

Molecular Interactions of Biglycan and Decorin with Elastic Fiber Components

BIGLYCAN FORMS A TERNARY COMPLEX WITH TROPOELASTIN AND MICROFIBRIL-ASSOCIATED GLYCOPROTEIN 1*

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The interactions of the dermatan sulfate proteoglycans biglycan and decorin have been investigated with the elastic fiber components, tropoelastin, fibrillin-containing microfibrils, and microfibril-associated glycoproteins (MAGP) 1 and 2. Both proteoglycans were found to bind tropoelastin and fibrillin-containing microfibrils but not MAGPs 1 and 2 in solid phase binding assays. The specificity of the binding of biglycan and decorin to tropoelastin was confirmed by co-immunoprecipitation experiments and by the blocking of the interactions with elastin-derived peptides. Isolated core proteins from biglycan and decorin bound to tropoelastin more strongly than the intact proteoglycans, and there were no differences in the tropoelastin binding characteristics of distinct glucuronate-rich and iduronate-rich glycoforms of biglycan. These findings indicated that the binding sites were contained in the protein cores of the proteoglycans rather than the glycosaminoglycan side chains. Scatchard analysis showed that biglycan bound more avidly than decorin to tropoelastin with K_d values estimated as 1.95×10^{-7} M and 5.3×10^{-7} M, respectively. In blocking experiments each proteoglycan showed extensive inhibition of binding of the other to tropoelastin but was most effective at blocking its own binding. This result suggested that biglycan and decorin had closely spaced but distinct binding sites on tropoelastin. Addition of the elastin-binding protein MAGP-1 to the assays enhanced the binding of biglycan to tropoelastin but had no effect on the decorin-tropoelastin interaction. Co-immunoprecipitation experiments showed that MAGP-1 interacted with biglycan but not decorin in the solution phase. The results indicated that biglycan specifically formed a ternary complex with tropoelastin and MAGP-1. Overall the study supports the concept that biglycan may have a specific role in the elastinogenic phase of elastic fiber formation.

The small dermatan sulfate proteoglycans biglycan and decorin are constituents of extracellular matrices in a wide range of tissues (1, 2). Structurally, these two proteoglycans represent the members of the class 1 subgroup of the small leucine-rich proteoglycan gene family. They exhibit the characteristic structure of a core protein with 10 leucine-rich repeat

domains flanked by cysteine-rich regions and the presence of one (decorin) or two (biglycan) chondroitin/dermatan sulfate side chains, of variable composition, attached to their N-terminal domains (2–4). Biglycan and decorin have distinct but overlapping temporal and spatial expression patterns and have individual roles in the regulation of matrix assembly, control of cell proliferation, and modulation of the activity of transforming growth factor β (2–4). Decorin has been shown to bind fibrillar collagen, resulting in the stabilization and correct organization of the fibrils during fibrillogenesis (5). Decorin null mice have abnormally fragile skin caused by the presence of irregular and loosely packed collagen fibers in this tissue (6). Biglycan has also been identified with developing collagen fibers, suggesting that it is also involved in collagen fiber assembly (7). Biglycan null mice have an osteoporosis-like phenotype, suggesting that the proteoglycan has a distinct function acting as a positive regulator of bone formation (8). Decorin has been demonstrated to retard the growth of a variety of cell types including many tumor cells via interaction with the epidermal growth factor receptor, whereas biglycan may be involved in regulation of hemopoiesis. Both proteoglycans have been shown to bind transforming growth factor β , and decorin has been demonstrated to neutralize the activity of this growth factor (2–4). Both decorin and biglycan also bind to the microfibrillar collagen VI within the N-terminal region of the triple helix (9).

Recent evidence suggests that biglycan and decorin may also be involved in elastic fiber biology. Immunohistochemical studies on human skin have suggested that decorin may be associated with fibrillin-containing, elastin-associated microfibrils, whereas biglycan may be associated with the elastin core of the elastic fibers (10, 11). Kielty *et al.* (12) have shown that chondroitinase AC treatment disrupts the bead component of fibrillin-containing microfibrils and that a chondroitinase AC-sensitive proteoglycan can be co-precipitated with fibrillin from smooth muscle cell conditioned medium. Most recently, Trask *et al.* (13) have presented evidence that decorin can be immunoprecipitated from chondrocyte-conditioned medium as a ternary complex with microfibrillar components, fibrillin-1 and MAGP-1.¹

Studies in our laboratory have shown distinct temporal expression patterns for decorin and glycoforms of biglycan during development of the highly elastic nuchal ligament in fetal bovines. Decorin expression was found to peak early in this tissue. However, the expression of a specific glycoform of biglycan correlated with the elastinogenic phase of elastic fiber formation, suggesting a role for biglycan in this stage of the process

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¹ The abbreviations used are: MAGP, microfibril-associated glycoprotein; BSA, bovine serum albumin; TBS, Tris-buffered saline.

(14). In the present study we have investigated the interaction of decorin and biglycan glycoforms with the elastin precursor tropoelastin, MAGPs, and isolated fibrillin-containing, elastin-associated microfibrils. The results show that both proteoglycans interact with tropoelastin and microfibrils and that biglycan forms a ternary complex with tropoelastin and MAGP-1. This is consistent with a specific role for biglycan in elastic fiber biology.

EXPERIMENTAL PROCEDURES

Materials—Specific polyclonal rabbit antibodies to bovine tropoelastin and MAGP-1 have been described previously (15). Biglycan, decorin, tropoelastin, and MAGPs 1 and 2 were purified from the nuchal ligament of 230-day-old fetal calves as described previously (14, 16). Bovine pepsin-treated type VI collagen was prepared as described elsewhere (17). Soluble elastin-derived peptides ($\alpha\beta$ elastin) were prepared from bovine aorta elastin as described by Cleary and Cliff (18). Chondroitinase ABC and hyaluronidase from streptomyces hyalurolyticus were purchased from Seikagaku Corp. (Tokyo, Japan).

