Chondroitin Sulfate Perlecan Enhances Collagen Fibre Formation

IMPLICATIONS FOR PERLECAN CHONDRODYSPLASIAS

Alexander J. Kvist1, Anna E. Johnson1,2, Matthias Mörgelin5, Erika Gustafsson5, Eva Bengtsson43, Karin Lindblom†, Attila Aszódi6, Reinhard Fässler6, Takako Sasaki2, Rupert Timp5, and Anders Aspberg††

From the Departments of 1Experimental Medical Sciences and 2Clinical Science Lund, Lund University, SE-22184 Lund, Sweden and 6Max Planck Institute for Biochemistry, D-82152 Martinsried, Germany

Inactivation of the perlecan gene leads to perinatal lethal chondrodysplasia. The similarity to the phenotypes of the Col2A1 knock-out and the disproportionate micromelia mutation suggests perlecan involvement in cartilage collagen matrix assembly. We now present a mechanism for the defect in collagen type II fibril assembly by perlecan-null chondrocytes. Cartilage perlecan is a heparan sulfate or a mixed heparan sulfate/chondroitin sulfate proteoglycan. The latter form binds collagen and accelerates fibril formation in vitro, with more defined fibril morphology and increased fibril diameters produced in the presence of perlecan. Interestingly, the enhancement of collagen fibril formation is independent on the core protein and is mimicked by chondroitin sulfate E but neither by chondroitin sulfate D nor dextran sulfate. Furthermore, perlecan chondroitin sulfate contains the 4,6-disulfated disaccharides typical for chondroitin sulfate E. Indeed, purified glycosaminoglycans from perlecan-enriched fractions of cartilage extracts contain elevated levels of 4,6-disulfated chondroitin sulfate disaccharides and enhance collagen fibril formation. The effect on collagen assembly is proportional to the content of the 4,6-disulfated disaccharide in the different cartilage extracts, with growth plate cartilage glycosaminoglycan being the most efficient enhancer. These findings demonstrate a role for perlecan chondroitin sulfate side chains in cartilage extracellular matrix assembly and provide an explanation for the perlecan-null chondrodysplasia.

The basement membrane heparan sulfate (HS)5 proteoglycan perlecan was first isolated from the murine Engelbrecht-Holm-Swarm tumor. Perlecan binds to extracellular matrix components integral to basement membranes, such as collagen type IV, nidogen, laminin, and fibronectin (1), as well as to extracellular matrix components outside the basement membrane (e.g. PRELP (proline/arginine-rich end leucine-rich repeat protein) and collagen type I) (2). Perlecan supports cell attachment by both binding and clustering integrins (3). Binding to growth factors has been shown for both the HS side chains (fibroblast growth factor-2) (4) and the core protein (progranulin) (5). Based on these interactions, perlecan is believed to have a role in basement membrane integrity. In addition, perlecan is expressed outside basement membranes (e.g. in cartilage) (6–9). Although identified as an HS proteoglycan, perlecan can also be partially substituted with chondroitin sulfate (CS) (9, 10).

The generation of perlecan-null mice revealed several intriguing findings (11, 12). Although mice lacking perlecan did develop grave disorders caused by compromised basement membrane strength or integrity (e.g. rupture of the pericardial sac), the initial assembly of basement membranes seemed to be without complication. Later studies have shown additional defects in the neuromuscular junctions (13), hyperplasia of the conotruncal endocardial cushions, and transposition of the great arteries (14) in the perlecan-null mice. A most striking finding was the severe skeletal defects exhibited, apparently caused by the lack of perlecan in developing cartilage. Following the publication of these results, at least two human hereditary diseases with skeletal abnormalities have been ascribed to an underlying scarcity or complete lack of perlecan, underscoring the relevance of this finding in the mouse model (15, 16).

In skeletal development, the deposition of a cartilaginous template precedes the formation of bones through endochondral ossification. The integrity of this template is a prerequisite for proper development of the skeleton. In the perlecan-null mice, the growth plate of the bone anlagen is disorganized, and the whole templates appear bent and distorted. In addition, the cartilage shows fewer and less organized collagen type II fibrils as well as decreased levels of proteoglycan staining, indicating a failure to organize the extracellular matrix (12, 17).

It has been suggested that this is due to impaired growth factor presentation and signaling in the absence of perlecan attachment sites; VB, perlecan globular domain V (CS/HS-substituted); CS, chondroitin sulfate; GAG, glycosaminoglycan; EM, electron microscopy.
Perlecan and Collagen Fibril Formation

(11). An alternative hypothesis is that the balance between collagen type II production and breakdown had shifted toward degradation in the perlecan-null mice (12). Perlecan could, for example, protect the cartilage extracellular matrix from degradation by binding and regulating the activities of matrix metalloproteases (17). Interestingly, the similarity of the perlecan-null skeletal phenotype to those of the Col2A1 null mice and the disproportionate micromelia mice (that carry a mutation in the collagen type II C-propeptide) suggested that perlecan, rather than protecting against collagen degradation, could be involved in collagen type II matrix assembly.

Mature collagen fibers may contain several different types of bound accessory proteins. They take part in the organization of these fibers and regulate links to other molecules, thereby contributing to the architecture of the fibrillar collagen network. Interestingly, some accessory molecules function as modulators that regulate the early steps in the assembly of collagen monomers to fibrils. For example, it has been found that extracellular matrix proteins, such as the tyrosine-rich acidic matrix protein (18) and the cartilage oligomeric matrix protein,6 accelerate the formation of collagen fibrils from monomers in vitro. Other molecules have the opposite effect and slow down fibril formation in vitro (e.g. decorin (19) and fibromodulin (20)). Apart from these accessory proteins, it is clear from several studies that other extracellular macromolecules, notably glycosaminoglycans, also may participate in regulating collagen fibril assembly (19, 21–24). The results from these investigations were, however, in some aspects contradictory, leaving some questions regarding the exact effects of these molecules.

Gene targeting of decorin, fibromodulin, and other members of the small leucine-rich repeat proteoglycan family leads to abnormal collagen fibrils and disturbed mechanical properties of the tissues (25). Thus, a picture is emerging where macromolecules in the vicinity of the cell regulate the early stages of collagen fibril formation.

We now show that perlecan is involved in cartilage matrix assembly by enhancing collagen fibril formation. We found that chondrocytes from perlecan-null mice have an impaired ability to organize collagen to fibrils compared with wild-type chondrocytes. We also confirm that perlecan in growth plate cartilage is a CS/HS proteoglycan and that the CS chains on perlecan greatly enhance the rate of fibril formation of collagen types I and II in vitro, whereas neither the HS-substituted form nor the core protein had any effect. We also show that CS-E, unlike other glycosaminoglycans tested, mimics the effect of perlecan. Finally, we confirm that CS purified from perlecan-containing fractions of cartilage extracts is enriched in 4,6-sulfated disaccharides and enhances collagen fibril formation. This suggests a mechanism for some of the skeletal defects seen in perlecan-null mice and human chondrodysplasias resulting from mutation of the perlecan gene (HSPG2).

