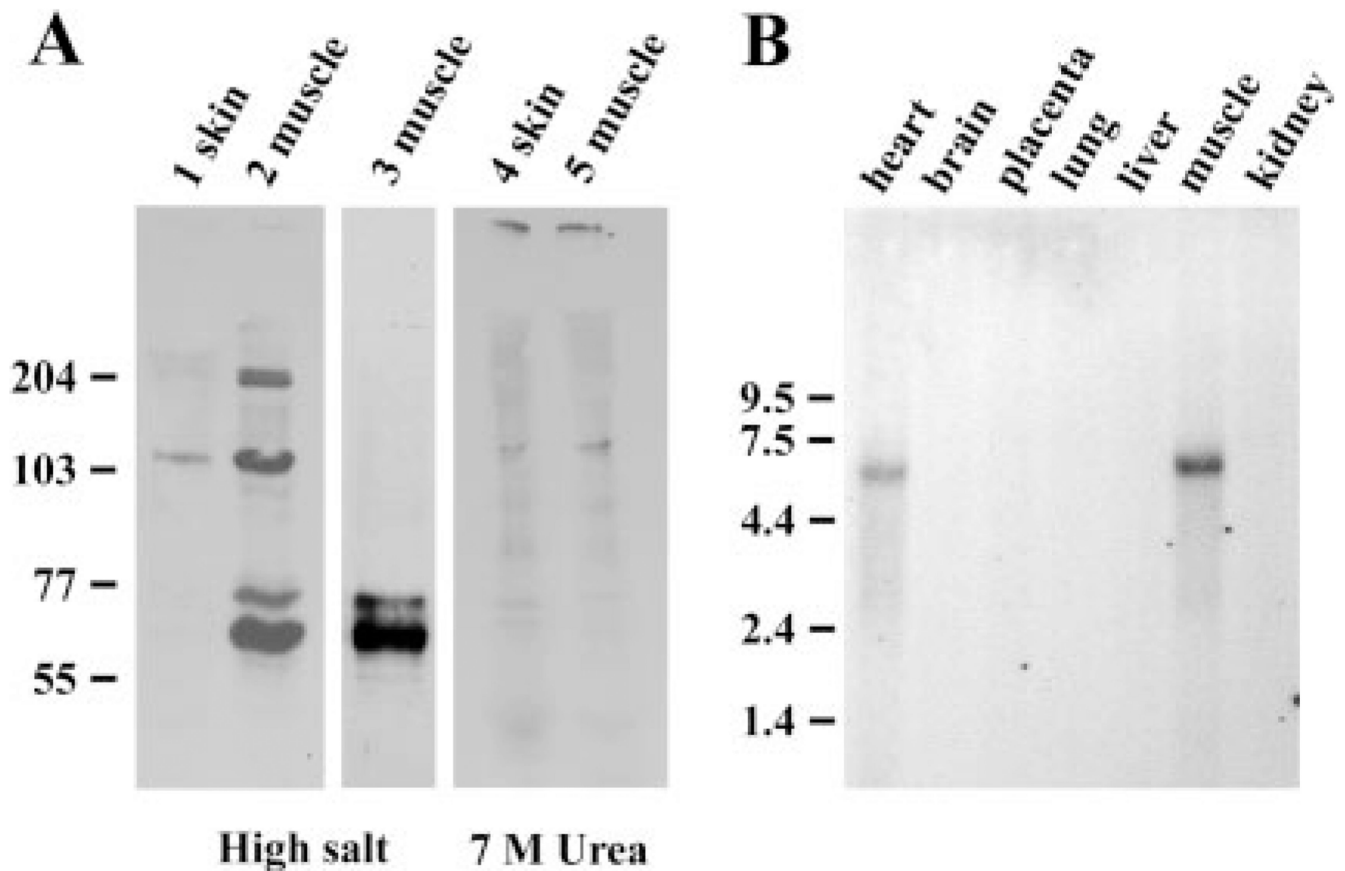


FIG. 6. Expression of Col XXII in tissues

A, immunoblot with pAb R34 of proteins extracted from mouse skin and muscle using high salt (*lanes 1–3*) and urea (*lanes 4 and 5*). The skin extracts contained relatively small amounts of Col XXII (*lane 1*). In contrast, in high salt extract of muscle, four distinct bands were detectable (*lane 3*). Treatment of the extract shown in *lane 2* with collagenase abolished the bands with higher molecular weight, indicating that they had a collagenous structure. Only the NC1 domain resisted the protease digestion (*lane 3*). As seen here, the NC1 domain occasionally migrated as a double band, probably due to differential glycosylation of the molecules (*lane 2 and 3*). Additional extraction of the tissues with 7 M urea did not result in recovery of more Col XXII from the skin (*lane 4*) or muscle (*lane 5*). *B*, Northern blot of Col XXII in different tissues. Only a single mRNA of 6.4 kb was detected on a human tissue blot. A strong signal was found in the heart and skeletal muscle but not in other organs such as brain, placenta, lung, liver, or kidney. In *A* molecular weight standards on the *left* are shown in kDa; in *B* the RNA size standards are in kb.

