Fibrillogenesis of Collagen Types I, II, and III with Small Leucine-Rich Proteoglycans Decorin and Biglycan

Timothy Douglas,* Sascha Heinemann, Susanne Bierbaum, Dieter Scharnweber, and Hartmut Worch

Institute of Material Science, Max Bergmann Center of Biomaterials, Technische Universität Dresden, 01069 Dresden, Germany

Received April 18, 2006; Revised Manuscript Received May 23, 2006

Collagen has found use as a scaffold material for tissue engineering as well as a coating material for implants with a view to enhancing osseointegration through mimicry of the bone extracellular matrix in vivo. The aim of this study was to compare the collagen types I, II, and III with regard to their ability to bind the small leucine-rich proteoglycans (SLRPs) decorin and biglycan during fibrillogenesis in vitro in phosphate buffer. In addition, the influence of SLRPs on the proportion of collagen molecules incorporated into fibrils during fibrillogenesis in vitro at high and low ionic strength was investigated, as were their effects on the morphology of collagen fibrils and the speed of fibrillogenesis. Considerably more biglycan than decorin was bound by all three collagen types. Collagen II bound significantly more SLRPs in fibrils than collagen I and III. Decorin and biglycan decreased the proportion of collagen molecules of all three collagen types incorporated into fibrils in similar fashion. Biglycan affected neither fibril diameter nor the speed of fibrillogenesis. Decorin reduced the fibril diameter of all three collagen types. The differences in SLRP-binding ability between collagen types could be of significance when selecting collagen type and/or SLRPs as scaffold materials for tissue engineering or implant coatings.

Introduction

Titanium and its alloys are widely used as orthopedic materials due to their high biocompatibility. As the success of an implant depends on the stability of its fixation in the surrounding bone, surface modifications which encourage osteoblast attachment, proliferation, and differentiation should be advantageous. One approach in surface modification is to mimic the extracellular matrix (ECM) of bone by coating implants with collagen, its main organic component. Collagens contain binding sites for osteoprogenitor cells, and in several publications, titanium or its alloys have been coated with collagen, which has improved osteoblast attachment, proliferation and differentiation in vitro, while increasing early bone remodeling, bone contact and formation of surrounding bone in vivo.[1−7]

Collagens are structural proteins of which 27 types have thus far been identified.[8−10] Among the fibril-building collagen types are I, II, and III, whose molecules consist of an uninterrupted triple helix of approximately 300 nm in length and 1.5 nm in diameter. Fibrils are formed by self-assembly from collagen molecules and demonstrate a cross-striated, *D*-periodic banding pattern, where $D = 67$ nm.[11] Collagen type I is the most abundant type in mammals, and is the type present in mineralized bone, but during bone development and repair, collagen types II and III are also expressed.[12,13]

The strategy of mimicking bone ECM can be taken a step further by incorporating small leucine-rich proteoglycans (SLRPs), which are found associated with collagen fibrils in vivo and are believed to regulate matrix assembly in several tissues.[14] SLRPs are proteoglycans (PGs), consisting of one or more glycosaminoglycan (GAG) chains attached to a protein core. These linear chains consist of repeating units of anionic, acidic sugars. Two members of the SLRP family which are found in bone, as well as a variety of tissues, are decorin and biglycan.[15,16] These belong to class I of the SLRPs and contain 10 leucine-rich repeats flanked by cysteine-rich regions. At the N-terminal domain, decorin is substituted with one GAG chain, biglycan with two.[14] These chains consist of either chondroitin sulfate (CS) or dermatan sulfate (DS).

Surfaces coated with collagen fibrils containing decorin showed significantly accelerated and enhanced formation of focal adhesions by osteoblasts compared to those without decorin.[17] In addition, studies have reported that decorin and biglycan are able to bind to and modulate the activity of the growth factors TGF-β1, which stimulates osteoblast proliferation and causes osteoblast chemotaxis, and BMP-4, which enhances osteoblast differentiation.[18−23] Therefore, as well as influencing cell behavior directly, these SLRPs could potentially function as growth factor reservoirs or modulators.

The aim of this study was to compare the collagen types I, II and III with regard to their ability to bind the SLRPs decorin and biglycan. To our best knowledge, no-one has yet attempted to quantify the amounts of SLRPs bound to fibrils of different collagen types as a result of fibrillogenesis in vitro. Bound SLRPs were quantified using two different colormetric assays which yielded similar results. The influence of SLRPs on the proportion of collagen molecules incorporated into fibrils during fibrillogenesis was also investigated. In addition, the changes in fibril morphology and kinetics of fibrillogenesis brought about by the addition of SLRPs were studied using atomic force microscopy (AFM) and turbidity measurements, respectively.