EXPERIMENTAL PROCEDURES

Isolation of Chondrocytes—Perlecan-deficient mice (12) were kept in accordance with local regulations. Null embryos and wild-type littermates (embryonic day 18.5) were dissected into ice-cold Dulbecco’s phosphate-buffered saline and decapitated. Thereafter, the rib cages were isolated, and individual ribs were dissected. After a 20–30-min initial collagenase digestion at 37 °C (Worthington grade 2; 2 mg/ml in Dulbecco’s modified Eagle’s medium with Glutamax-1, penicillin, and streptomycin), the ribs were dissected free from remaining muscle tissue, perichondrium, and bone, transferred to fresh collagenase, and digested for 4 h at 37 °C. The digested tissue was pipetted into a single cell suspension, sieved through a 70-μm mesh, and diluted in Dulbecco’s modified Eagle’s medium containing 20% fetal calf serum. After washing away the collagenase by centrifugation, the cells were seeded (30,000 cells/cm²) and expanded in monolayer culture for a period shorter than 2 weeks in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum before transferring to alginate culture (see below).

Alginate Bead Culture—Chondrocytes were taken into suspension by trypsin/EDTA digestion of the monolayer culture and embedded in alginate beads (26). Briefly, the cells were resuspended (10⁵ cells/ml) in Ham’s F-12 medium and mixed with an equal volume 2% alginate in 0.15 M NaCl prewarmed to 37 °C. Alginate beads were formed through dropping the cell suspension into 102 mM CaCl₂ in 0.15 M NaCl through a 23-gauge needle. After 10 min, the beads were washed twice in Ham’s F-12 medium and finally cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 containing 5% fetal calf serum with penicillin and streptomycin.

Immunofluorescence Staining—Alginate bead cultures were fixed overnight at 4 °C in 95% ethanol with 1% acetic acid, dehydrated in 99.5% ethanol and xylene, and embedded in low melting temperature paraffin. Sections (5 μm) were deparaffinated and rehydrated in alcohols, and nonspecific antibody binding was blocked with 1% normal donkey serum (Jackson Immunoresearch Laboratories). The sections were stained with mouse monoclonal antibody M2 139 against rat collagen type II (27) in 1% donkey normal serum followed by fluorescein isothiocyanate-conjugated anti-mouse IgG secondary antibodies preadsorbed against immunoglobulins from other species than the primary target antibody (Jackson Immunoresearch Laboratories) and mounted in Vectashield (Vector Laboratories). The fluorescence was documented through a × 100 Plan-Neofluar (numerical aperture 1.30) oil immersion objective in an Axioplan 2 imaging microscope equipped with an Axiocam HR digital camera using the Axiovision 4.2 software (Carl Zeiss). The TIFF files were identically adjusted using the Level tool in Photoshop 7.0.

Transmission Electron Microscopy—Alginate cultures were sequentially washed in serum-free Ham’s F-12 medium and twice in cadecylate-Ca buffer (0.15 m sodium cacodylate, 25 mM CaCl₂, pH 7.4) and fixed overnight at room temperature in 2.5% glutaraldehyde and 1.5% paraformaldehyde in cacodylate-Ca buffer. After washing four times in cadecylate-Ca buffer, the beads were postfixed for 2 h at room temperature in 1% Oso₄, 25 mM CaCl₂, dehydrated through 50, 70, 96%, and 99.5% eth-

6 K. Halász, M. Mórgelin, and D. Heinégård, unpublished data.
anol (2 × 15 min each) and acetone (2 × 5 min), and embedded in Epon resin. Sections (50 nm) were cut using an ultramicrotome with a diamond knife, and structures were visualized in a Jeol JEM 1230 transmission electron microscope and captured with a Gatan Multiscan 791 digital camera.

**Extraction and Partial Purification of Cartilage Perlecan—**
Calf legs were obtained from the local abattoir, and the articular cartilage and distal growth plate of the metacarpal bone were dissected and immediately frozen on dry ice. After pulverization in liquid nitrogen, the cartilage from 20 calf bones was extracted with 12 ml of extraction buffer (4 M guanidine hydrochloride, 50 mM sodium acetate, pH 5.8, containing 5 mM benzamidine hydrochloride, 0.1 M 6-aminohexanoic acid, and 5 mM N-ethylmaleimide) per g of tissue powder at 4 °C for 24 h. After removing the remaining insoluble material by centrifugation at 20,000 × g at 4 °C for 30 min, the proteins and proteoglycans in the extracts were fractionated by CsCl density gradient centrifugation with a starting density of 1.5 g/ml under dissociative conditions in 4 M guanidine hydrochloride as described elsewhere (28). The gradient tube was divided into four equal fractions using a Beckman tube slicer, and aliquots of the fractions were analyzed for the presence of perlecan by Western blotting. The top fraction (D4) was found to contain perlecan and subjected to another dissociative CsCl density gradient centrifugation with a starting density of 1.35 g/ml and fractionated by slicing, and the resulting D4.D4 and D4.D3 fractions were found to contain perlecan by Western blotting.

**Analysis of Cartilage Perlecan Glycosaminoglycans—**
Aliquots of the top fraction from the sequential CsCl density gradient centrifugation of calf growth plate and calf articular cartilage extracts were dialyzed against 10 mM HEPES, 3 mM calcium acetate, 0.1% Triton X-100 and digested with chondroitinase ABC (protease-free; Seikagaku), heparitinase type III (Seikagaku), or a combination of both enzymes for 4 ha at 37 °C in HEPES, 150 mM NaCl, 2 mM EDTA, 0.05% Surfactant P20, pH 7.5) were injected over the surface at 20 μl/min for 180 s, and the association and dissociation were followed in a BIAcore 2000 instrument. The surface was regenerated by injecting 20 μl of 10 mM HEPES, 1 M NaCl, 2 mM EDTA, 0.05% P20, pH 7.5.

**In Vitro Collagen Fibril Formation Assay—**
Bovine skin pepsin-extracted collagen type I (Vitrogen 100; Nutacan BV, Leimuiden, The Netherlands) and bovine pepsin-extracted tracheal cartilage collagen type II (19) were used in fibril formation assays (20). Briefly, a solution of collagen monomers (330 nM) was brought to neutral pH by the addition of an appropriate volume of 0.012 M NaOH and buffered by 20 mM HEPES, 150 mM NaCl, pH 7.4. Perlecan fragments were added at concentrations equimolar to that of collagen or at one-tenth the molar concentration of collagen. After mixing vigorously and briefly, the samples were transferred to cuvettes and incubated at 37 °C (collagen type I) or 35 °C (collagen type II) in a water-jacketed cuvette holder in the spectrophotometer, and the absorbance due to light scattering at 400 nm (collagen type I) or 313 nm (collagen type II) was monitored over a duration of 5–18 h.