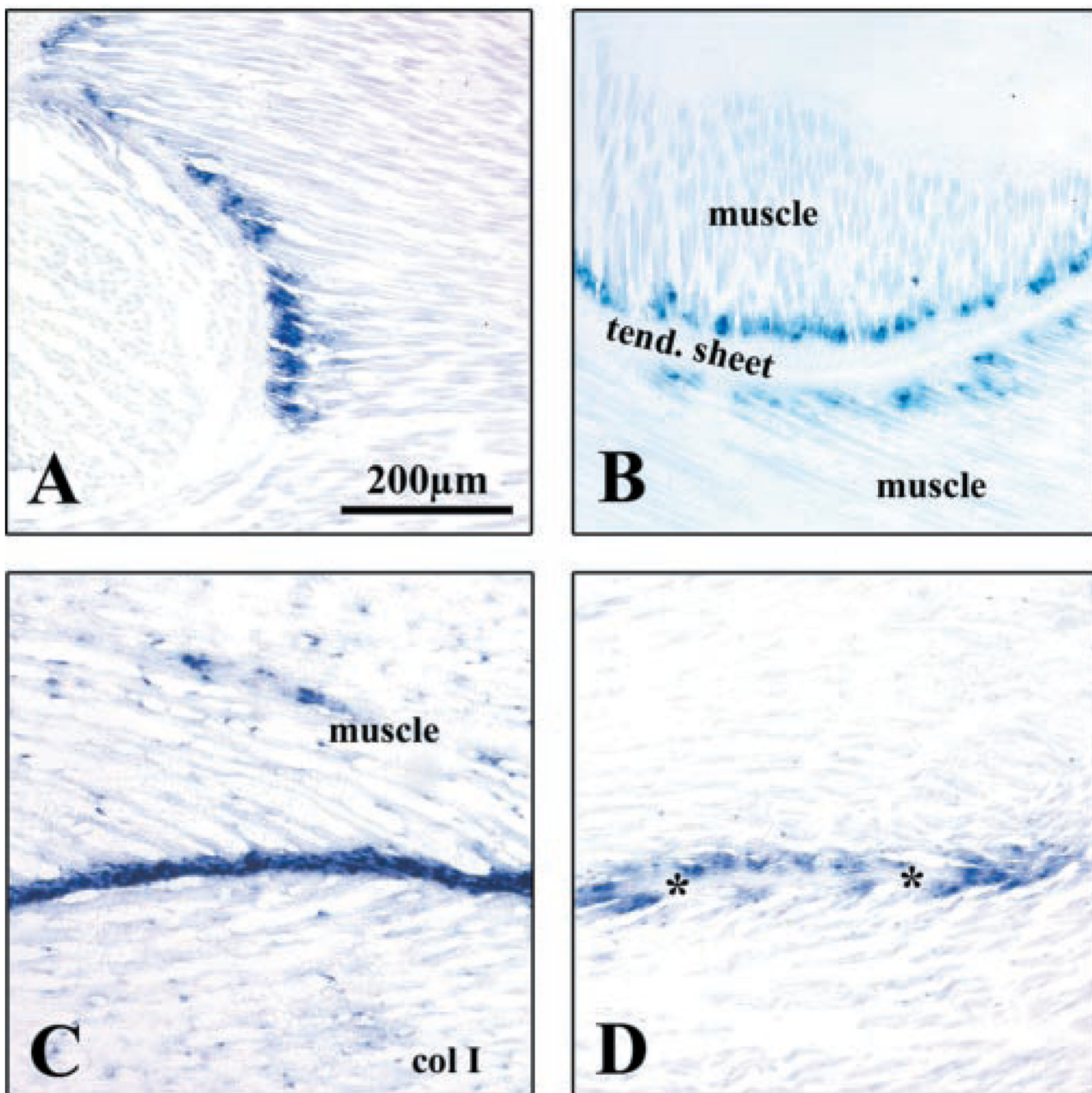


FIG. 7. *In situ* hybridization

A, Col XXII mRNA was detected in the muscle close to the rib. The staining is restricted to a narrow band of cells, and no labeling was detected within the bone or in other locations within the muscle. B, muscle cells at the attachment site to aponeurosis, which separates muscle compartments, express Col XXII mRNA. C, for comparison, collagen I mRNA is mainly synthesized by fibroblasts of the aponeurosis between the muscles. D, hybridization of a parallel section of C for Col XXII mRNA shows that Col XXII is not synthesized by the tendon fibroblasts (*asterisks*) but can be detected only in the muscle cells.