Materials and Methods

Materials. All chemicals, including the pepsin-treated collagen types I (bovine skin), II (bovine tracheal cartilage), and III (human placenta),

* To whom correspondence should be addressed. E-mail: Timothy.Douglas@mailbox.tu-dresden.de.

10.1021/bm0603746 CCC: $33.50
Published on Web 07/01/2006

as well as decorin (bovine articular cartilage, molecular weight (MW) approximately 100 kD, of which ca. 40 kD is core protein and ca. 60 kD GAG chain) and biglycan (bovine articular cartilage, MW 200−350 kD, of which ca. 45 kD is core protein and 155−295 kD GAG chains), were obtained from Sigma-Aldrich Chemie GmbH, Germany. All chemical reagents used were reagent grade and all solutions were prepared with high purity deionized water. Spectroscopic measurements were made using a Tecan Spectrafluor Plus spectrometer. Turbidity measurements were conducted using a Perkin-Elmer UV−vis spectrometer.

Preparation of Fibrils. Fibrils from collagen types I, II, and III were prepared according to the method of Williams.[24] Briefly, collagen was dissolved at 1 mg/mL in 10 mM acetic acid overnight at 4 °C. Aliquots were mixed on ice with equal volumes of double concentrated fibrillogenesis buffer (50 mM sodium dihydrogenphosphate and 10 mM potassium dihydrogenphosphate at pH 7.4 to yield 60 mM phosphate in total, with or without 270 mM NaCl) in 1.5 mL microcentrifuge tubes (Brandt, Germany). SLRPs were added to the reaction solution before the start of fibrillogenesis. Fibril formation took place at 37 °C overnight. Fibrils were subsequently separated by centrifugation for 15 min at 10 000*g*. Supernatant and pellet were retained for analysis.

Protein Determination in Supernatant and Pellet. Collagen concentration in supernatants was determined using a protocol based on the method of Lowry.[25] 200 μL supernatant was mixed with 1 mL of a freshly prepared solution formed by diluting 2% (w/w) Na_2CO_3 in 0.1 M NaOH with 0.5% $CuSO_4 \cdot 5\ H_2O$ (w/w) in 1% (w/w) $Na_3C_6H_5O_7 \cdot 2\ H_2O$ by a factor of 50. After 15 min of mixing, 100 μL of Folin and Ciocalteu's Phenol reagent was added and the mixture vigorously shaken. After subsequent incubation in the dark for 90 min, absorbance was measured at 700 nm. A calibration curve derived from solutions of the respective collagen type in 10 mM acetic acid ranging between 0 μg/mL and 500 μg/mL subjected to the same procedure enabled determination of collagen concentration. All experiments were performed in triplicate.

The mass of collagen in the pellet was calculated by subtracting the mass of collagen detected in the supernatant from the initial mass of collagen before fibrillogenesis.

SLRP Determination by Dimethylmethylene Blue (DMMB) Assay. SLRPs were quantified using a protocol based on the method of Chou.[26] Briefly, pellets in microcentrifuge tubes were resuspended in 500 μL of a 0.1 mg/mL Papain solution in Hank's balanced salt solution (HBSS) using pulses from an ultrasound horn (UP 100H, Dr. Hieschler GmbH, Germany) at cycle = 1 and 100% amplitude for 3 s. Thereafter digestion took place at 60 °C for 24 h. After digestion, 40 μL of the solution was transferred to a 96-well Nunc microplate and reacted with 250 μL of 1,9-dimethymethylene blue (DMMB) solution composed of 21 mg of DMMB, 5 mL of absolute ethanol, and 2 mg of sodium formate per 1 L with the pH adjusted to 1.5 using 6 M HCl. The amount of SLRPs was determined by measuring the absorbance at 590 nm and comparing it to a calibration curve consisting of SLRP solutions in HBSS with concentrations ranging from 0 to 100 μg/mL. All experiments were performed in triplicate.

SLRP Determination by Hexosamine Assay. SLRPs were quantified using a protocol based on the Swann-Elson hexosamine assay as applied by Yannas.[27] In brief, pellets in microcentrifuge tubes were resuspended in 500 μL of 6 M HCl and hydrolyzed at 105 °C for 6 h. HCl was removed by drying over NaOH pellets under vacuum. The residue was dissolved in 1.25 mL of dest. aqua. A total of 1 mL of this solution was mixed with 1 mL of a solution A (1.25 M Na_2CO_3, 390 mM acetyl acetone) and incubated at 95 °C for 1 h. After cooling, 5 mL of ethanol was added, followed by 1 mL of a solution B (178 mM Ehrlich's Reagent (*p*-dimethylaminobenzaldehyde), 8.46 M ethanol (50% pure ethanol by volume), 3 M HCl). The resulting mixture was left to stand for 1 h and absorbance was measured at 540 nm. Calibration samples consisting of SLRP masses ranging from 0 to 100 μg were subjected to the same treatment. All experiments were performed in triplicate.