In experiments with purified GAGs, the concentration of added carbohydrate was 0.78 μg/ml, equivalent to the amount of CS (measured as hexosamine) (30) on 33 nM perlecan domain IB. Bovine lung heparan sulfate was from Akzo-Nobel. Keratan-2-sulfate, dermatan sulfate, CS-4, and CS-6 were from the NIH Standard (33), whereas CS-D and CS-E were from Seikagaku. An additional CS-D preparation was a kind gift from Dr. Nikos Karamanos. Dextran sulfate (~4 sulfates/disaccharide eq) was from Amersham Biosciences.

**Hydroxyproline Assay—**
Collagen type I fibril formation reactions were performed as described above. Duplicate samples were incubated at 37 °C in screw cap 1.5-ml microtubes (Sarstedt). At each time point (0, 60, 120, and 180 min), samples were centrifuged at 20,800 × g for 30 min at room temperature, and the supernatants were transferred to fresh screw cap Eppendorf tubes. After hydrolysis in 6 M HCl (110 °C, 16 h),
hydroxyproline was assayed as described (34), and the concentration of collagen was calculated assuming a collagen type I hydroxyproline content of 13.4% (35).

Negative Staining Electron Microscopy—Negative staining electron microscopy of collagen fibrils and data evaluation were performed as previously described (36). Collagen fibril diameter measurements were performed using the interactive measurement tool of Axiovision 4.2 (Carl Zeiss). Raw data were obtained as width counted in number of pixels and was transformed to metric measurements.

Chondroitin Sulfate Composition—Characterization of CS with regard to sulfation was performed according to Lauder et al. (37). Briefly, samples were digested with 1 units/mg chondroitinase ABC (protease-free; Seikagaku) at 37 °C for 15 h. For disaccharide composition analysis of cartilage CS, purified glycosaminoglycans were digested with 1.7 units/mg each of chondroitinase ABC and chondroitinase ACII (protease-free; Seikagaku) at 37 °C for 24 h with a second addition of enzyme after 18 h. After inactivation of the enzyme (100 °C, 2 min) and reduction by sodium borohydride (final concentration 25 mM), the oligosaccharides were desalted on a Biogel P2 column equilibrated in 25 mM ammonium acetate, lyophilized, and dissolved in H2O. The oligosaccharides were separated at 1 ml/min on a 250 × 4-mm CarboPac PA-1 (Dionex) with a 50 × 4-mm pre-column maintained at 30 °C. The eluents used were 0.1 M NaOH (A) and 1.3 M NaCl (B) in 0.1 M NaOH, and the oligosaccharides were eluted with a gradient of 0 min/2% B to 12 min/2% B to 62 min/46% B to 70 min/87% B to 76 min/100% B to 80 min/100% B and detected by their absorbance at 232 nm. The elution position of reduced disaccharides was determined using standards (Seikagaku), and the relative abundance of different oligosaccharides was calculated by automatic integration of the peak area under curves.

β-Elimination of CS-substituted Perlecan Domain I—Chondroitin sulfate-substituted perlecan domains I and V (IB and VB, respectively) in 20 mM HEPES, 150 mM sodium chloride, pH 7.4 (HBS), were incubated at 4 °C for 16 h with equal volumes of 0.5 M sodium hydroxide (final concentration 0.25 M NaOH). After removal of the sodium hydroxide by dialysis against the HBS buffer (Microdialyzer 100; Pierce), the samples were used in collagen fibril formation experiments.

To confirm β-elimination, the GAG chains released from perlecan fragment IB were biotinylated on their reducing end and visualized by Western blotting. The GAGs were suspended in HBS, the sample pH was adjusted to 5.5 by the addition of 0.27 μl of 1.74 M acetic acid, and GAGs were biotinylated with EZ-Link LC biotin hydrazide (Pierce) according to the manufacturer’s protocol. Non-β-eliminated fragment IB in HBS was included as a control and subjected to pH correction and all subsequent steps in the biotinylation protocol. The biotinylated samples were precipitated at 4 °C for 16 h in 10 volumes of 96% ethanol with sodium acetate (50 mM) and centrifuged at 14,000 × g for 20 min at 4 °C. The supernatant was removed, and any remaining solvent was evaporated in a SpeedVac. After separation by SDS-PAGE (4–16%) and electrotransfer onto a 0.2-μm Fluorotrans polyvinylidene difluoride membrane ( Pall), the membranes were probed with horseradish peroxi-

dase-conjugated streptavidin (DAKO) or with an anti-perlecan domain I antibody using chemiluminiscent detection.

RESULTS

Perlecan-null Chondrocytes Have Defective Collagen Networks—We were interested in determining whether perlecan-null chondrocytes have a compromised ability to form a collagen type II network. Chondrocytes were isolated from wild type and perlecan-deficient embryos (12) and cultured in alginate beads (26), which prevents dedifferentiation (38) (Fig. 1). In
alginate bead culture, the wild-type chondrocytes formed a capsule that radiated a halo of fine fibrils staining for collagen type II (Fig. 1, A and B). Very few perlecan-deficient chondrocytes actually formed a collagen capsule, although they expressed the chondrocyte marker protein COMP (cartilage oligomeric matrix protein) (not shown), indicating that they retained the chondrocyte phenotype (39). Interestingly, the few capsules formed by perlecan-null chondrocytes lacked the collagen type II fibril halo (Fig. 1, E and F).

Transmission electron microscopy of chondrocytes in alginate beads confirmed these findings and simultaneously demonstrated the phenomenon in more detail. Wild-type cells assemble collagen monomers to fibrils just outside the cell (Fig. 1, C and D), whereas no such structures were observed in perlecan-null cells (Fig. 1, G and H). Note the high levels of thin fibrillar elements surrounding the perlecan-deficient chondrocyte, indicating that collagen is synthesized and partially assembled.

**Perlecan from Embryonic Cartilage is CS-substituted**—Perlecan was partially purified from guanidine hydrochloride extracts of calf growth plate (Fig. 2, A and C) or articular cartilage (Fig. 2, B and D) by sequential CsCl density gradient centrifugation. Perlecan prepared in this manner was digested with chondroitinase ABC, heparitinase, or a combination of these, electrophoresed on a 3–10% gradient SDS-polyacrylamide gel, and subsequently immunoblotted with antibodies against perlecan (Fig. 2, A and B). Chondroitinase ABC digestion of fractions containing perlecan from growth plate led to decreased high molecular weight smears and the appearance of lower molecular weight bands (Fig. 2A). The effect of chondroitinase digestion on corresponding fractions from articular cartilage was less distinct (Fig. 2B). Heparitinase digestion has comparable effects on both preparations. This confirms earlier observations that the perlecan species in developing cartilage is partially substituted with CS (9).