Fibrillin-containing microfibrils were prepared using a method based on that of Kielty *et al.* (19). Briefly, frozen nuchal ligament tissue from a 200-day-old fetus (10 g) was crushed in liquid nitrogen and washed (2×50 ml) with ice-cold Tris buffer, pH 7.4, containing 0.4 M NaCl, together with protease inhibitors, ϵ -amino caproic acid (10 mM), EDTA (2 mM), *N*-ethylmaleimide (2 mM), and phenylmethylsulfonyl fluoride (1 mM), and then once with the same buffer containing CaCl_2 (10 mM) and lacking EDTA (50 mM). The residue was resuspended in calcium-supplemented buffer (40 ml) containing 2500 units of highly purified bacterial collagenase (Sigma type VII) and incubated for 2 h at 37 °C followed by 18 h at 4 °C. The digest was treated with excess EDTA and centrifuged. The supernatant was subjected to gel filtration on a column (2.5×98 cm) of Sepharose CL-2B equilibrated in Tris/NaCl buffer at a flow rate of 20 ml/h. The void volume peak (V_0) was pooled, adjusted to 10 mM of CaCl_2 , and incubated with 1 unit of streptomyces hyaluronidase for 18 h at 4 °C. The V_0 sample was adjusted to 1.35 g/ml by the addition of CsCl and subjected to density gradient centrifugation in a 70.1 Ti head (Beckman) at 40,000 rpm ($110,000 \times g$) for 72 h. The gradient was divided into 14 fractions, and fibrillin-containing microfibrils were detected in the middle of the gradient (density 1.37–1.40 g/ml) by dot blotting with anti-fibrillin-1 antibodies. The fractions containing microfibrils were pooled and dialyzed into Tris-buffered saline. Type VI collagen was detected in fractions of lower density (approximately 1.30 g/ml).

For the binding experiments, biglycan and decorin were radiolabeled with Na^{125}I (Amersham Biosciences, Inc.) using Iodo beads (Pierce) as described previously (16). Briefly, each proteoglycan (100 μg) was reacted with a washed Iodo bead and 1 mCi of ^{125}I for 5 min in 50 mM Tris buffer, pH 7.4, containing 0.3 M guanidinium chloride. Free radiolabel was removed by gel filtration through Sephadex G-10. The specific activities of decorin and biglycan were both 5.0×10^6 dpm/ μg of core protein unless stated otherwise. Core proteins of biglycan and decorin were prepared by digestion of the ^{125}I -labeled proteoglycans with chondroitinase ABC as described by Reinboth *et al.* (14).

Binding Assays—Solid phase binding assays were performed on microtiter plates (Nunc Immunosorb modules) using a method described previously (16), with ^{125}I -labeled proteoglycan in the liquid phase and test proteins coated on the wells. Incubations were for 3 h at 37 °C, and binding of ^{125}I -labeled proteoglycan to each well was directly measured by a Wallac 1261 Multi- γ counter. Dissociation constants were measured using the method of Bidanset *et al.* (20).

Co-immunoprecipitation assays were performed as follows. The test protein (2 μg of tropoelastin or 1 μg of MAGP-1) was incubated with 5×10^5 dpm of ^{125}I -labeled biglycan or decorin for 4 h at 37 °C in 50 μl of TBS (10 mM Tris buffer, pH 7.4 containing 0.15 M NaCl and protease inhibitors, 2 mM EDTA, 1 mM benzamide, 1 mM ϵ -amino-*n*-caproic acid, and 0.5 mM phenylmethylsulfonyl fluoride) containing 2 μg of BSA. Control incubations contained no test protein. A specific rabbit polyclonal antiserum (10 μl) raised to the test protein was then added, and the incubation was continued for 18 h at 4 °C with gentle shaking. To ensure full recovery of the immunoprecipitate, protein A-Sepharose (30 μl) was added, and incubation was continued for 1 h at room temperature with gentle shaking. The immunoprecipitate-protein A-Sepharose complex was recovered by centrifugation ($3000 \times g$ for 10 min) and resuspended in 100 μl of TBS containing 0.05% Tween 20. The complex was washed by centrifugation three times through 200 μl of 1 M sucrose in TBS/Tween. The bound ^{125}I proteoglycan was eluted from

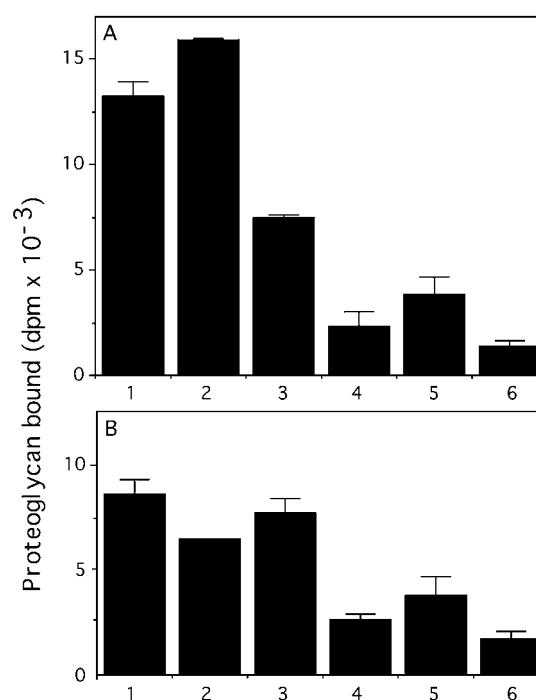


FIG. 1. Solid phase binding of biglycan and decorin to elastic fiber components. Purified elastic fiber components, tropoelastin, MAGP-1, and MAGP-2 (each 100 ng/well) and fibrillin-containing microfibrils (1 μg /well) were coated onto microtiter plates. Pepsin-treated type VI collagen (800 ng/well) and BSA (100 ng/well) were also coated onto wells as positive and negative controls, respectively. After blocking, 2.3×10^5 dpm of ^{125}I -labeled biglycan (A) or decorin (B) (each of specific activity $5 \times 10^6/\mu\text{g}$) was added, and the wells were incubated at 37 °C for 3 h. After washing, binding was measured by direct γ counting. Columns 1, pepsin VI collagen; columns 2, tropoelastin; columns 3, fibrillin-containing microfibrils; columns 4, MAGP-1; columns 5, MAGP-2; columns 6, BSA. The means \pm S.D. of quadruplicate determinations are shown.

the complex by resuspension in nonreduced electrophoresis buffer (30 μl) and heating to 100 °C for 2 min. Each sample was centrifuged to remove the Sepharose beads, and then β -mercaptoethanol was added to a final concentration of 2%. The samples were reheated for 2 min to reduce disulfide bonds and then analyzed for their ^{125}I -labeled proteoglycan content by γ counting and SDS-PAGE/autoradiography as described previously (15, 16). Each co-immunoprecipitation was performed in quadruplicate.