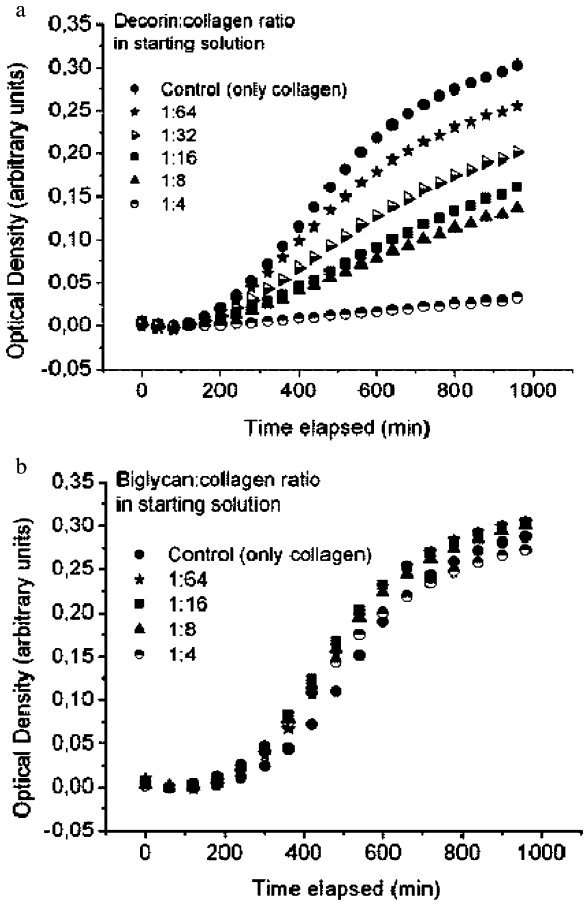

Figure 1. Measurement of turbidity changes during fibrillogensis of collagen type I in 30 mM phosphate buffer with 135 mM NaCl at different SLRP:collagen w/w ratios in the starting solution; (a) with decorin; (b) with biglycan.

Examination of Morphology by AFM. Collagen coatings on titanium-sputtered glass disks were prepared by resuspending pellets after centrifugation in 300 μL of fibrillogenesis buffer using an ultrasound horn as described above. The collagen fibril suspension was transferred dropwise onto smooth titanium-sputtered glass disks suitable for AFM under a laminar flow hood. After 30 min, the disks were rinsed three times with distilled water and air-dried. Fibril morphology was assessed by atomic force microscopy using a Bioscope instrument (Digital Instruments/Veeco) in tapping mode and aluminum reflex coated silicon tips. All images were obtained at a scan rate of 1.2 Hz, scanning 512 lines per image. Both height and amplitude images were captured simultaneously. For the AFM examination, fibrils were formed at SLRP:collagen ratios in the starting solution of 1:7 (w/w).

Investigation of Kinetics of Fibrillogenesis by Turbidity Measurements. Collagen was dissolved at 1 mg/mL in 10 mM acetic acid overnight at 4 °C. Aliquots were mixed on ice with equal volumes of double concentrated fibrillogenesis buffer (50 mM sodium dihydrogenphosphate, 10 mM potassium dihydrogenphosphate and 270 mM NaCl at pH 7.4 to yield 60 mM phosphate with 135 mM NaCl) in 1.0 mL quartz cuvettes (Hellma, Germany). SLRPs were added to the reaction solution before the start of fibrillogenesis. Fibril formation took place at 37 °C over a period of 1000 min. Optical density was measured at 313 nm every 30 s.