**Perlecan Binds to Collagen Type I via Chondroitin Sulfate Chains**—Given that perlecan in developing cartilage contains CS chains and that the collagen network in perlecan-null mouse cartilage appears compromised, we next studied the binding between perlecan substituted with different types of GAG and collagen. Using surface plasmon resonance, recombinant domains of perlecan (Fig. 3) were injected over collagen type I immobilized to a C1 sensor chip. Recombinant perlecan domain I substituted with HS alone (IA; Fig. 4) showed no binding. In recombinant domain Imut, the serine GAG attachment sites were mutated to alanine residues, and thus this fragment is not substituted. No binding between domain Imut and collagen type I could be detected (Fig. 4C).

**CS/HS-substituted Perlecan Accelerates Collagen Fibril Formation**—To determine whether perlecan had any effect on collagen matrix assembly, we performed in vitro collagen fibril formation assays, monitoring the increase in turbidity in collagen solutions after triggering these for fibril formation with or without perlecan fragments present (Fig. 5). Inclusion of the CS/HS-carrying recombinant perlecan domain IB was shown to greatly enhance this process, both accelerating the rate of collagen type II fibrillogenesis and apparently also increasing the amount of fibrils formed (Fig. 5A). Perlecan domain I substituted with HS only (IA) and the domain I core protein (Imut) showed no effect, neither enhancing nor inhibiting fibril formation. Corresponding experiments using the CS/HS-substituted domain V (Fig. 5B) or domain V fragments (not shown) showed similar promotion of fibril formation, although less pronounced. Collagen type I fibril formation proceeds more rapidly than collagen type II, but also here the CS-substituted perlecan domain I induced a dramatic increase in fibril formation (Fig. 5C). Hydroxyproline content in the pellets of in vitro fibril formation reactions confirmed that the increased turbidity corresponded to an increase in the amount of collagen and thus in the rate of assembly (Fig. 5D).
Electron Microscopy of Collagen Fibrils Formed in the Presence of Perlecan—The events monitored in the spectrophotometer were visualized by negative staining electron microscopy of the material from the fibril formation reactions (Fig. 6). In contrast to control reaction with collagen type I alone (Fig. 6A), the addition of CS/HS-substituted domain I (IB) to a solution of monomeric collagen type I molecules organizes these to fibrils almost instantaneously (Fig. 6B). Intermediate fibrils can be found even at the earliest time points, and fibrils are prominent already after 20 min of incubation. The fibrils formed in the presence of domain IB appear more regular, with few straggling monomers and a highly regular banded pattern. Indeed, the mature fibrils formed after 20 min in the presence of domain IB (Fig. 6B) are more evenly formed than those formed after a 320-min incubation in the absence of perlecan (Fig. 6A).

Electron microscopy of experiments performed with collagen type II alone (Fig. 6C) or with added domain IB (Fig. 6D) shows similar acceleration of fibril formation and increased regularity in fibril banding pattern as for collagen type I.

Measuring the diameters of a large number of collagen fibrils confirmed the acceleration of fibril formation by perlecan (Fig. 7). Thin collagen type I fibrils were rapidly formed in the presence of domain IB, thicker fibrils appeared after 20 min, and fibril thickness generally increased more rapidly over time in the presence of domain IB (Fig. 7A). Again a similar acceleration by perlecan of fibril assembly was observed for collagen type II (Fig. 7B).

Chondroitin Sulfate Chains Are Sufficient for Enhancing Collagen Fibril Formation—To determine whether the fibril formation enhancement by CS-substituted perlecan domain I is
Perlecan and Collagen Fibril Formation

A

![Turbidity vs. Time](image)

B

![Turbidity vs. Time](image)

C

![Turbidity vs. Time](image)

D

![Graph](image)

**FIGURE 5.** **CS-substituted perlecan domain I accelerates in vitro collagen fibril formation.** Pepsin-extracted type II collagen (A and B) or type I collagen (C and D) were diluted in HEPES buffer (pH 7.5) and mixed with recombinant perlecan domain I (A and C) or domain V (B). The turbidity of the solution was monitored in a spectrophotometer. Note the pronounced shortening of the lag phase, acceleration of turbidity increase, and elevated plateau level in the experiments with CS-substituted perlecan (domains IB and VB). 

- **A** and **B**: Turbidity at 400 nm vs. time (minutes).
- **C**: Turbidity at 400 nm with domain IB.
- **D**: Graph showing collagen in pellet (% of total collagen) vs. time (minutes).

- **Domain IB**: + IB (0.1x)
- **Col II control**: + IA (1x) + Imut (1x)
- **VA (1x)**
- **VB (1x)**
- **Col II control**: + IA (1x) + Imut (1x)
- **Col II control**: + IA (1x) + Imut (1x)

- **Time (minutes)**: 0, 200, 400, 600, 800, 1000
- **Turbidity 400 nm**: 0.01, 0.02, 0.04, 0.06, 0.08

**in vitro** collagen fibril formation assays (data not shown), suggesting that the specific disaccharide sequence may be more important than the overall degree of sulfation.

**Oversulfated Chondroitin Sulfate E Enhances Collagen Fibril Formation**—The enhanced collagen fibril formation seen after β-elimination of the perlecan fragments suggested that the GAG chains are sufficient to enhance fibril formation and that the core protein is not involved. Indeed, previous studies by Öbrink (23) demonstrated that GAG chains can affect collagen fibril formation independently of a core protein. To verify that this was the case, we assayed a number of different isolated GAGs for their effect on collagen fibril formation. As shown in Fig. 10A, heparan sulfate, keratan sulfate, and dermatan sulfate had no effect on fibril formation. The synthetic carbohydrate polymer dextran sulfate was also without effect, showing that an overall high charge density in itself is not sufficient for enhancement of collagen fibril formation (Fig. 10A). Interestingly, the oversulfated CS-E (enriched in 4,6-disulfated disaccharide units) markedly enhanced collagen fibril formation, dependent on clustering of the GAG chains, the chains were released from the core protein through β-elimination. The release of side chains was confirmed by biotinylation experiments. Immunoblotting showed that no GAG-substituted perlecan remained after β-elimination (Fig. 8A), and hydrazine biotinylation confirmed the release of free GAG chains in the β-elimination reaction mixture (Fig. 8B). In collagen fibril formation experiments, the control incubation appeared to lower the activity of fragments IB and VB (Fig. 8, C and D), although fragment IB was not seen to change size as compared with untreated IB (Fig. 8A). In contrast, release of the CS/HS chains from perlecan domain I or V by β-elimination resulted in increased enhancement of collagen fibril formation compared with the untreated material (Fig. 8, C and D).