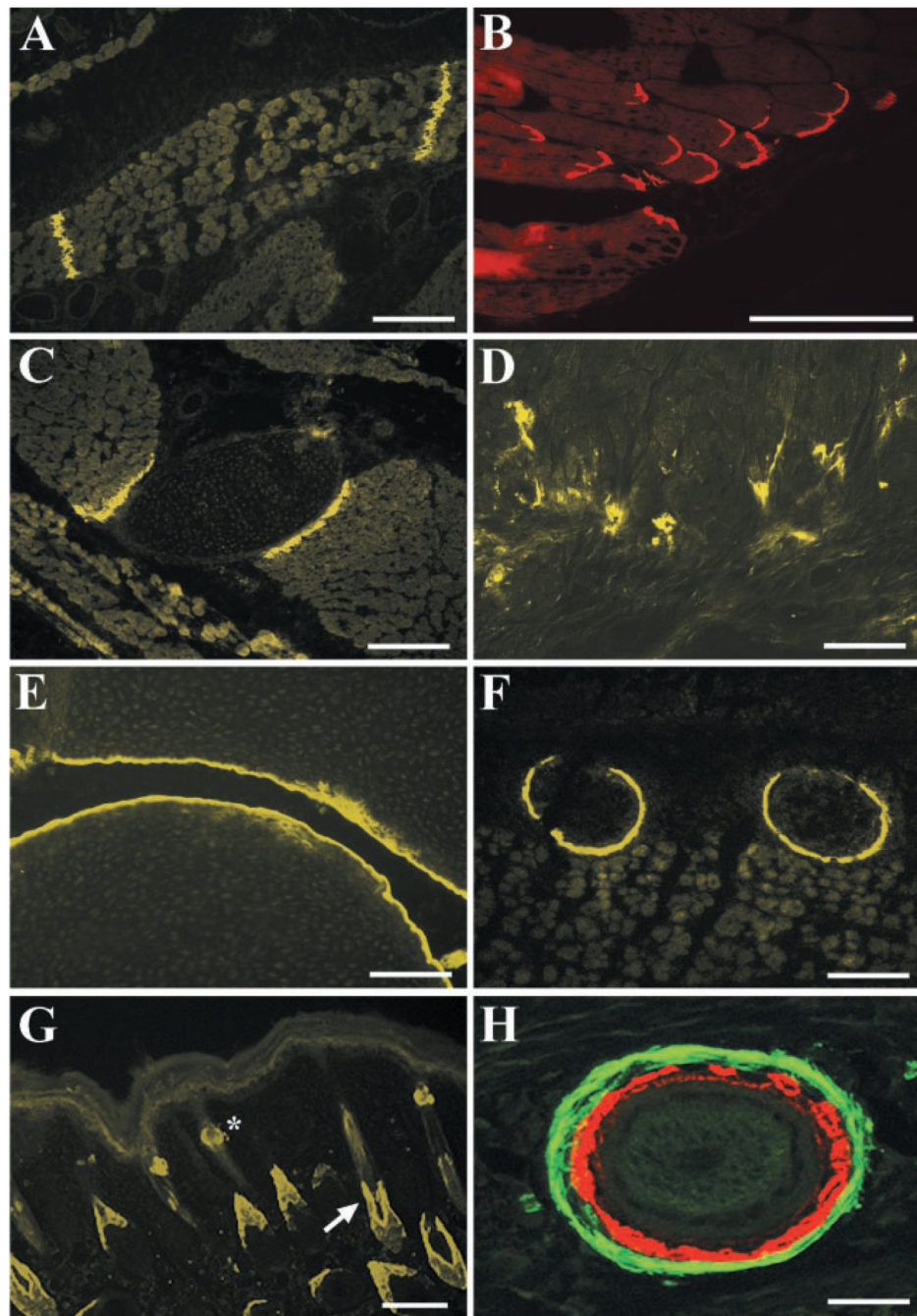


FIG. 8. Col XXII is deposited at tissue junctions

A, in the muscle of newborn mice, Col XXII staining is seen in tendinous sheets separating muscle compartments. *B*, at the insertion sites of muscle into tendon, Col XXII immunoreactivity is limited to the MTJ. *C*, at the outer body wall Col XXII is localized at their attachment sites of intercostal muscles to the ribs. *D*, in the heart, Col XXII is present at the chordae tendineae insertion sites into the muscle of the right ventricle. *E*, on both sides of the joint a narrow band close to the articular surface is positive for Col XXII. *F*, the cartilaginous part of ribs, close to the sternum, is positive for Col XXII staining (cross-section). *G*, at the lower portion of hair follicles (*arrow*), a limited, sheath-like Col XXII staining is observed. The staining of sebaceous gland is also evident (*asterisk*). *H*, cross-

section through the lower part of the dermis reveals ring-like Col XXII staining (*red*) around a hair follicle. The myofibroblasts surrounding the hair follicle were stained with antibodies to α -actinin (*yellow*). *Bars: A–G, 100 μ m; H, 20 μ m.*