Results

Turbidity Measurements. Turbidity changes during fibrillogensis of collagen type I in 30 mM phosphate buffer with 135 mM NaCl at different SLRP:collagen w/w ratios in the starting solution can be seen in Figure 1. The final optical density

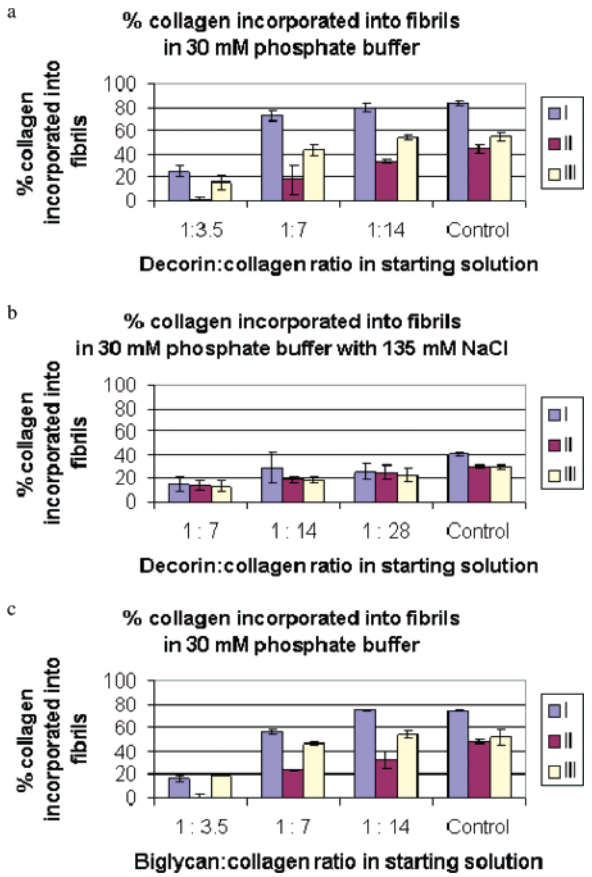

Figure 2. Effect of the small leucine-rich proteoglycans (SLRPs) decorin and biglycan on incorporation of collagen types I, II, and III into fibrils during fibrillogenesis at different SLRP:collagen w/w ratios in the starting solution; (a) with decorin in 30 mM phosphate buffer; (b) with decorin in 30 mM phosphate buffer with 135 mM NaCl; (c) with biglycan in 30 mM phosphate buffer.

decreased with increasing decorin:collagen ratio (Figure 1a). At a decorin:collagen ratio of 1:8, the opitical density had fallen to half the value for the control sample. At a ratio of 1:4, there was almost no increase in optical density due to fibrillogenesis. In contrast, increasing the biglycan:collagen ratio did not lead to an appreciable decrease in the final optical density nor did it significantly reduce the speed of fibrillogenesis (Figure 1b). It is possible that with increasing decorin concentration the time required to reach maximum optical density is increased. However, this is uncertain since it is unclear whether control samples had reached their maximum optical density after 1000 min. Biglycan concentration did not affect the speed of fibrillogenesis, even at a biglycan:collagen ratio of 1:4.

Influence of SLRPs on Proportion of Collagen Incorporated into Fibrils. The effect of SLRPs on the incorporation of collagen molecules of types I, II, and III into fibrils during fibrillogenesis at different SLRP:collagen w/w ratios is shown in Figure 2. All experiments were performed in triplicate; error bars show standard deviation. For all three collagen types, the proportion of collagen incorporated into fibrils decreased with increasing SLRP:collagen ratios in the starting solution, both with and without 135 mM NaCl. By increasing the ratio of SLRP to collagen to 1:3.5 (w/w) in 30 mM phosphate buffer without NaCl, the proportion of type I incorporated into fibrils from solution decreased from approximately 80% to 35% and 75% to 20% for decorin and biglycan, respectively, whereas the proportion of type III incorporated sank from roughly 50% to 20% for both decorin and biglycan. The proportion of type

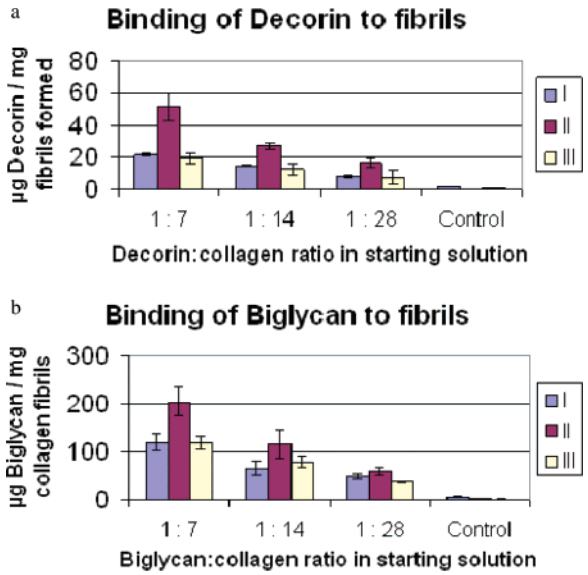

Figure 3. Quantification of SLRPs bound per mg collagen fibrils of collagen types I, II, and III during fibrillogenesis in 30 mM phosphate buffer at different SLRP:collagen w/w ratios in the starting solution by dimethylmethylene blue (DMMB) assay according to the method of Chou;[26] (a) with decorin; (b) with biglycan. All experiments were performed in triplicate; error bars show standard deviation.

