βig-h3 Interacts Directly with Biglycan and Decorin, Promotes Collagen VI Aggregation, and Participates in Ternary Complexing with These Macromolecules*

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Recombinant human βig-h3 was found to bind 125I-labeled small leucine-rich proteoglycans (SLRPs), biglycan, and decorin, in co-immunoprecipitation experiments. In each instance the binding could be blocked by an excess of the unlabeled proteoglycan, confirming the specificity of the interaction. Scatchard analysis showed that biglycan bound βig-h3 more avidly than decorin with $K_a$ values estimated as $5.88 \times 10^{-7}$ and $1.02 \times 10^{-7}$ M, respectively. In reciprocal blocking experiments both proteoglycans inhibited the other binding to βig-h3 indicating that they may share the same binding site or that the two binding sites are in close proximity on the βig-h3 molecule. Since βig-h3 and the SLRPs are known to be associated with the amino-terminal region of collagen VI in tissue microfibrils, the effects of including collagen VI in the incubations were investigated. Co-immunoprecipitation of 125I-labeled biglycan incubated with equimolar mixtures of βig-h3 and pepsin-collagen VI was increased 6-fold over βig-h3 alone and 3-fold over collagen VI alone. Similar increases were also observed for decorin. The findings indicate that βig-h3 participates in a ternary complex with collagen VI and SLRPs. Static light scattering techniques were used to show that βig-h3 rapidly forms very high molecular weight complexes with both native and pepsin-collagen VI, either alone or with the SLRPs. Indeed βig-h3 was shown to form a complex with collagen VI and biglycan, which appeared to be much more extensive than that formed by βig-h3 with collagen VI and decorin or those formed between the collagen and βig-h3, biglycan, or decorin alone. Biglycan core protein was shown to inhibit the extent of complexing of βig-h3 with native and pepsin-collagen VI suggesting that the glycosaminoglycan side chains of the proteoglycan were important for the formation of the large ternary complexes. Further studies showed that the direct interaction between βig-h3 and biglycan and between biglycan and collagen VI were also important for the formation of these complexes. The globular domains of collagen VI also appeared to have an influence on the interaction of the three components. Overall the results indicate that βig-h3 can differentially modulate the aggregation of collagen VI with biglycan and decorin. Thus this interplay is likely to be important in tissues such as cornea where such complexes are considered to occur.

Transforming growth factor-β (TGF-β)1-inducible gene-h3 (βig-h3) (also known variously as MP78/70 (1, 2), RGDCAP (3), and keratoepithelin (4)), is an extracellular matrix protein expressed in a wide variety of tissues including developing nuchal ligament, aorta, lung, kidney, and cartilage; and mature cornea, skin, bladder, and bone (5–11). The name βig-h3 stems from its identification and cloning as a major TGF-β-responsive gene in A549 lung adenocarcinoma cells (12, 13). βig-h3 protein is 76–78 kDa in size and contains four repeat domains, with homology to the insect protein fasciclin, and 11 cysteine residues most of which are clustered in a distinct amino-terminal region. The βig-h3 molecule appears to undergo partial processing at the carboxyl-terminal end to yield a 68–70-kDa isoform (13). βig-h3 has been shown to bind in vitro to a number of other matrix components including fibronectin, laminin, and several collagen types (14, 15). In addition, βig-h3 has multiple cell-adhesion motifs within the fasciclin-like domains that can mediate interactions with a variety of cell types via integrins $\alpha_3\beta_1$ (16, 17), $\alpha_1\beta_1$ (18), or $\alpha_2\beta_5$ (19). The precise functions of βig-h3 are unknown but it has been proposed that it may act as a cell adhesion molecule (19) and as a multifunctional linker protein interconnecting different matrix molecules to each other and to cells (5, 9). Recent evidence suggests that βig-h3 may be particularly important for skeletal muscle cell adhesion at the myotendinous junction (18), and for the induction of keratinocyte differentiation (20). The protein also appears to be involved in endothelial cell-matrix interactions during vascular remodeling and angiogenesis (21) and as a negative regulator of mineralization during cartilage differentiation and osteogenesis (22–24). Mutations in the human βig-h3 gene (TGFβII) have been linked to several autosomal dominant corneal dystrophies (4) characterized by severe visual impairment resulting from the progressive accumulation of βig-h3-containing protein deposits in the corneal matrix (25, 26).