RESULTS

Binding of Biglycan and Decorin to Elastic Fiber Components—Radiolabeled biglycan and decorin of equivalent specific activities were tested for binding to elastic fiber components using a solid phase assay (Fig. 1). Both proteoglycans showed significant binding in the solid phase to tropoelastin and fibrillin-containing microfibrils but not to MAGPs 1 and 2, compared with the BSA control. Interestingly the binding of decorin was much less extensive than that of biglycan to tropoelastin, suggesting that the latter was the stronger of the two interactions. As a measure of the significance of the interaction, biglycan bound more extensively to tropoelastin than to type VI collagen, previously known to bind biglycan and decorin (20), which was used as a positive control for the binding assay. To confirm that the interactions of biglycan and decorin with tropoelastin were specific, co-immunoprecipitation experiments were performed using radiolabeled proteoglycans and anti-tropoelastin antibodies. As with the solid phase assays, the tropoelastin interaction with biglycan was more extensive than with decorin, with about twice as much biglycan being specifically precipitated as decorin (Fig. 2). SDS-PAGE and autoradiographic analysis of the immunoprecipitates confirmed that the

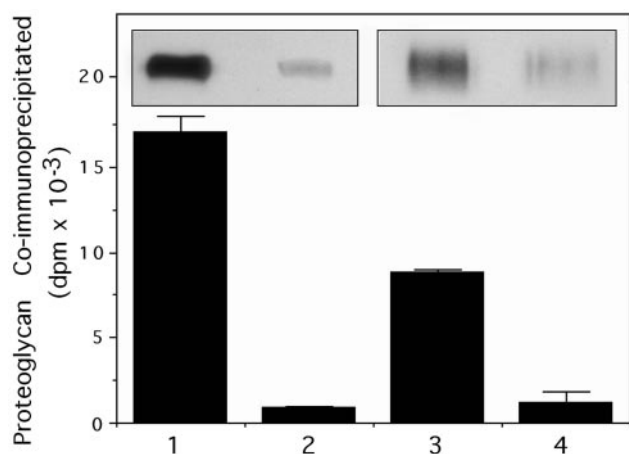


FIG. 2. Biglycan and decorin co-immunoprecipitate with tropoelastin. ^{125}I -Labeled biglycan or decorin (5×10^4 dpm) in TBS (50 μl) was incubated with 2 μg of tropoelastin or BSA control for 4 h at 37 $^\circ\text{C}$. Proteoglycan bound to the tropoelastin was then recovered by the addition of anti-tropoelastin antiserum (10 μl) followed by protein A-Sepharose (30 μl). Co-immunoprecipitated ^{125}I -labeled proteoglycan was measured by direct γ counting (columns 1–4). The means \pm S.D. of quadruplicate immunoprecipitations are shown. Column 1, biglycan with tropoelastin; column 2, biglycan with BSA; column 3, decorin with tropoelastin; column 4, decorin with BSA. SDS-PAGE on 12% gels and autoradiography confirmed that the precipitated counts were associated with the proteoglycan (insets). Left inset, biglycan (about 220 kDa); right inset, decorin (about 120 kDa).

radioactivity was bound to the intact proteoglycans. Biglycan was detected as a diffuse species with an average apparent molecular mass of approximately 220 kDa, whereas decorin was evident as a diffuse 120-kDa band (Fig. 2, insets).

Biglycan and Decorin Specifically Bind to Tropoelastin via Their Core Proteins—The binding of both proteoglycans to tropoelastin was shown to be proportional to the amount of tropoelastin coated on the wells, relatively insensitive to the presence or absence of calcium ions (Fig. 3, A and B) and saturable (Fig. 3C). Moreover, the binding of both proteoglycans to tropoelastin could be blocked by preincubation of the radiolabeled proteoglycan with an excess of an elastin-derived peptide mixture (Fig. 4). This finding indicates that the binding site(s) for biglycan and decorin are also present in mature elastin as well as its precursor, tropoelastin.

To determine whether the binding of biglycan and decorin to tropoelastin was via their protein cores or via the glycosaminoglycan side chains, the solid phase binding assay was repeated using ^{125}I -labeled core proteins prepared by chondroitinase ABC digestion of the radiolabeled proteoglycans (Fig. 5). The isolated core protein from biglycan showed strong binding to tropoelastin, which was more extensive than the intact proteoglycan of the same specific activity (Fig. 5, columns 1 and 2). This finding indicates that the interaction of biglycan with tropoelastin is via the core protein and that the glycosaminoglycan side chains appear to have an inhibitory effect on the interaction. The inhibitory effect may be due to the repulsion of the highly negatively charged glycosaminoglycan chains limiting the density of biglycan molecules attaching to the tropoelastin substrate. Similarly the decorin core protein also bound more extensively than intact decorin to tropoelastin, indicating that decorin also bound the elastin precursor via the protein core (Fig. 5, columns 3 and 4). Consistent with earlier results, it was evident that the binding of decorin or its isolated core protein to tropoelastin was not as extensive as that of biglycan or its core protein.

We have previously shown that a specific glucuronate-rich glycoform of biglycan is expressed in nuchal ligament during elastinogenesis, which is very distinct from glycoforms from