II incorporated in 30 mM phosphate buffer without NaCl fell from 60% to 0% for both SLRPs.

The addition of 135 mM NaCl to the fibrillogenesis buffer significantly reduced the proportion of collagen incorporated into fibrils for all three types. However, this reduction was less pronounced for collagen II than for types I and III. Additionally, the higher ionic strength of the fibrillogenesis buffer resulted in an almost complete equilibration of the behavior of the three collagens with respect to the amount incorporated into fibrils. However, in contrast to experiments without NaCl, where only a small decrease was observed before a sudden drop from 1:7 to 1:3.5, the decline up to 1:7 was more pronounced at 135 mM NaCl, by more than a factor of 2 for types I and III.

SLRP Binding to Fibrils. The results of the quantification of SLRPs immobilized per mg of collagen fibrils of collagen types I, II, and III during fibrillogenesis at different SLRP:collagen w/w ratios are shown in Figure 3. All experiments were performed in triplicate; error bars show standard deviation. A higher mass of SLRP was bound per mg of collagen fibrils formed by collagen II than collagen I and collagen III at all SLRP:collagen ratios. The relative difference between collagen II and collagens I and III increases with SLRP:collagen ratio and was most pronounced at a ratio of 1:7. In the case of decorin (Figure 3a), collagen II bound approximately twice as much as collagen I and III at a ratio of 1:28, but roughly 2.5 times as much at 1:7. In the case of biglycan (Figure 1b), type II bound only about 1.25 times as much as types I and III at 1:28 and 1.5 times as much at 1:14, but roughly twice as much at 1:7. No difference was seen between collagen I and collagen III. For all three collagen types, more biglycan was bound per mg of collagen fibrils formed than decorin. The difference between amounts of biglycan and decorin bound was more pronounced for collagen I and III than collagen II.

The results obtained by dimethylmethylene blue (DMMB) assay (Figure 4a) correlated well with those obtained by hexosamine assay (Figure 4b). All experiments were performed in triplicate; error bars show standard deviation.

Morphology. The morphology of collagen fibrils of collagen types I, II, and III in the absence and presence of SLRPs

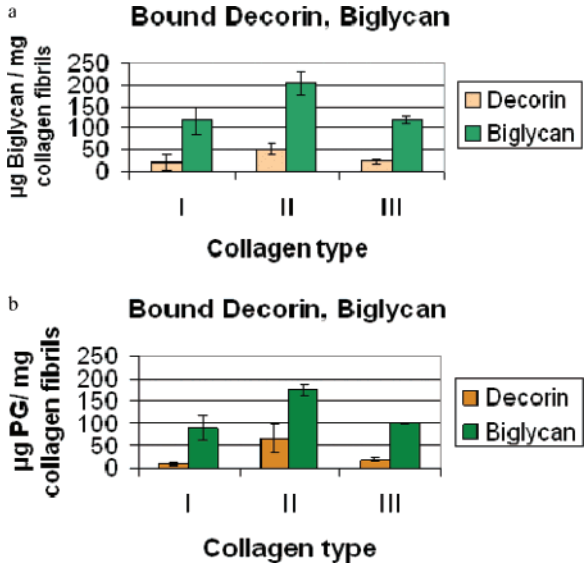

Figure 4. Comparison of quantification of SLRPs bound per mg collagen fibrils of collagen types I, II, and III during fibrillogenesis in 30 mM phosphate buffer by (a) dimethylmethylene blue (DMMB) assay according to the method of Chou;[26] (b) hexosamine assay according to the method of Yannas.[27] SLRP:collagen w/w ratio in the starting solution = 1:7. All experiments were performed in triplicate; error bars show standard deviation.