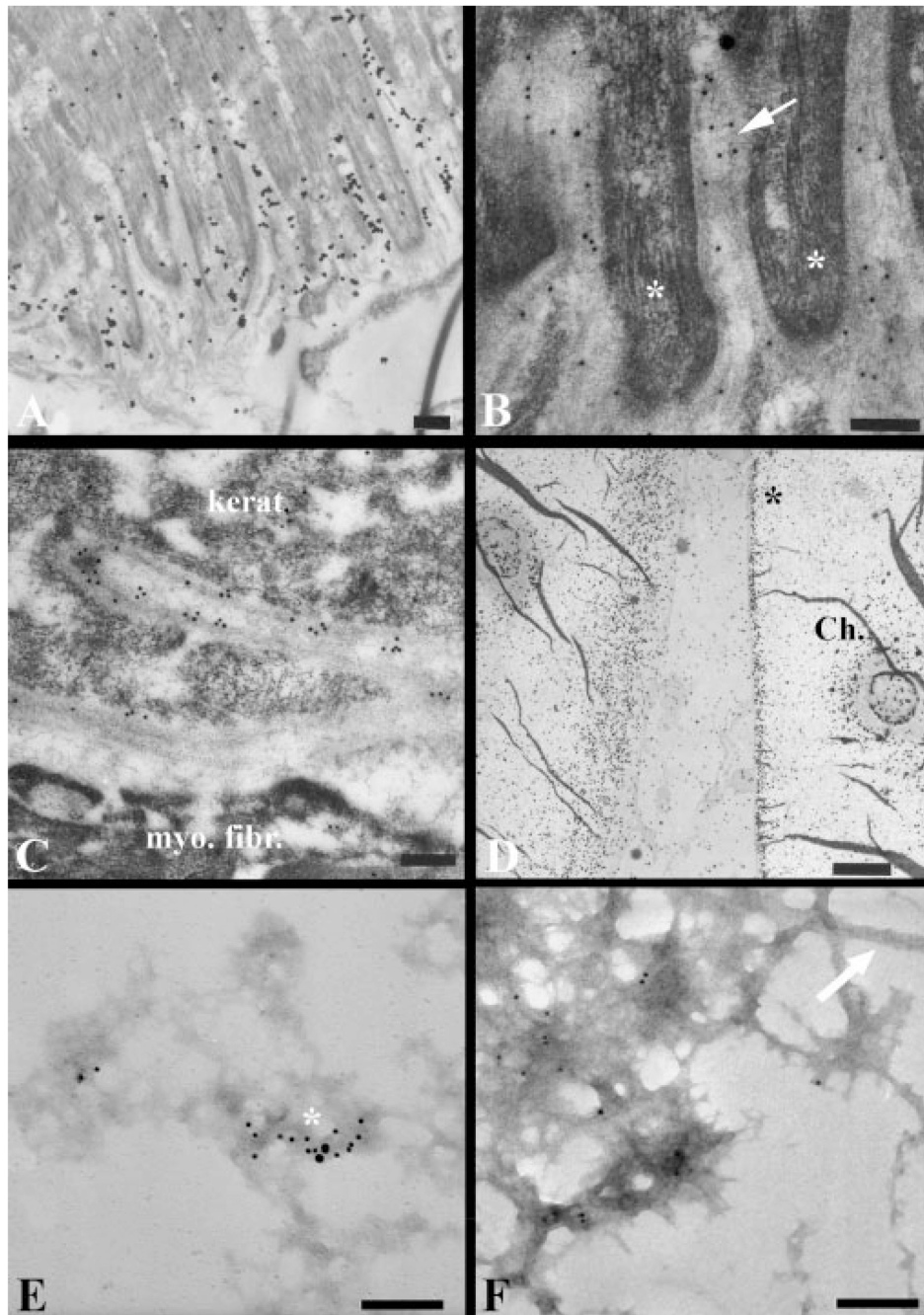


FIG. 9. Ultrastructural localization of Col XXII *in situ*

Ultrathin sections were incubated with pAb R34, and the bound antibodies were visualized with a colloidal gold-labeled second antibody. *A* and *B*, the basement membrane zone of the myotendinous junction contains Col XXII. In *A*, the finger-like insertions of the myocytes into the tendon (*asterisk*) are surrounded by a basement membrane, which is decorated with gold particles (*arrow*). In *B*, a larger magnification shows the gold labeling of the lamina densa of the basement membrane. *C*, around the hair follicles, Col XXII is localized along the partially interdigitated basement membrane along the follicle wall, between the myofibroblasts and the keratinocytes (*kerat.*, keratinocytes; *myo. fibr.*, myofibroblast). Most gold particles are associated with the lamina densa. *D*, in the joint, Col XXII is deposited

close to the articular surface (*Ch.*, chondrocyte). *E* and *F*, native fibrils were extracted from articular cartilage. Immunogold particles representing Col XXII were not associated directly with cross-banded collagen-containing fibrils (*F*, *white arrow*) but were localized to the amorphous fibrillin-containing microfibrillar material (*E*: *white asterisk*, large particles: Col XXII, *small particles*, fibrillin). *Bars*: A–D, 200 nm; *E* and *F*, 250 nm.