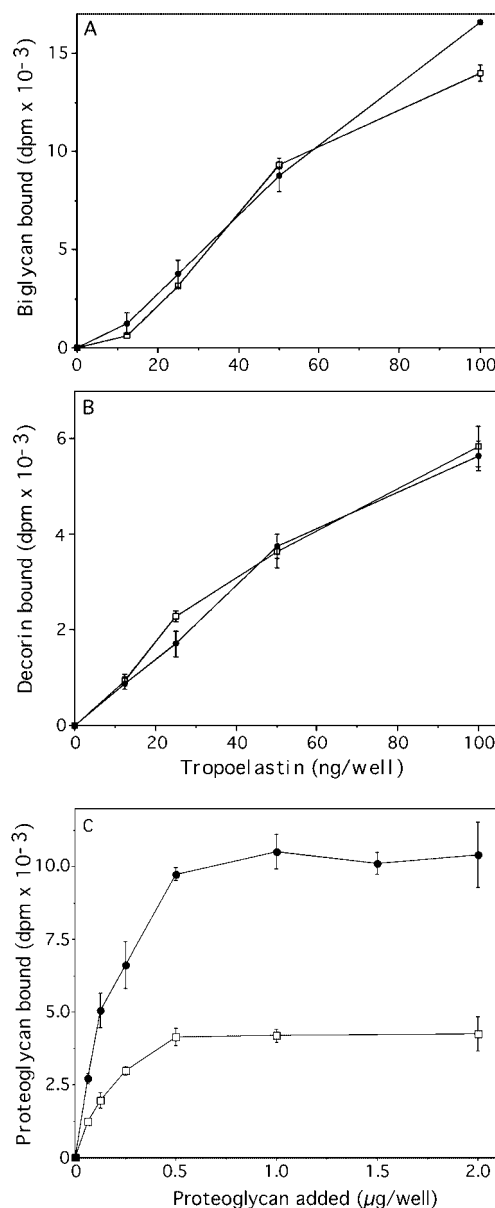


FIG. 3. Biglycan and decorin binding is proportional to amount of tropoelastin, is not calcium-dependent, and is saturable. A and B, proportional, non-calcium-dependent binding. Tropoelastin was coated in serial dilution on rows of wells of a microtiter plate. After blocking, each row was incubated at 37 $^\circ\text{C}$ for 3 h with 2.3×10^5 dpm of ^{125}I -labeled proteoglycan and either biglycan (A) or decorin (B) in the presence (circles) or absence (squares) of 0.9 mM CaCl_2 . After washing, specific binding was measured by direct γ counting. The means \pm S.D. of quadruplicate determinations are shown. C, saturation curves. Tropoelastin or BSA (100 ng/well) was coated onto the wells of a microtiter plate. After blocking, the wells were incubated at 37 $^\circ\text{C}$ for 3 h with increasing concentrations of ^{125}I -labeled proteoglycan, either biglycan (circles) or decorin (squares). The specific activity of each proteoglycan was 1×10^6 dpm/ μg . After washing, specific binding was measured by direct γ counting. The means \pm S.D. of quadruplicate determinations are shown.

earlier or later stages in development of the tissue (14). To determine whether biglycan isoforms with different glycosaminoglycan side chains had any influence on binding to tropoelastin, biglycan from the nuchal ligament of 230-day-old fetuses was compared with biglycan derived from adult bovines, which has a much higher iduronate content (Fig. 6). The result showed no major difference in binding of the two glycoforms of biglycan over a range of tropoelastin densities in the microtiter wells. Thus major differences in the iduronate-glucuronate ra-

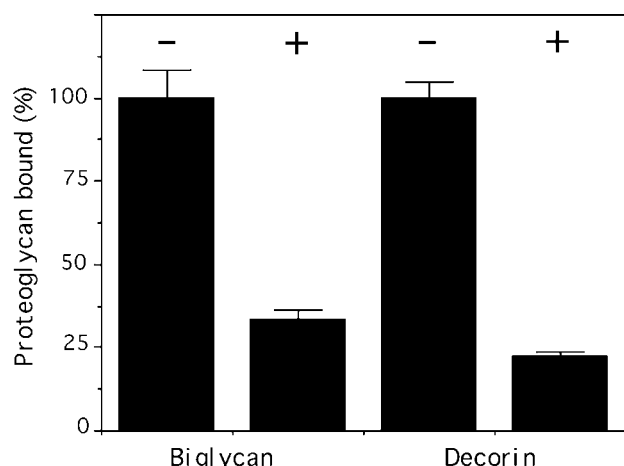


FIG. 4. Elastin-derived peptides block biglycan and decorin binding to tropoelastin. Tropoelastin (100 ng/well) was coated onto microtiter plates. After blocking, the wells were then incubated for 3 h at 37 °C with 2.3×10^5 dpm of 125 I-labeled biglycan or decorin that had been preincubated in the presence (+) or absence (-) of elastin-derived peptides equivalent to 3 μ g/well. After washing, binding to the wells was measured by direct γ counting. The binding is expressed as a percentage of the binding of the untreated proteoglycan to tropoelastin. The means \pm S.D. of quadruplicate determinations are shown.

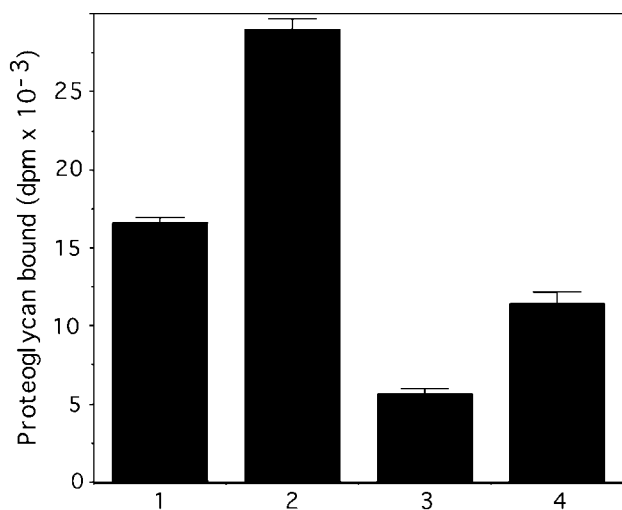


FIG. 5. Biglycan and decorin binding to tropoelastin via their protein cores. Tropoelastin (100 ng) was coated onto wells of a microtiter plate. After blocking, the wells were incubated for 3 h at 37 °C with 1.9×10^5 dpm of 125 I-labeled proteoglycan or core protein. Lane 1, biglycan; lane 2, biglycan core protein; lane 3, decorin; lane 4, decorin core protein. After washing, specific binding to the wells was measured by direct γ counting. The means \pm S.D. of quadruplicate determinations are shown.

tios within the glycosaminoglycan structures did not appear to alter the tropoelastin binding properties of biglycan.

Measurement of K_d Values for the Interactions of Biglycan and Decorin with Tropoelastin—To determine the relative affinities of the binding of biglycan and decorin to tropoelastin, their dissociation constants were measured using a method described previously (16). Increasing amounts of 125 I-labeled proteoglycan were added to wells coated with tropoelastin. After incubation, the amounts of bound and unbound proteoglycan were determined. Specifically bound proteoglycan was calculated as the amount bound to the wells coated with tropoelastin minus that bound to BSA-coated wells. The binding curves for biglycan are shown in Fig. 7A. The K_d value for biglycan binding to tropoelastin was calculated using Scatchard analysis. For each biglycan concentration, the ratio of specifically bound counts to unbound counts was plotted

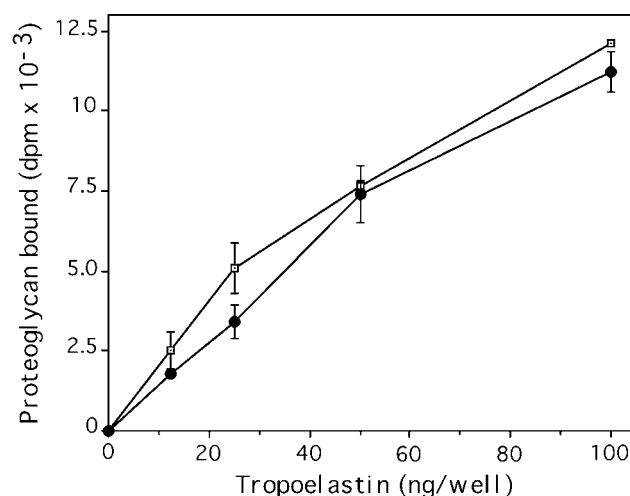


FIG. 6. Distinct glycoforms of biglycan show equal binding to tropoelastin. Tropoelastin was coated in serial dilution on rows of wells of a microtiter plate. After blocking, the wells were incubated for 3 h at 37 °C with 1.9×10^5 dpm of 125 I-labeled biglycan glycoforms, either from 230-day-old fetal (squares) or adult (circles) nuchal ligament. After washing, specific binding to the wells was measured by direct γ counting. The means \pm S.D. of quadruplicate determinations are shown.

against the amount of biglycan bound. The K_d of the biglycan-tropoelastin interaction was calculated as 1.95×10^{-7} M from the slope of the resulting straight line, which equals $-1/K_d$ assuming one binding site/molecule (Fig. 7B). Similarly the binding curves and Scatchard analysis for the decorin-tropoelastin are shown in Fig. 7 (C and D, respectively). The K_d for the decorin-tropoelastin interaction was measured as 5.3×10^{-7} M. This result confirms that biglycan binds more avidly than decorin to tropoelastin.