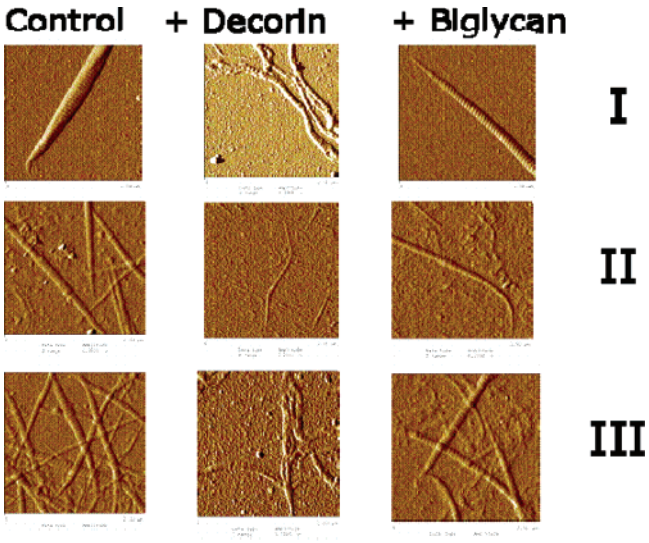

Figure 5. Morphological examination of fibrils of collagen types I, II, and III formed in 30 mM phosphate in the absence and presence of SLRPs (SLRP:collagen ratio 1:7) by atomic force microscopy (AFM). Columns from left to right: fibrils without SLRP; with decorin; with biglycan. Rows from top to bottom: collagen type I; type II; type III. SLRP:collagen w/w ratio in the starting solution = 1:7. Area shown = 2.5 μm × 2.5 μm except for collagen type III with decorin (area shown = 2.0 μm × 2.0 μm).

obtained by AFM are shown in Figure 5. The images in Figure 5 are representative for the samples. In the absence of SLRPs, fibrils of collagen types II and III, which were approximately 170 nm in width, were shorter and markedly thinner than those of type I, which showed thicknesses of approximately 250 nm. Collagen II fibrils did not appear thinner than those of collagen III in control samples, but type II fibrils appeared to be marginally thinner than type III fibrils in samples containing decorin (approximately 90 nm for collagen II in comparison to 140 nm for collagen III). Furthermore, collagen II fibrils were shorter and displayed more pointed, tapered tips. Type I fibrils containing decorin were thinner than those without SLRPs, with widths of approximately 150 nm. Addition of decorin caused fibrils of all three collagen types to become thinner. In contrast, no appreciable change in fibril diameter was observed after fibrillogenesis with biglycan. Banding was conserved after addition of SLRPs.

Discussion

The aim of this study was to characterize fibrils of collagen types I, II, and III containing SLRPs with regard to the amount of SLRPs bound, the effect of SLRPs on the fibril morphology, and formation kinetics as well as the proportion of collagen incorporated into fibrils.

A. SLRP Binding to Fibrils. A1. Collagen Types I−III Bind More Biglycan than Decorin. Considerably more biglycan than decorin was bound by all three collagen types at all SLRP:collagen ratios (Figure 3). To explain these differences, it is necessary to consider the nature of SLRP:collagen interactions, which are believed to be mediated both by the core protein and the GAG chains.

Interactions between core protein and collagen have been demonstrated by immunostaining of the core protein on fibrils[28,29] and binding of biotinilated decorin core and recombinant unglycosylated decorin as well as radioactively labelled recombinant biglycan to collagen fragments and fibrils, respectively.[29,30] A total of 55% of the amino acids in the protein cores of decorin and biglycan are identical,[31] and it has been suggested that biglycan and decorin core proteins compete for identical or adjacent binding sites on collagen I fibrils.[29] Assuming that the same number of sites are available for core-mediated biglycan and decorin binding and that equal molar amounts of decorin and biglycan do indeed bind to fibrils via the protein core, the higher mass of biglycan bound may be at least partly explained by biglycan's higher molecular weight (200−350 kD compared to 100 kD for decorin) due to the presence of two, longer GAG chains. As more than approximately 3.5 times more biglycan than decorin was bound by type II and at least 3.5 times more by I and III at SRLP:collagen ratios of 1:/, (Figure 4) the difference in molecular weight alone does not explain the differences, assuming equal molar core-mediated binding. Furthermore, the dissociation constant for biglycan from collagen I fibrils in phosphate-buffered saline (PBS) is 2 orders of magnitude higher than that of decorin,[29] which suggests that biglycan binds more weakly via the core protein than decorin. Therefore, possible differences in GAG chain-mediated binding between biglycan and decorin should be considered.