*Perlecan CS Chains Are Highly Sulfated*—Aggrecan makes up the overwhelming majority of CS in cartilage. To elucidate any structural differences between aggrecan and perlecan glycans, GAG chains from different sources were digested by chondroitinase ABC, separated by high pressure anion exchange chromatography on a CarboPac PA-1 column, and the position of eluted components was detected by monitoring absorbance at 232 nm (37). Fig. 9 displays the profile of the oversulfated chondroitin sulfate CS-D, where peaks indicate the elution position of the different di-, tetra-, and hexasaccharides (Fig. 9A). Comparison of the carbohydrate profiles of perlecan domain V6B (a CS-carrying fragment of domain V that enhances collagen fibril formation), CS-D, CS-6, and aggrecan revealed that all samples are similar in the early part of the spectrum (not shown). In contrast to aggrecan from calf nasal cartilage (Fig. 9B) or calf growth plate (not shown), the recombinant perlecan CS chains from domain V6B contain highly sulfated CS disaccharides similar to CS-D, eluting late in the separation (Fig. 9B).

In order to compare the CS profiles of different proteins, the area under the peaks as a fraction of the total area under peaks was calculated (Table 1). CS-D had a high ratio of high sulfate disaccharides, whereas aggrecan had a low such ratio. The recombinant chondroitin sulfate perlecan domains V6B, V4B, and IB all displayed an intermediate level of high sulfate. A chondroitin sulfate-substituted recombinant laminin β1 chain fragment produced in the same cell culture system displayed high sulfate ratios similar to those of the recombinant perlecan domains (Table 1). Interestingly, this fragment had no effect in collagen fibril formation assays (data not shown), suggesting that the specific disaccharide sequence may be more important than the overall degree of sulfation.

**Perlecan and Collagen Fibril Formation**—The enhanced collagen fibril formation seen after β-elimination of the perlecan fragments suggested that the GAG chains are sufficient to enhance fibril formation and that the core protein is not involved. Indeed, previous studies by Öbrink (23) demonstrated that GAG chains can affect collagen fibril formation independently of a core protein. To verify that this was the case, we assayed a number of different isolated GAGs for their effect on collagen fibril formation. As shown in Fig. 10A, heparan sulfate, keratan sulfate, and dermatan sulfate had no effect on fibril formation. The synthetic carbohydrate polymer dextran sulfate was also without effect, showing that an overall high charge density in itself is not sufficient for enhancement of collagen fibril formation (Fig. 10A). Interestingly, the oversulfated CS-E (enriched in 4,6-disulfated disaccharide units) markedly enhanced collagen fibril formation,
with a distinct decrease in lag phase duration, increased fibril formation rate, and elevated plateau level (Fig. 10B), and hydroxyproline determination confirmed increased insoluble collagen after CS-E stimulation (Fig. 10C). Surprisingly, none of the other tested CS variants with different sulfation patterns, including two different preparations of the oversulfated CS-D (enriched in 2,6-disulfated disaccharide units), enhanced collagen fibril formation, suggesting that a specific structure or sequence of disaccharides may be involved.

Glycosaminoglycans from Perlecan-enriched Cartilage Fractions Stimulate Collagen Fibril Formation and Include Highly Sulfated Chondroitin Sulfate—Chondroitin sulfate substitution of perlecan is evident in developing cartilage (9). Thus, glycosaminoglycans isolated from immature cartilage may stimulate collagen fibril formation via the chondroitin sulfate side chains as described above.

To investigate this claim, a total glycosaminoglycan preparation was isolated from perlecan-containing cesium chloride D4 samples from cow articular cartilage, calf articular cartilage, and calf growth plate cartilage. Control samples were prepared from cesium chloride D1 fractions mainly containing aggrecan and without detectable perlecan.

The addition of control D1 GAG chains to the collagen fibril formation assay caused no effect on the rate of collagen fiber assembly (Fig. 11A). In contrast, the addition of GAG chains from D4 samples containing perlecan CS chains led to a marked stimulation of collagen fibril formation (Fig. 11B). The addition of GAGs from cow and calf articular cartilage led to intermediate stimulation, whereas the addition of GAGs from growth plate cartilage led to a significantly higher level of stimulation.

Interestingly, chondroitin sulfate chains from cesium chloride D4 fractions containing no perlecan contained barely detectable levels of 4,6-disulfated disaccharides (Table 2). On the other hand, cesium chloride D4 fractions containing perlecan had CS chains with significantly higher abundance of the 4,6-disulfated disaccharides. Furthermore, the levels of this disulfated disaccharide were similar in cow and calf articular cartilage but approximately twice as high in the growth plate cartilage sample.

Thus, the levels of 4,6-disulfated disaccharide incorporation in CS chains correlate with the degree of stimulation of collagen fibril formation. This demonstrates that a high content of 4,6-disulfated disaccharide, such as that found in CS-E and in perlecan-enriched cartilage samples, is essential for chondroitin sulfate enhancement of collagen fibril formation.

DISCUSSION
Two human genetic disorders with skeletal manifestation caused by perlecan mutations have been identified. The Schwartz-Jampel syndrome results from mutations leading to truncation and reduced levels of perlecan (15, 40), whereas the Silverman-Handmaker type of dyssegmental dysplasia is caused by a functional null mutation of perlecan (16). The findings in the latter correspond to those in the perlecan-null mouse, and neither mice nor humans carrying functional null mutations in perlecan survive birth.

Although it was known previously that perlecan is present in cartilage, its importance for skeletogenesis was not recognized until the production of perlecan-null mice. These mice fail to organize the extracellular matrix and to form an adequate cartilage template and therefore display severe skeletal defects with disorganized growth plates and bent long bones (11, 12). The mechanistic explanation for the skeletal phenotype remains unclear. One possibility is that the lack of perlecan may...
interface with growth factor signaling (11). Alternatively, lack of perlecan could lead to a shifted cartilage extracellular matrix homeostasis with increased collagen degradation. We now show that perlecan is involved in cartilage extracellular matrix homeostasis, albeit affecting collagen matrix assembly rather than degradation.

In vitro fibril formation experiments reported in this study show that perlecan stimulates the formation of collagen fibrils from monomers, and this supports the hypothesis of a failure of organization on the level of the collagen network. Light microscopy and transmission electron microscopy studies on perlecan-null chondrocytes confirm the importance of perlecan for cartilage collagen matrix assembly. This finding offers a mechanism for the skeletal phenotype displayed by perlecan-deficient mice.