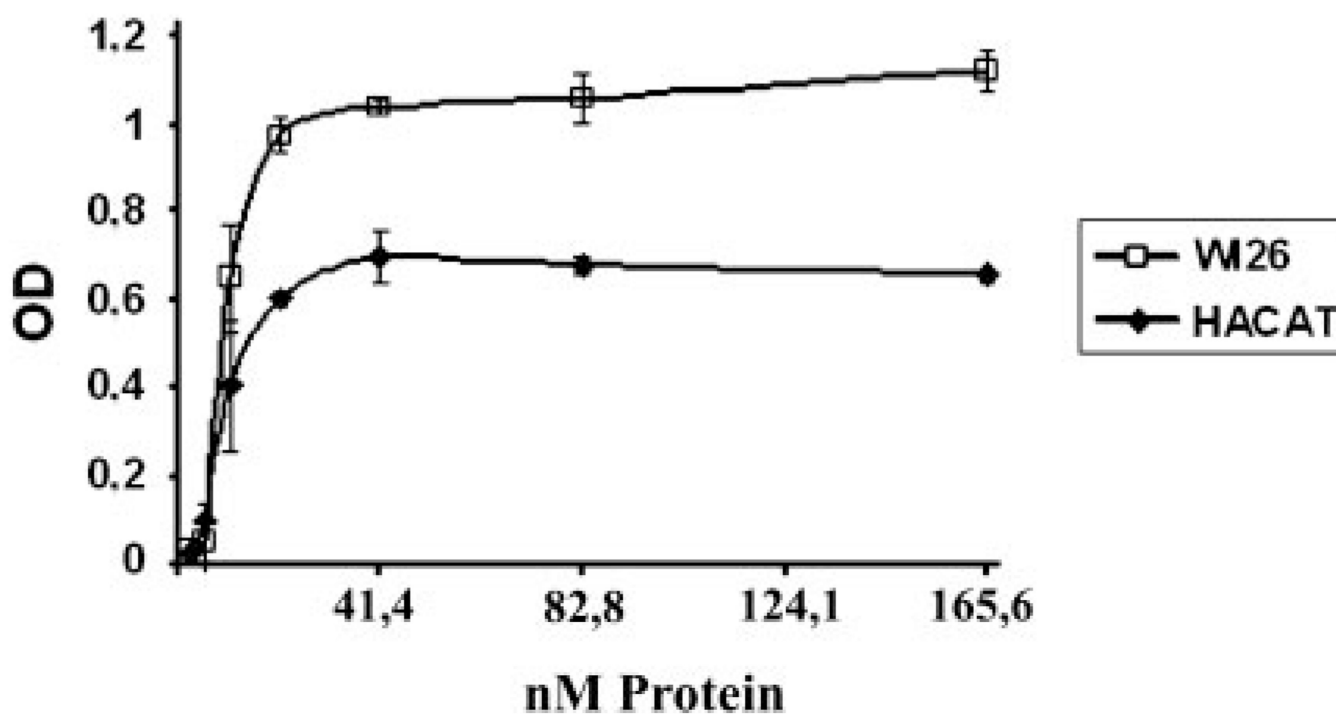


FIG. 10. Cell binding of Col XXII

The fibroblast cell line Wi-26 and the keratinocyte cell line HACAT were tested for their binding to recombinant Col XXII. The wells were coated with different amounts of full-length Col XXII, and the cells were allowed to attach for 30 min. After washing, the adherent cells were quantitated as described under "Experimental Procedures." At a coating concentration of 40 nM Col XXII, cell binding reached saturation levels. *OD*, optical density.