Biglycan and Decorin Have Distinct Binding Sites on Tropoelastin—Inhibition experiments were undertaken to determine whether decorin could block the interaction of biglycan with tropoelastin. Using the solid phase binding assay, wells coated with tropoelastin were preincubated with increasing concentrations of unlabeled decorin, biglycan, or BSA, washed, and incubated with 125 I-labeled biglycan (Fig. 8A). Decorin was found to inhibit biglycan binding to tropoelastin but not as effectively as biglycan itself, at any given concentration. Preincubation with BSA was found to have no effect on the interaction (not shown). In the reciprocal experiment, tropoelastin-coated wells were preincubated with unlabeled decorin, biglycan, or BSA and then incubated with 125 I-labeled decorin (Fig. 8B). Both proteoglycans were found to inhibit decorin binding to tropoelastin. Interestingly in this instance, decorin was more effective than biglycan at inhibiting the interaction. If the two proteoglycans shared the same binding site, then it would be expected that the molecule with the highest affinity, biglycan, would be most effective at blocking the binding of both biglycan and decorin. However, each proteoglycan was most effective at blocking its own binding, indicating that the two proteoglycans do not share an identical binding site. However, the sites appear to be in close proximity to each other because the binding of one of the proteoglycans significantly inhibited the binding of the other.

MAGP-1 Forms a Ternary Complex with Biglycan and Tropoelastin—To determine whether MAGP-1 binding to tropoelastin affected its interactions with biglycan and decorin, the solid phase inhibition assays were repeated using tropoelastin-coated wells that had been preincubated with increasing amounts of MAGP-1 (Fig. 9). Interestingly, MAGP-1 pretreatment did not block the binding of biglycan to the tropoelastin but enhanced binding by up to 40%. Maximum bi-

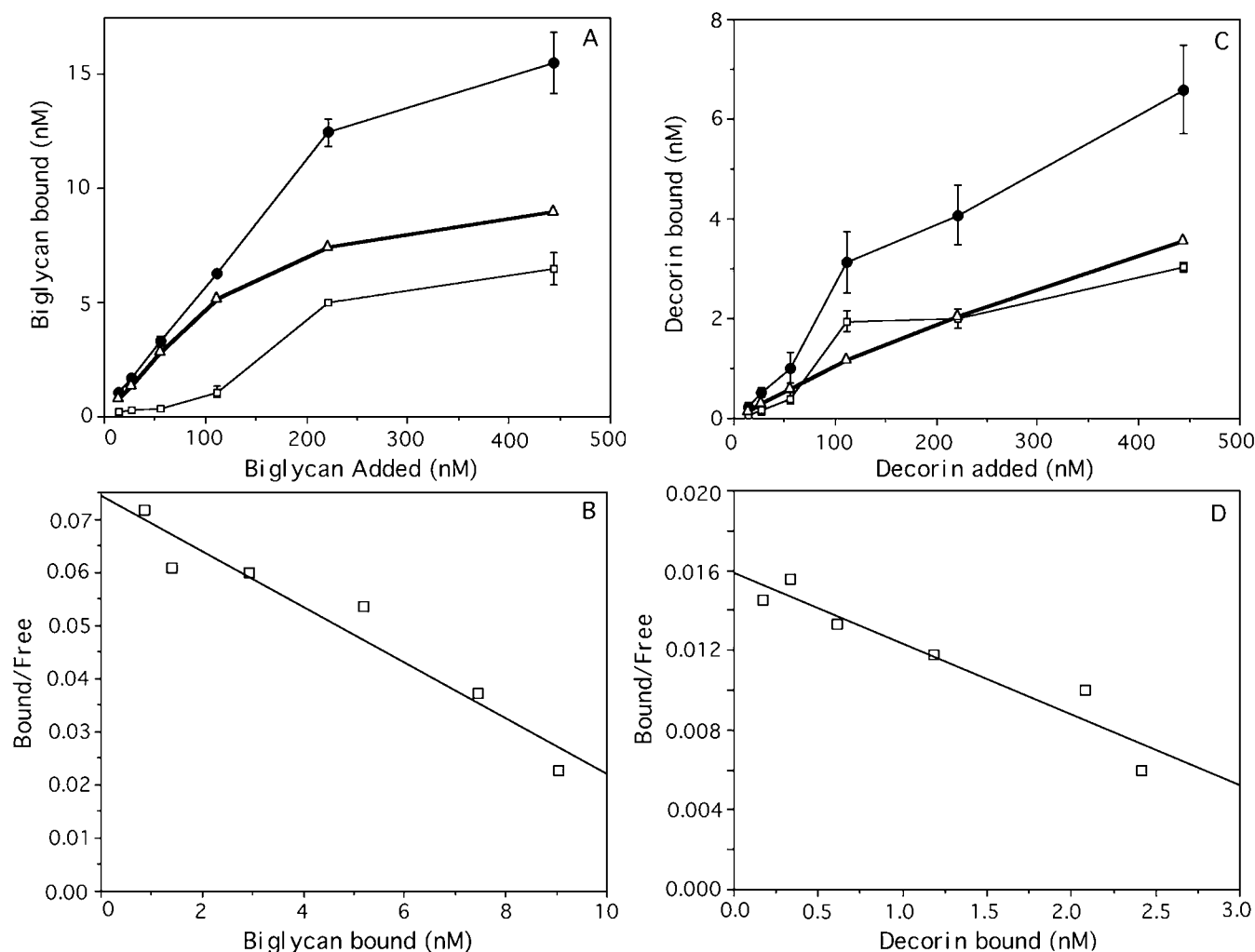


FIG. 7. Calculation of K_d values for the interactions of biglycan and decorin with tropoelastin. A, microtiter plates coated with tropoelastin (circles) or BSA (squares) (100 ng/well) were incubated for 3 h at 37 °C with ^{125}I -labeled biglycan at concentrations of 0–1 $\mu\text{g}/\text{well}$ (specific activity, 5.0×10^{-6} dpm/ μg). The liquid phase was then removed, the wells were washed, and the amount of bound and unbound radioactivity in each well was determined by γ counting. Specific binding (triangles) was calculated as the amount of biglycan bound to tropoelastin-coated wells minus that bound to the corresponding BSA-coated wells. The means \pm S.D. of quadruplicate determinations are shown. B, data from A expressed using Scatchard analysis to determine K_d value for biglycan interaction with tropoelastin. Slope of line = $-1/K_d$. C, a similar experiment to A except ^{125}I -labeled decorin (specific activity, 5.0×10^{-6} dpm/ μg) was used in place of biglycan. D, data from C expressed using Scatchard analysis to determine K_d value for the interaction of decorin with tropoelastin.