Perlecan is known to exist in two forms. It was originally isolated and described as an HS proteoglycan but was later found to exist also in a form substituted with both CS and HS (9, 10). Our studies demonstrate discrete roles for HS perlecan and CS/HS perlecan. Whereas CS/HS perlecan will organize collagen monomers to fibrils in a highly efficient manner, HS perlecan is inert in the same experiment. The data presented here suggest a new role for perlecan in cartilage, where CS perlecan, present in growth plate cartilage, facilitates the organization of collagen to mature fibrils.

In early in vitro experiments, mesenchymal cells plated on perlecan displayed condensation (8), and it was postulated that perlecan would be important for chondrogenesis in vivo. Experiments located the condensation activity of perlecan to domain I (41). Although GAG substitution was necessary for this effect,
Perlecan and Collagen Fibril Formation

since domain Imut did not support condensation, this appears to be dependent on HS substitution, since both domains IB and IA had comparable activity. However, chondrocyte differentiation proceeds in perlecan-null mice, as determined by expression of chondrocyte markers, such as collagen type II, COMP, and matrilin-3 (12), and cartilage and bone are formed. Thus, it appears that this role of perlecan may be compensated for by other components in cartilage.

Defects in cartilage in perlecan-null mice arise later (after condensation) and result in disorganization of the matrix, particularly in the growth plate. Within the growth plate, the organization gets progressively more compromised, moving from resting via proliferating to the hypertrophic zone. In the hypertrophic zone, the collagen fibrils are shorter in length and present at a greatly reduced density compared with wild type (17). In the present study, few of the perlecan-null chondrocytes in culture stained positive for collagen fibrils, reflecting the in vivo situation. Thus, although greatly reduced, some fibril deposition appears possible. This probably reflects the inherent ability of collagen monomers to form fibrils. In addition, other enhancers of collagen fibril formation may also compensate to some degree for the lack of perlecan.

The lack of collagen fibrils is not due to decreased production of collagen type II. The perlecan-null chondrocytes have increased general biosynthesis and in particular increased mRNA for collagen type II a1, COMP, and matrilin-3 as compared with wild type cells (12). The perlecan-null chondrocytes show a more synthetic morphology with prominent rough ER and more developed plasma membrane folds (12). We have observed in electron microscopy both a prominence of apparently distended rough ER and an increased number of vesicles in perlecan-null chondrocytes (not shown). As seen in Fig. 1, D and H, it appears that collagen is present, although no major fibrils are seen to be forming.

The bent shape of the perlecan-null long bones probably results from the mutant cartilage templates being unable to withstand mechanical load. Indeed, dissecting perlecan-null cartilage templates was difficult, since the cartilage tissue was extremely soft compared with wild type (not shown). Interestingly, the collagen-tailed form of acetylcholinesterase in the neuromuscular junction is normally anchored to perlecan, and mice lacking perlecan fail to retain acetylcholinesterase in these synapses (13). Thus, the cartilage templates of the mutants are presumably subject to more extended muscular contraction than in the wild type. This may well be an aggravating factor in the skeletal malformation of the perlecan-null mice. Indeed, cartilage-specific conditional perlecan knock-out mice produced using Col2A1-Cre deletion of a floxed perlecan allele

FIGURE 9. The recombinant perlecan fragments carry highly sulfated CS chains. High pressure anionic exchange chromatography of CS digested with chondroitinase ABC produces a CS fingerprint of di-, tetra-, and hexasaccharides with different sulfation patterns. A, the fingerprint of the highly sulfated CS form CS-D with the disaccharide peaks labeled and the unlabeled peaks corresponding to tetra- and hexasaccharides. B, the high sulfate region of the overlaid CS fingerprints of CS-D, the collagen fibril formation enhancing CS-substituted perlecan domain V6B, CS-6, and calf aggrecan. In contrast to aggrecan, the perlecan domain CS chains contain oversulfated disaccharides. The traces in B were displaced along the y axis to facilitate comparison.

TABLE 1

| Sample                        | Relative amount of chondroitin sulfate disaccharide (percentage of total area under curve) |
|-------------------------------|------------------------------------------------------------------------------------------|
|                               | ΔDi-0S | ΔDi-4S | ΔDi-6S | ΔDi-2,4,6S | ΔDi-2,6S | ΔDi-4,6S | ΔDi-2,6S | ΔDi-2,4,6S | Othera | Oversulfatedb |
|-------------------------------|--------|--------|--------|------------|----------|----------|----------|------------|--------|--------------|
| CS-6 (NIH Std)                | 0      | 1.5    | 52.5   | 0          | 0        | 0        | 0        | 0          | 46.0   | 0            |
| CS-6 (shark)                  | 0      | 2.0    | 60.1   | 0          | 0        | 0        | 0        | 0          | 39.7   | 0            |
| CS-D                          | 0.1    | 10.3   | 32.0   | 0.1        | 5.0      | 3.1      | 57.6     | 8.2        |
| Aggrecan, c-sarc             | 0.6    | 60.0   | <0.1   | 0          | 0        | 0        | 0        | 39.4       |
| Aggrecan, calf nasal         | 0      | 46.9   | 15.1   | 0.2        | 0        | 0        | 38.0     | 0.2        |
| Perlecan domain IB             | 0      | 30.8   | 33.8   | 0.3        | 1.5      | 0.3      | 35.4     | 2.1        |
| Perlecan domain V4B           | 0.3    | 18.6   | 39.7   | 0.5        | 1.9      | 0.3      | 41.4     | 2.7        |
| Perlecan domain V6B            | 0.5    | 23.0   | 31.1   | 0.3        | 1.6      | 0.2      | 45.4     | 2.1        |
| Laminin B1 IV 3b-1           | 0      | 24.6   | 33.3   | 0.4        | 0.7      | 0.3      | 42.1     | 1.4        |

a Tetra- and hexasaccharides resulting from cleavage with chondroitinase ABC, protease-free (Seikagaku).
b Sum of ΔDi-4S, ΔDi-2,4,6S, and ΔDi-2,6S.
c From rat chondrosarcoma.
d CS/HS perlecan domain I expressed in 293 cells (30).
e CS/HS perlecan domain I expressed in 293-EBNA cells (31).
f CS/HS perlecan domain V E3,4LG3 expressed in 293-EBNA cells (31).
g CS perlecan domain V E1,2LG2E3,4LG3 expressed in 293-EBNA cells (31).
h CS laminin B1 domain IV expressed in 293-EBNA cells (47).
show a phenotype with chondrodysplasia but no bending of the long bones.7

In vitro, perlecan enhances both collagen type I and type II fibril formation. We have shown that growth plate cartilage expresses the CS/HS-substituted form of perlecan, which is active in fibrillogenesis. It is possible that expression of this variant of perlecan is limited to cartilage and that perlecan elsewhere has a predominant substitution with heparan sulfate, which is inactive in fibrillogenesis. Experiments documenting the expression pattern of CS-perlecan would increase our understanding.