Interactions of GAG chains of biglycan and decorin with collagen have been demonstrated by the fact that precipitation of collagen by a CS-substituted SLRP was less effective after removal of the GAG chains.[32] Furthermore, a significant reduction in decorin binding after GAG chain removal and inhibition of decorin and biglycan binding by sulfate and CS as well as an increase in phosphate concentration, presumably by competition with the GAG chains of these PGs for binding sites on collagen, have been observed.[33] GAGs are believed to bind to collagen ionically; Öbrink[34,35] reported that the binding of CS to collagen molecules was highly dependent on ionic strength and that DS and CS binding to collagen was abolished with increasing ionic strength. Binding of radioactively labeled biglycan was completely inhibited in 30 mM phosphate and 140 mM NaCl, and in view of the fact that decorin and biglycan are substituted by GAG chains consisting of CS or DS, it was suggested that biglycan binds to collagen I primarily through its GAG chain.[33]

Collagen I has been reported to have different affinities for different GAGs, with DS having a higher affinity than CS.[36] In

a study by Öbrink, roughly twice as many moles of DS as CS were bound per mole of collagen despite the GAGs having similar molecular weights.[35,37] Although the decorin and biglycan used in this study come from the same tissue, bovine articular cartilage, it is possible that the composition of the GAG chains differs. In cartilage, a mixture of DS- and CS-substituted forms are present.[38]

Therefore, it could be speculated that GAG chains of decorin and biglycan with differing proportions of CS and DS would demonstrate different affinities for different collagen types.

Subtraction of the MW of decorin's core protein (40 kD) from the total MW (100 kD) yields an average GAG chain length of approximately 60 kD. For biglycan, the core protein MW is 45 kD and the total MW 200−350 kD, giving a MW of the two chains combined of 155−305 kD, or 77.5−152.5 kD per chain, assuming that both chains have equal lengths. This exceeds the GAG chain MW of decorin. MacPherson suggested that GAGs might "bridge" basic regions in neighboring collagen fibrils and thus exert a stabilizing effect and that a higher GAG chain length would result in a more stable interaction.[39] Öbrink examined binding of DS and CS preparations with differing molecular weights to collagen molecules and found that more of the high molecular weight DS and CS was bound.[35,37] Hence, GAG-mediated binding for biglycan may be higher thanks to its longer chains.

A2. Collagen Type II Binds More Biglycan and Decorin than Types I and III. More SLRPs were bound by collagen II than collagen I and III (Figures 3 and 4). Fibrils of collagen II were markedly thinner than those of collagen I (Figure 5). When the collagens are in fibrillar form, the thinner quaternary structure of collagen II would result in a higher surface area than collagen I and might allow increased PG and GAG binding, as suggested by Thalhammer.[40] This by itself does not explain why collagen III binds similar amounts of SLRP to collagen I and not collagen II, although the fibrils of type II are not significantly thicker than those of type III. It may be that collagens I, II, and III have different inherent affinities for GAGs. GAG has resisted removal from collagen-II-rich bovine tracheal cartilage by washing with high ionic strength solution, and collagen II precipitated from solution by addition of CS bound more than collagen I.[41,42] Since collagen II is found in tissues with a high PG or GAG content, such as cartilage, the ability to bind a larger amount of SLRP might be physiologically useful. This is supported by the observation that collagen types I, II, and III tend to be associated with different GAGs in vivo.[43] Therefore, it could be postulated that type II has an inherently higher affinity for GAG than types I and III, which would mean increased GAG-mediated binding of SLRPs.

B. Influence of SLRPs on Proportion of Collagen Incorporated into Fibrils, Fibril Morphology, and Fibril Formation Kinetics. Both decorin and biglycan decreased the proportion of collagen incorporated into fibrils (Figure 2).

Decorin plays a role in the lateral growth of collagen fibrils and is believed to regulate fibril diameter in vitro.[44] Due to the structural homology of decorin and biglycan, a similar role for biglycan could be envisaged. By binding to collagen fibrils, SLRPs may limit lateral growth by sterically hindering the approach of further collagen molecules through the GAG chains if bound via the core protein, or through the protein if bound via the GAG chains. In addition, the anionic GAG chains on SLRPs bound to different fibrils may repel each other. Both of these would hinder fibril growth. It would be expected that both steric hindrance and anionic repulsion would be greater in the case of biglycan as it has twice as many and longer GAG chains than decorin. Since no difference can be observed in the amounts of collagen incorporated at equal decorin and biglycan ratios, biglycan does not seem better at hindering fibril formation despite the higher chain length and presence of a second chain. Either the structural differences in biglycan are irrelevant or biglycan binds differently to fibrils, perhaps to a greater extent via the GAG chains, which would affect steric hindrance and anionic repulsion. Formation of collagen II fibrils at 30 mM phosphate without NaCl is more strongly hampered at all SLRP:collagen ratios, but especially at lower ratios (1:7 and 1:14) (Figure 2a,c). This increased hindrance may be due to the higher mass of decorin and biglycan bound per mg of collagen II fibrils formed relative to types I and III. It could be postulated that the hindering effect of SLRPs is less significant at 135 mM NaCl due to the higher ionic strength. This is supported by the absence of a sudden drop from 1:7 to 1:3.5 for collagen I and III at 135 mM NaCl (Figure 2b). The amount of decorin bound by collagen II at 1:7 with 135 mM NaCl did not significantly differ from that bound without NaCl. Perhaps increased steric hindrance and ionic repulsion caused by bound SLRPs at 30 mM without NaCl can be compensated by ionic repulsion caused by the presence of 135 mM NaCl.