glycan binding was observed to tropoelastin that had been pretreated with 25-fold excess of MAGP-1 (2.5 μg of MAGP-1 to 100 ng of tropoelastin), suggesting that saturation of MAGP-1-binding sites on the tropoelastin had occurred at this ratio. In contrast, the MAGP-1 pretreatment had no effect on the binding of decorin to tropoelastin. These findings suggested that MAGP-1 was not blocking the binding sites for decorin and biglycan on tropoelastin, and thus MAGP-1 appeared to have a binding site on the tropoelastin that was distinct from the biglycan- and decorin-binding sites.

In additional experiments to confirm that MAGP-1 did not compete with biglycan for binding to tropoelastin, excess (2.5 $\mu\text{g}/\text{well}$), MAGP-1 was included in the biglycan-containing liquid phase of the tropoelastin binding assay. Rather than reduce biglycan binding, the inclusion of MAGP-1 caused a 2.5-fold increase in the binding of the proteoglycan to tropoelastin substrate (Fig. 10A). Control wells coated with BSA showed no increase in background biglycan binding in the presence of MAGP-1. The large increase in biglycan binding to tropoelastin substrate suggested that the MAGP-1 and biglycan had formed a complex in the liquid phase that had subsequently bound to the tropoelastin. Thus it was evident that biglycan, tropoelas-

tin, and MAGP-1 had specifically formed a ternary complex. In a similar experiment, the inclusion of excess MAGP-1 in the liquid phase did not significantly alter decorin binding to tropoelastin substrate (Fig. 10B).

Biglycan Binds to MAGP-1 in the Liquid Phase—To determine whether biglycan or decorin binds to MAGP-1 in the liquid phase, co-immunoprecipitation experiments with anti-MAGP-1 antiserum were performed (Fig. 11). Six-fold more biglycan was co-immunoprecipitated with MAGP-1 than in the control reactions where MAGP-1 had been replaced with BSA, confirming that biglycan does specifically bind to MAGP-1 in solution. In contrast, no specific co-immunoprecipitation of decorin with MAGP-1 was detected.

DISCUSSION

Elastic fibrogenesis is a poorly understood process that is considered to involve the deposition of the elastin precursor tropoelastin into a framework of fibrillin-containing microfibrils (21, 22). In addition to tropoelastin and fibrillins 1 and 2 (23–28), a number of other macromolecules have been identified that may be involved in this process. These include microfibril-associated proteins MAGPs 1 and 2 (13, 15, 29–34); the

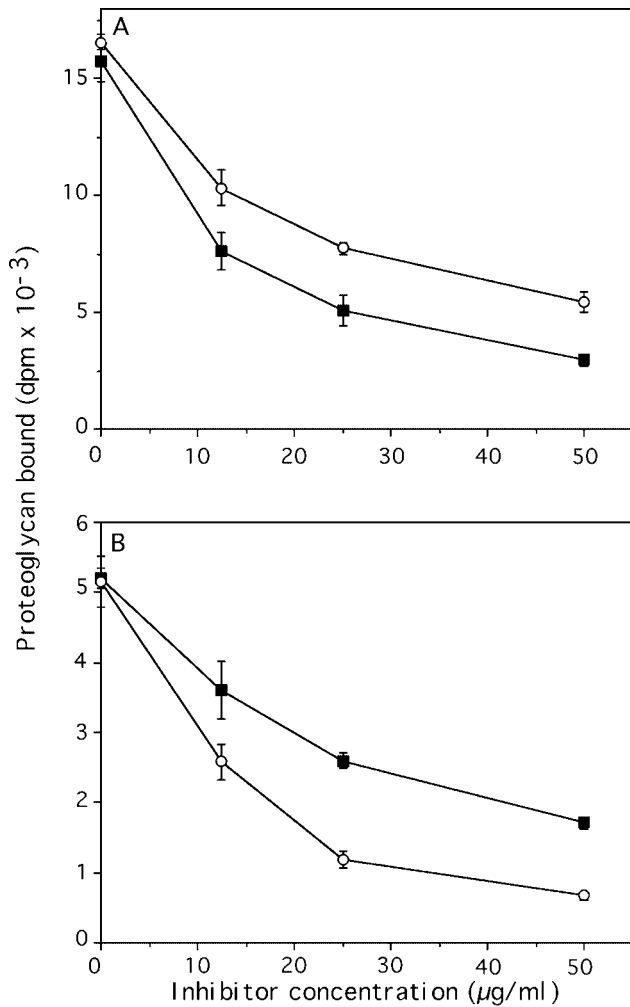


FIG. 8. Biglycan and decorin inhibit binding of each other to tropoelastin. Tropoelastin (100 ng/well) was coated onto microtiter wells. After blocking, the wells were incubated for 1 h at 37 °C with increasing amounts of unlabeled biglycan or decorin (0–5 $\mu\text{g/well}$). After washing, the wells were incubated for 3 h at 37 °C with 1.9×10^5 dpm of ^{125}I -labeled biglycan (A) or decorin (B). After further washing, the effect of unlabeled decorin (circles) and biglycan (squares) on the binding of ^{125}I -labeled proteoglycan was measured by direct γ counting. The means \pm S.D. of quadruplicate determinations are shown.

67-kDa cell surface elastin-binding protein (35); the elastin-microfibril interface protein emilin (36); the fibrillin-like latent transforming growth factor B-1 binding proteins (37, 38); and the small dermatan sulfate proteoglycans decorin and biglycan (13, 14). Recently Trask *et al.* (13) demonstrated the importance of sulfation for the assembly of elastin, fibrillin-1, and MAGP-1 into extracellular matrix, and this finding suggested that a proteoglycan(s) is involved in elastic fiber assembly. However, the significance of biglycan and decorin in this process is yet to be determined.