To elucidate the function of βig-h3 within the extracellular matrix, studies in our laboratory have focused on the localization, molecular forms, and matrix interactions of βig-h3 within various tissues. Ultrastructural localization studies on developing tissues showed that, in most instances, βig-h3 was loosely associated with collagen fibers although, in developing kidney, labeling was also observed close to the tubular and capsular basement membranes. Double immunolabeling experiments with antibodies to βig-h3 and collagen VI indicated that much of the βig-h3 was associated with collagen VI microfibrils rather than the collagen fibers themselves (5). The collagen V1 microfibrils, 3–10 nm in diameter, exhibit a characteristic, double-headed period of about 100 nm (27). In some tissues collagen VI appears to form additional structures including thicker cross-banded fibrils and hexagonal networks (28, 29). The precise functions of collagen VI are unclear but

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1 The abbreviations used are: TGF-β, transforming growth factor-β; βig-h3, transforming growth factor-β-inducible gene-h3; BSA, bovine serum albumin; GAG, glycosaminoglycan; rβig-h3, recombinant βig-h3; SLRP, small leucine-rich repeat proteoglycan; TBS, Tris-buffered saline.
the protein is considered to be important for tissue architecture, interlinking structural components of the matrix, and for cell-matrix interactions (27). Mutations in collagen VI genes (COL6A1, COL6A2, and COL6A3) have recently been linked to the muscle wasting diseases, Bethlem myopathy and Ullrich dystrophy (30, 31).

To define the relationship of βig-h3 with collagen VI we have isolated collagen VI microfibrils from collagenase-treated nuchal ligament and demonstrated that βig-h3 is covalently attached to collagen VI at regular intervals along at least some of the microfibrils (32). The binding site is located close to the amino-terminal end of the collagen VI molecule. Additional binding assays have demonstrated that βig-h3 binds in vitro to collagen VI, but in a non-covalent manner (32). The βig-h3 attachment site on collagen VI appears to be close to those documented for the small leucine-rich proteins (SLRP), decorin and biglycan, and matrilins (33–35). SLRPs, in particular biglycan, have been shown to influence the aggregation and organization of collagen VI into networks in vitro, mimicking those found in some tissues, particularly cornea (28, 34).

In the present study we investigated the influence of rβig-h3 on collagen VI aggregation and its interactions with decorin and biglycan in vitro. We have shown that rβig-h3 directly binds to biglycan and decorin and in turn forms ternary complexes with collagen VI and these SLRPs. Furthermore, rβig-h3 promotes the rapid aggregation of collagen VI tetramers into very large assemblies in vitro and differentially influences the aggregation of the collagen with the two SLRPs. The findings indicate that βig-h3 is likely to be involved in the modulation of collagen VI-proteoglycan interactions during development of a range of tissues including cornea. It is possible that disruption of the normal interactions of βig-h3 with collagen VI and/or SLRPs may be important for the development of βig-h3-linked corneal dystrophies.

**Experimental Procedures**

**Materials**—Biglycan and decorin were purified from the nuchal ligaments of 230-day-old fetal calves as described previously (36). Human rβig-h3 and pepsin-treated collagen VI were prepared and purified as described previously (32, 37). Purified rβig-h3 was stored at 4 °C in 50 mM Tris buffer, pH 7.4, containing 500 mM NaCl to prevent self-aggregation prior to aggregation experiments. Native collagen VI was purified from fetal bovine skeletal muscle. The frozen tissue was crushed and extracted at 4 °C for 18 h in 5 volumes of TBS, pH 7.4, containing proteinase inhibitors and 0.1% Nonidet P-40. The non-solubilized material was then extracted extensively over 48 h with 0.6 M KCl, containing inhibitors (5 × 10 volumes) to solubilize cytoskeletal proteins. The residue was rinsed with collagenase buffer (32) and digested sequentially with highly purified bacterial collagenase (7500 units/ml) at 37 °C for 24 h and then hyaluronidase (200 units/ml) for a further 24 h. The supernatant was subjected at 4 °C to CsCl equilibrium density gradient centrifugation at 30,000 rpm (g = 193,000), with initial density of 1.325 g/ml, for 72 h in a fixed angle head (70.1 Ti) in a Beckman L7-55 ultracentrifuge. The fractions containing collagen VI microfibrils were pooled and purified further on a second CsCl gradient with initial density of 1.27 g/ml. Fractions containing collagen VI microfibrils were dialyzed into TBS, pH 7.4, containing 400 mM NaCl, and final purification was performed by fast protein liquid chromatography on a Superose 6 column (1.5 × 25 cm). Proteinase inhibitors were included throughout the purification process. Purified native and pepsin-collagen VI were depolymerized by dialysis into 100 mM sodium citrate buffer, pH 4.0 (34). Any remaining aggregates were removed from the tetromers by centrifugation at 15,000 × g for 10 min. Specific polyclonal rabbit antibodies to βig-h3 and type VI collagen have been described previously (32, 37). Anti-His<sub>6</sub> antibody was purchased from Qiagen. Chondroitinase ABC was purchased from Seikagaku Corp. (Tokyo, Japan).