Our findings are in agreement with early observations (42) that a proteoglycan preparation from an 18-year-old human would not increase fibrillogenesis of collagen type II, whereas such a preparation from 0–3-year-old humans would. This activity was lost after digestion with chondroitinase ABC. Interestingly, in the experiments by Kuijer et al. (42), no proteoglycan was found associated with the collagen pellet formed in fibrillation. In our EM experiments, perlecan domain I is seen as phase-bright round dots. These are found in association with the early fibrils but not with later fibrils in collagen type I fibrillation (Fig. 6, compare 0 min with 320 min). This raises the possibility that the perlecan GAG effect on collagen fibril formation may be of a catalytic character. Further experiments are needed to clarify this.

Perlecan domain V is also substituted with GAG, although the actual number of chains varies from 0 to at least 2 (2, 43). Recombinant perlecan domain V was, however, not as efficient in enhancing collagen fibril formation as domain I. This is interesting in light of results published by Rossi et al. (44). Mice genetically engineered to lack exon 3 of perlecan lack the GAG substitution sites in domain I. Perlecan produced by mutant fibroblasts is mainly chondroitin sulfate-substituted, indicating that domain V is present and substituted. These mice show an eye phenotype but have not been reported to display any cartilage phenotype. This suggests that in vivo the domain V may be sufficient to organize the collagen fibrils. Careful ultrastructural analysis of the cartilage and growth plate in these animals and studies in aged mice to reveal possible susceptibility to osteoarthritis would be interesting.

The fibril formation-enhancing activity of CS/HS perlecan domain I (IB) was shown to depend on GAG substitution, since domain I core protein (Imut) did not enhance fibril formation. We initially hypothesized that the core protein could be important by clustering the CS chains to the termini of the perlecan molecule, bringing several collagen molecules into proximity and perhaps in register with each other and thus accelerate fibril formation. This would have explained the lower degree of

7 A. Aszödi and R. Fässler, unpublished data.
Perlecan and Collagen Fibril Formation

Disaccharide composition of chondroitin sulfate extracted from cartilage

| CsCl fraction                  | Relative amount of chondroitin sulfate disaccharide (percentage of total chondroitin sulfate) |
|-------------------------------|---------------------------------------------------------------------------------------------|
|                               | ΔDi-0S | ΔDi-4S | ΔDi-6S | ΔDi-2,6S | ΔDi-4,6S | ΔDi-2,4,6S | Tetra-/Hexasaccharides |
| Cow articular cartilage D1a   | 1.8    | 31.8   | 66.3   | 0        | 0.03     | 0          | 0.1               |
| Calf articular cartilage D1a  | 9.8    | 54.1   | 35.7   | 0.04     | 0.1      | 0          | 0.2               |
| Calf growth plate D1a         | 11.2   | 68.8   | 19.9   | 0        | 0.1      | 0          | 0                 |
| Cow articular cartilage D4b  | 15.1   | 57.2   | 25.5   | 0.14     | 1.8      | 0          | 0.3               |
| Calf articular cartilage D4b  | 17.5   | 59.8   | 21     | 0        | 1.6      | 0          | 0.2               |
| Calf growth plate D4b         | 14.7   | 65     | 16.4   | 0        | 2.7      | 0          | 0.8               |
|                               | c      | b      | c      |           |          |            |                   |
|                               | a      | b      | c      |           |          |            |                   |
|                               |         | c      | c      |           |          |            |                   |
|                               |         |         |         |           |          |            |                   |
|                               |         |         |         |           |          |            |                   |
|                               |         |         |         |           |          |            |                   |

*Chondroitin sulfate di-, tetra-, and hexasaccharides constituted on average 21% of the total area under the curve.

a No perlecan detected by Western blot.

b Containing perlecan as determined by Western blot.

c Containing perlecan as determined by Western blot.

c Containing perlecan as determined by Western blot.

c Containing perlecan as determined by Western blot.

c Containing perlecan as determined by Western blot.

Collagen fibril formation enhancement observed with recombinant perlecan domain V, since this fragment was shown to carry a single CS chain (31). Clustering appears, however, not to be important, since releasing the CS chains by β-elimination augmented the enhancement of fibril formation.

The idea that CS perlecan is active in cartilage is surprising, since the overwhelming majority of cartilage proteoglycan is made up of aggrecan, which is massively substituted with chondroitin sulfate. This indicated that the structure of the GAG chains was critical for function. In analysis of the composition of the GAG chains on recombinant perlecan and aggrecan, it was found that the perlecan chains were more highly sulfated than those from aggrecan. Indeed, purified oversulfated CS from squid (CS-E, containing 4,6-disulfated disaccharides) was a very efficient enhancer of collagen fibril formation, whereas other CS or GAG preparations had no effect. The inability of CS-D (containing 2,6-disulfated disaccharides) or dextran sulfate (~4 sulfates/disaccharide eq) to enhance fibril formation suggests that the effect depends on specific motifs found in CS-E and perlecan CS.

Investigation of the sulfation pattern of CS chains extracted from cartilage demonstrated that CS from perlecan-containing CsCl density gradient centrifugation fractions incorporated significantly higher levels of 4,6-disulfated disaccharides compared with CsCl fractions with no perlecan present, where 4,6-disulfated disaccharides were barely detectable. Furthermore, only samples containing a significant proportion of 4,6-disulfated disaccharides stimulated collagen fibril formation, and the degree of stimulation correlated with the proportional content of this disaccharide. The level of 4,6-disulfated disaccharides were highest in growth plate cartilage, which may reflect the higher anabolic activity of this developing cartilage.

Biological roles for highly sulfated CS have been described. For example, it has been shown (45) that CS-E can promote neurite outgrowth, indicating that the ultrastructure of sulfation is important. The mechanism for fibril formation enhancement involves at least an initial binding to collagen, as suggested by EM and BIAcore results. Interestingly, CS-E has been shown to bind various collagens, and the minimal sequence for CS-E binding to collagen type V has been determined (46). It would be of interest to identify the CS structure important for the collagen type I and II fibril formation-enhancing effect.

Thus, the fine structure of the perlecan GAG chains appears to be important for collagen fibril formation enhancement, but the exact mechanism by which the perlecan CS proteoglycan binds collagen and enhances fibril formation remains to be clarified.

In conclusion, we have demonstrated a novel role for CS-substituted perlecan in cartilage where this proteoglycan variant enhances collagen type II fibril formation. GAG substitution of a core protein is a complex process, and the cell devotes considerable resources to the sugar modification of proteins. The complexity and variation of these modified structures invite speculation as to their biological relevance. These experiments present an example of diverse biological roles for the same core protein depending on the nature of the GAG with which it is substituted.