We have recently shown that the expression of a specific glycoform of biglycan correlates with elastinogenesis during development of the highly elastic tissue, nuchal ligament (14). To investigate further the possible role of biglycan and its relative decorin in elastinogenesis, we have focused in this study on the identification and characterization of molecular interactions of these dermatan sulfate proteoglycans with tropoelastin, purified fibrillin-containing microfibrils, and MAGPs 1 and 2. Both biglycan and decorin showed specific, saturable, calcium-independent binding to tropoelastin in solid phase assays and co-immunoprecipitation experiments. Soluble elastin peptides were shown to block the interactions, indicating that

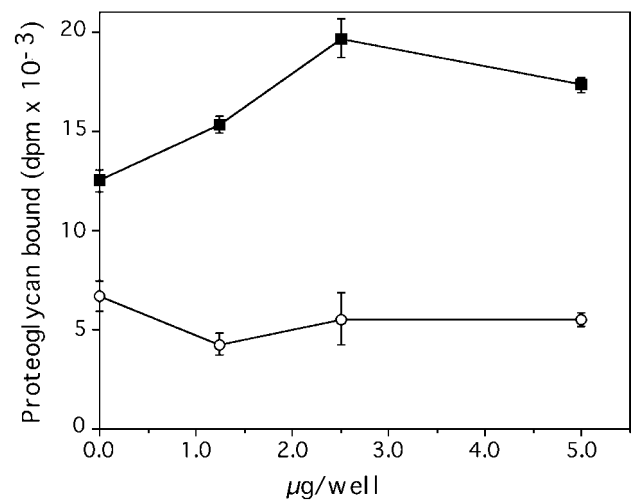


FIG. 9. Preincubation of solid-phase tropoelastin with MAGP-1 enhances biglycan binding to tropoelastin. Tropoelastin (100 ng/well) was coated onto microtiter wells. After blocking, the wells were incubated for 1 h at 37 °C with increasing amounts of MAGP-1 (0–5 $\mu\text{g/well}$). After washing the wells were incubated for 3 h at 37 °C with 1.9×10^5 dpm of ^{125}I -labeled biglycan or decorin. After further washing the binding of ^{125}I -labeled biglycan (squares) or decorin (circles) was measured by direct γ counting. The means \pm S.D. of quadruplicate determinations are shown.

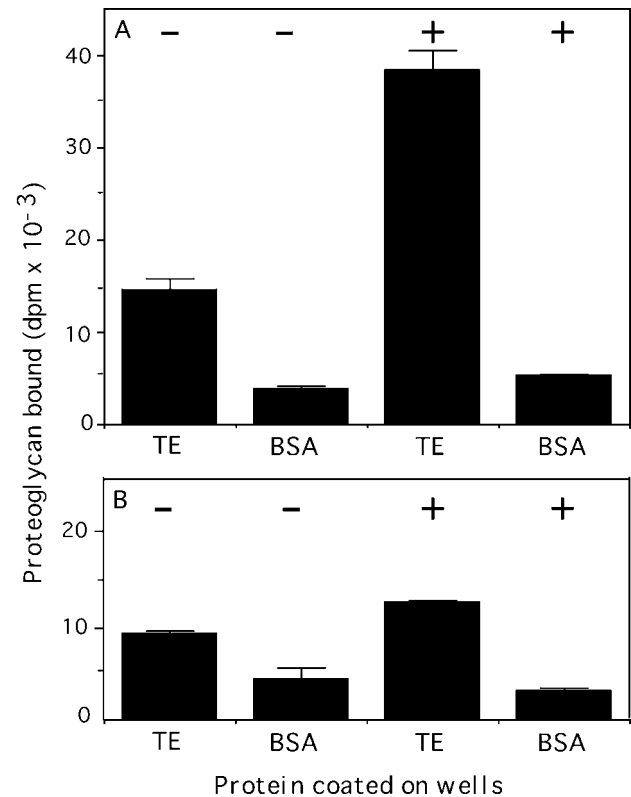


FIG. 10. Addition of MAGP-1 to biglycan in the liquid phase enhances its binding to tropoelastin. Microtiter wells were coated with tropoelastin (TE) or BSA (100 ng/well). After blocking, the wells were incubated for 3 h at 37 °C with 1.9×10^5 dpm of ^{125}I -labeled biglycan (A) or decorin (B) in the presence (+) or absence (-) of MAGP-1 (2.5 $\mu\text{g/well}$). After washing, the binding of ^{125}I -labeled proteoglycan to the wells was measured by direct γ counting. The means \pm S.D. of quadruplicate determinations are shown.

the binding sites for biglycan and decorin are also present in mature elastin as well as in the tropoelastin precursor. Both proteoglycans were shown to interact with tropoelastin via their core proteins rather than their glycosaminoglycan side

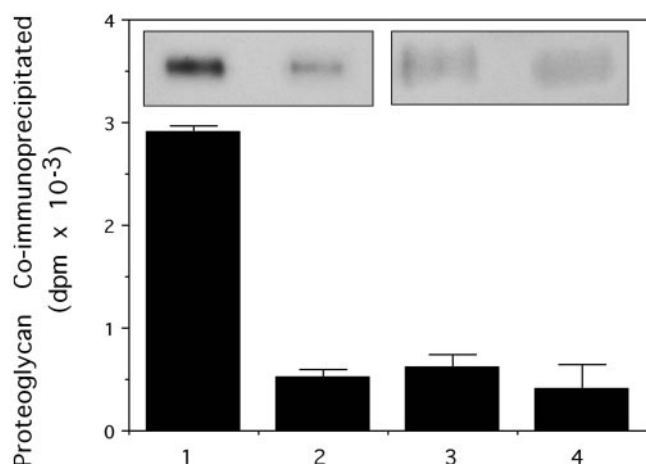


FIG. 11. **Biglycan co-immunoprecipitates with MAGP-1.** ¹²⁵I-labeled biglycan or decorin (5×10^4 dpm) in TBS (50 μ l) was incubated with 1 μ g of MAGP-1 or BSA control for 4 h at 37 °C. Proteoglycan bound to the MAGP-1 was then recovered by the addition of anti-MAGP-1 antiserum (10 μ l), followed by protein A-Sepharose (30 μ l). Co-immunoprecipitated ¹²⁵I-labeled proteoglycan was measured by direct γ counting (columns 1–4). The means \pm S.D. of quadruplicate immunoprecipitations is shown. Column 1, biglycan with MAGP-1; column 2, biglycan with BSA; column 3, decorin with MAGP-1; column 4, decorin with BSA. SDS-PAGE on 12% gels and autoradiography confirmed that the precipitated counts were associated with the proteoglycan (insets). Left inset, biglycan (about 220 kDa); right inset, decorin (about 120 kDa).