Decorin caused a significant thinning of fibrils of types I, II, and III (Figure 5).

It has been shown that decorin reduces fibril diameter.[45] However, biglycan did not lead to an appreciable reduction of fibril diameter.

The differing effects of decorin and biglycan on fibril morphology are reflected in the results of the turbidity study (Figure 1). The decrease in the optical density with increasing decorin:collagen ratios can be ascribed to two possible influences: decreasing fibril diameter and decreasing proportion of collagen incorporated into fibrils. As Figure 2b shows, incorporation of collagen I into fibrils in the presence of 135 mM NaCl at a decorin:collagen ratio of 1:7 is approximately 50% of that in the absence of decorin. The 50% drop in the amount of fibrils would explain the 50% drop in optical density after 1000 min at decorin:collagen = 1:8; however, the final optical densities at 1:16 and 1:32 are similar to those at 1:8 (Figure 1a). This suggests that not only is the amount of fibrils lower but also that the fibril diameter has decreased. In contrast, biglycan did not cause the total optical density to decrease, even at a biglycan:collagen ration of 1:4 (Figure 1b), suggesting that the amount of fibrils remains constant up to 1:4. If the effects of decorin and biglycan on the proportion of collagen incorporated into fibrils at 30 mM phosphate with 135 mM NaCl were to resemble those at 30 mM phosphate without NaCl (Figure 2a,c), a similar 50% drop in the amount of fibrils at biglycan:collagen = 1:7 would be expected.

One possible explanation for the differences between decorin and biglycan may be that decorin binds to fibrils during fibril formation, hindering lateral growth. This would lead to a reduced fibril diameter and lower optical density. However, biglycan may bind to fibrils more slowly, after formation is already complete. In this case, fibrillogenesis would not be decelerated and fibril diameter would not be reduced, leading to a higher optical density than for decorin. It could be speculated that biglycan would bind differently due to a weaker association between core protein and collagen and stronger GAG chain-mediated binding. This would be supported by studies showing that deglycosylated core protein seems to be responsible for retardation of fibrillogenesis.[46,47] An alternative explanation is that biglycan binding has been prevented by the presence of 135 mM NaCl; under similar fibrillogenesis conditions, namely at 30 mM phosphate and 140 mM NaCl, biglycan binding was reported to have been completely inhibited.[33] Yet another explanation may be that the decorin and biglycan used were substituted with GAG chains of differing compositions. Dif-

fering influences of DS- and CS-substituted SLRPs have already been reported, with decorin and biglycan with DS chains delaying lateral fibril growth and CS-substituted forms accelerating it.[48,49]

The reduction in fibril diameter caused by decorin would also result in a higher surface area, which would be expected to encourage decorin binding relative to biglycan. The fact that significantly more biglycan is bound than decorin at 30 mM phosphate despite the higher surface area of fibrils containing decorin suggests that fibril surface area exerts less of an influence than other factors described above, namely GAG chain length and GAG chain composition.

The composition of PGs from cartilage depends on donor age and the joint and layer of cartilage they are obtained from.[50] Therefore, further investigation is necessary to determine whether collagen and SLRPs from other sources yield similar results, as collagen-SLRP interactions may be specific for tissue of origin.[47,48]

Summary

More biglycan than decorin is bound in fibrils of collagen I, II, and III as a result of fibrillogenesis, and collagen II binds more decorin and biglycan than collagen I and III in 30 mM phosphate buffer at low ionic strength without NaCl. Both decorin and biglycan reduce the proportion of collagen of all three collagen types incorporated into fibrils during fibrillogenesis to a comparable extent in 30 mM phosphate, and an increased ionic strength due to the presence of 135 mM NaCl leads to a reduction in the proportion of collagen incorporated into fibrils. Banding of fibrils is preserved in the presence of SLRPs. Decorin reduces the fibril diameter of all three collagen types, but no change in fibril thickness was detected after addition of biglycan.

Acknowledgment. The authors thank the German Ministry for Education and Research (BMBF) for financial support and Dr. Thomas Hanke for help and discussions.

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BM0603746