Acknowledgments—We thank Dr. Dick Heinegård for reagents, helpful suggestions, and critical reading of the manuscript. We are also grateful to Maria Baumgarten for skillful negative staining work and Rita Wallén (EM-unit, Cell and Organism Biology, Lund University) for help with electron microscopy. A monoclonal antibody against collagen type II was a kind gift from Dr. John A. Mo (Lund University), and disulfated CS disaccharides and CS-D were kind gifts from Dr. Nikos Karamanos (University of Patras, Greece).

REFERENCES
1. Timpl, R., and Brown, J. C. (1996) BioEssays 18, 123–132
2. Bengtsson, E., Mörghelin, M., Sasaki, T., Timpl, R., Heinegård, D., and Aspegren, A. (2002) J. Biol. Chem. 277, 15061–15068
3. Brown, J. C., Mörghelin, M., Göhring, W., Yamada, Y., and Timpl, R. (1997) Eur. J. Biochem. 250, 39–46
4. Aviezer, D., Hecht, D., Safran, M., Eisinger, M., David, G., and Yayon, A. (1994) Cell 79, 1005–1013
5. Gonzalez, E. M., Mongiat, M., Slater, S. J., Baffa, R., and Ioizzo, R. V. (2003) J. Biol. Chem. 278, 38113–38116
6. Sandaraj, N., Fite, D., Ledbetter, S., Chakravarti, S., and Hassell, J. R. (1995) J. Cell Sci. 108, 2663–2672
7. Handler, M., Yurchenco, P. D., and Ioizzo, R. V. (1997) Dev. Dyn. 210, 130–145
8. French, M. M., Smith, S. E., Akanbi, K., Sanford, T., Hecht, J., Farach-Carson, M. C., and Carson, D. D. (1999) J. Cell Biol. 145, 1103–1115
9. Govindraj, P., West, L., Koob, T. J., Neame, P., Doege, K., and Hassell, J. R. (2002) J. Biol. Chem. 277, 19461–19469
10. Couchman, J. R., Kapoor, R., Stahan, M., and Wu, R. R. (1996) J. Biol. Chem. 271, 9595–9602
11. Arikawa-Hirasawa, E., Watanabe, H., Takami, H., Hassell, J. R., and Yamada, Y. (1999) Nat. Genet. 23, 354–358
12. Costell, M., Gustafsson, E., Aszödi, A., Mörghelin, M., Bloch, W., Hunziker, E., Addicks, K., Timpl, R., and Fässler, R. (1999) J. Cell Biol. 147, 1109–1122
13. Arikawa-Hirasawa, E., Rossi, S. G., Rotundo, R. L., and Yamada, Y. (2002) Nat. Neurosci. 5, 119–123
14. Costell, M., Carmona, R., Gustafsson, E., Gonzalez-Iriarte, M., Fassler, R., and Munoz-Chapuli, R. (2002) Circ. Res. 91, 158–164
15. Nicole, S., Davoine, C. S., Topaloglu, H., Cattolico, L., Barral, D., Brightton, P., Hamida, C. B., Hammouda, H., Cruaud, C., White, P. S., Samson, D., Urtizberea, J. A., Lehmann-Horn, F., Weissenbach, J., Gentili, F., and Fontaine, B. (2000) Nat. Genet. 26, 480–483
16. Arikawa-Hirasawa, E., Wilcox, W. R., Le, A. H., Silverman, N., Govindraj, P., Hassell, J. R., and Yamada, Y. (2001) Nat. Genet. 27, 431–434
17. Gustafsson, E., Aszodi, A., Ortega, N., Hunziker, E. B., Denker, H. W., Werb, Z., and Fassler, R. (2003) Ann. N. Y. Acad. Sci. 995, 140–150
18. MacBeath, J. R., Shackleton, D. R., and Hulmes, D. J. (1993) J. Biol. Chem. 268, 19826–19832
19. Vogel, K. G., Paulsson, M., and Heinegård, D. (1984) Biochem. J. 223, 587–597
20. Hedbom, E., and Heinegård, D. (1989) J. Biol. Chem. 264, 6898–6905
21. Gavriel, P., and Kagan, H. M. (1988) Biochemistry 27, 259–270
22. Mathews, M. B., and Decker, L. (1968) Biochem. J. 109, 517–526
23. Obrink, B. (1973) Eur. J. Biochem. 145, 3–78
24. Mo, J. A., and Holmdahl, R. (1996) J. Immunol. 157, 2440–2448
25. Lauder, R. M., Huckerby, T. N., and Nieduszynski, I. A. (2000) Glycobiology 10, 393–401
26. Houselmann, H. J., Fernandes, R. J., Mok, S. S., Schmid, T. M., Block, J. A., Aydelotte, M. B., Kue ttner, K. E., and Thonar, E. J. (1994) J. Cell Sci. 107, 17–27
27. Zaucke, F., Dinser, R., Maurer, P., and Paulsson, M. (2001) Biochem. J. 358, 17–24
28. Arikawa-Hirasawa, E., Le, A. H., Nishino, I., Nonaka, I., Ho, N. C., Franchomano, C. A., Govindraj, P., Hassell, J. R., Devaney, J. M., Spranger, J., Stevenson, R. E., Iannaccone, S., Dalakas, M. C., and Yamada, Y. (2002) Am. J. Hum. Genet. 70, 1368–1375
29. French, M. M., Gomes, R. R., Jr., Timpl, R., Hook, M., Czymmek, K., Farach-Carson, M. C., and Carson, D. D. (2002) J. Bone Miner. Res. 17, 48–55
30. Kuijer, R., van de Stadt, R. I., de Koning, M. H., van Kampen, G. P., and van der Korst, J. K. (1988) Connect. Tissue Res. 19, 277–297
31. Tapanadechopone, P., Hassell, J. R., Rigatti, B., and Couchman, J. R. (1999) Biochem. Biophys. Res. Commun. 265, 680–690
32. Rossi, M., Morita, H., Sormunen, R., Airenne, S., Kreivi, M., Wang, L., Fukai, N., Olsen, B. R., Tryggvason, K., and So ininen, R. (2003) EMBO J. 22, 236–245
33. Hinck, M., Mikami, T., Faisser, A., Vilela-Silva, A. C., Pavao, M. S., and Sugahara, K. (2003) J. Biol. Chem. 278, 43744–43754
34. Takagaki, K., Munakata, H., Kakizaki, I., Iwafune, M., Itabashi, T., and Endo, M. (2002) J. Biol. Chem. 277, 8882–8889
35. Sasaki, T., Mann, K., Miner, J. H., Miosge, N., and Timpl, R. (2002) Eur. J. Biochem. 269, 431–442