chains. Indeed variations in the glycosaminoglycan composition did not appear to directly affect binding to tropoelastin because the glucuronate-rich glycoform of biglycan, specifically expressed during the elastinogenesis phase in nuchal ligament development (14), was found to bind equally strongly to tropoelastin as the iduronate-rich glycoform found in the adult tissue. Therefore these variations must serve other purposes within the tissue. Scatchard analysis showed that biglycan had greater affinity than decorin for tropoelastin with K_d values measured as 1.95×10^{-7} M and 5.3×10^{-7} M, respectively. The interactions are of moderately high affinity and are of comparable strength to the tropoelastin-MAGP-1 interaction (K_d of 2.6×10^{-7} M) measured previously (16). Reciprocal blocking experiments showed that biglycan and decorin could inhibit binding of the other proteoglycan to tropoelastin. However, biglycan was more effective at blocking its interaction with tropoelastin, and decorin was more effective at blocking its binding to tropoelastin. This finding suggests that the two proteoglycans have binding sites in close proximity on tropoelastin but that they are not identical binding sites. The demonstration that biglycan specifically binds to tropoelastin more strongly than decorin is consistent with previous ultrastructural immunolocalization studies that showed specific staining for biglycan on the amorphous elastin component of elastic fibers (10). Interestingly, in the same study, decorin was preferentially identified in association with the fibrillin-containing microfibrillar component of these fibers. Decorin has recently been shown to bind to the fibrillin-1 component of the microfibrils (13). In our studies both decorin and biglycan were found to interact with isolated microfibrils in solid phase binding assays, to a similar extent. This finding contrasts with the differential immunostaining observed in tissues (10) and may be explained by the masking of biglycan epitopes in tissues and/or the presence of additional matrix constituents in tissues that may inhibit the interaction of biglycan with the microfibrils.

Because MAGP-1 has also been shown to bind tropoelastin (16, 29, 34), we determined whether MAGP-1 could act as an

inhibitor and block the interactions of biglycan and decorin with tropoelastin in the solid phase assays. However, when tropoelastin-coated wells were pretreated with MAGP-1, subsequent biglycan binding to the wells was enhanced by up to 40% in wells where MAGP-1 binding to tropoelastin had reached saturation (above 2.5 μ g of MAGP-1/well). This suggested either that MAGP-1 binding was causing a conformational change in tropoelastin leading to its increased avidity for biglycan or that biglycan was also binding to MAGP-1, even though no interaction had been detected in direct solid phase binding assays. However, biglycan binding to tropoelastin substrate was even more extensively enhanced (by about 250%) when the experiment was repeated with 2.5 μ g of MAGP-1 incorporated into the liquid phase of the assay rather than as a pretreatment of the solid phase. This finding indicated that the increased binding of biglycan to the substrate was most likely due to binding to MAGP-1 in the solution phase followed by the binding of the aggregate to the tropoelastin coating on the wells. Because MAGP-1 is known to self-aggregate (29), it seems likely that aggregates of multiple MAGP-1 and biglycan molecules (containing 2–3 biglycan molecules) had formed in the liquid phase, and some of these had bound to the tropoelastin molecules in the solid phase. In contrast to previous solid phase assays that detected no interaction between biglycan and MAGP-1, co-immunoprecipitation experiments using anti-MAGP-1 antiserum confirmed that biglycan and MAGP-1 do interact in solution. Thus it was evident from the above results that biglycan forms a ternary complex with MAGP-1 and tropoelastin.

In contrast to the biglycan-tropoelastin interaction, MAGP-1 showed no effect on the binding of decorin to tropoelastin, indicating that the binding sites for MAGP-1 and decorin are not close together on the tropoelastin molecule and confirming that decorin does not significantly interact with MAGP-1 in the solid phase binding or immunoprecipitation assays. The latter finding appears to contrast with the study of Trask *et al.* (13). These workers demonstrated that decorin could be co-immunoprecipitated with MAGP-1 from the conditioned medium of chondrocyte cultures. However, in the same study, both macromolecules were shown to complex with fibrillin-1. It seems likely that these immunoprecipitates containing MAGP-1 and decorin also contained fibrillin-1, and thus it is possible that the decorin was binding the fibrillin-1 rather than MAGP-1 in the complexes. Recent evidence indicates that the MAGP-1-binding site is in the N-terminal region of fibrillin-1 (34) and is thus distinct from the more central decorin-binding region described Trask *et al.* (13).

The differential binding of biglycan and decorin to tropoelastin and MAGP-1 suggests that the proteoglycans are likely to possess distinct functions in elastic fiber biology. MAGP-1 is associated with the “bead” regions of the fibrillin-containing microfibrils (39), and it has been suggested that MAGP-1 may act as a binding protein for the deposition of tropoelastin onto the surface of the microfibrils during elastinogenesis (21, 29). The specific complexing of biglycan to MAGP-1 and tropoelastin suggests that biglycan may also be involved in this process. However, recent evidence indicates that tropoelastin can bind directly to the fibrillin-1 component of the microfibrils, suggesting that elastin deposition may occur independently of MAGP-1 (40). In addition, it has recently been shown that biglycan null mice have no obvious disturbance of elastic fiber assembly (8), suggesting that any role for biglycan in this process is likely to be limited. Alternatively, the biglycan may be involved in the stabilization of the maturing fiber, perhaps by mediating interactions of elastin with elements of the surrounding matrix. The glycosaminoglycan side chains of the specific glucuronate-

rich glycoform expressed during elastinogenesis may be involved in this mediation because they do not appear to be directly involved in binding to tropoelastin (see above).

The role(s) for decorin in elastic fiber biology is equally unclear. Decorin has now been shown to bind tropoelastin (see above) and fibrillin-1 (13), and its expression appears to peak in the early stages of elastic tissue development (14). Thus it is possible that this proteoglycan is involved in the stabilization of the fibrillin-containing microfibrils and the deposition of tropoelastin onto the microfibrils in the early stages of elastinogenesis. However, it should be noted that decorin null mice appear to have no obvious elastic fiber abnormalities (6). Perhaps another member of the small leucine-rich proteoglycan family, such as biglycan, may be able to substitute for decorin in these mice. The elucidation of the full roles of biglycan and decorin in elastic fiber biology remains a difficult but intriguing challenge.

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**GLYCOBIOLOGY AND
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BIGLYCAN FORMS A TERNARY
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AND MICROFIBRIL-ASSOCIATED
GLYCOPROTEIN 1**

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