**Iodination of Biglycan and Decorin**—Biglycan and decorin (100 μg) were radiolabeled with Na<sup>125</sup>I (Amersham Biosciences) using IO-DIOD-BEADS (Pierce). Each proteoglycan was reacted with an IO-DIOD-BEAD and 0.75 mCi of <sup>125</sup>I for 5 min in 50 mM Tris buffer, pH 7.4, containing 0.3 M guanidinium chloride. Bound and free radiolabels were separated by gel filtration through Sephadex G-10. The specific activities of biglycan and decorin were both 3.4 × 10<sup>6</sup> dpm/μg of core protein unless stated otherwise. Core proteins of biglycan and decorin were prepared by digestion of the <sup>125</sup>I-labeled proteoglycans with chondroitinase ABC as described previously (36).

**Co-immunoprecipitation Assays**—Co-immunoprecipitation assays were performed as described previously (35). Briefly, the test protein (1 μg of rβig-h3 or 2 μg of collagen VI) was incubated with 7 × 10<sup>6</sup> dpm of <sup>125</sup>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, EDTA (2 mM), benzamidine (1 mM), e-amino-n-caproic acid (1 mM), and phenylmethylsulfonyl fluoride (0.5 mM)) containing 2 μg of BSA. Control incubations contained no test protein. Specific rabbit polyclonal anti-serum (10 μl) raised against collagen VI was added and 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 (3,000 × 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 <sup>125</sup>I-proteoglycan was eluted from the complex by resuspension in non-reduced 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 <sup>125</sup>I-labeled proteoglycan content by γ counting and SDS-PAGE/autoradiography as described previously (36). Each co-immunoprecipitation was performed in triplicate. The protocol was slightly modified for estimation of K<sub>d</sub> values, see Fig. 2 for details.

**Static Light Scattering Analysis of Collagen VI Aggregation**—An argon laser (Laser model 95, Laser Laser Inc.) was operated at a wavelength of 488 nm with an output power of 300 milliwatts. Light was focused into a sample cell containing a solution of the test macromolecules. The sample cell was maintained at 15 °C by a temperature controller (Endocal model RTE-5DD, Nelsab Instruments). The light scattered by the sample was detected at a scattering angle of 90°. Scattered light was detected by the single photon-counting photomultiplier tube (model EM1 9863B/350) with an aperture setting of 800 μm. The output pulses from the photomultiplier were passed through an amplifier discriminator to a digital correlator (model BI-2030AT, Brookhaven Instruments Inc.). The correlator was set to run continuously with a sample time of 1 μs and displayed the average count rate that was recorded every 30 s. Each experiment was commenced by mixing native or pepsin-collagen VI tetramers (120 nm) with rβig-h3 (120 nm) and/or a proteoglycan (biglycan or decorin (480 nm)) in TBS, directly in the cell.

**Analysis of Collagen VI-containing Aggregates by SDS-PAGE**—Pepsin-treated and native collagen VI samples were incubated in TBS containing ovalbumin (120 nm) at 4 °C for 4 h with rβig-h3 only or with rβig-h3 plus biglycan or decorin at the concentrations indicated above. The samples were centrifuged at 18,000 × g for 20 min and the supernatants were removed. The pellets were rinsed with TBS and dissolved in electrophoresis buffer. The proteins in the supernatants were recov-
**RESULTS**

**βig-h3 Binds to Biglycan and Decorin via Their Core Proteins**—Co-immunoprecipitation experiments were performed using $^{125}$I-labeled biglycan and decorin to determine whether βig-h3 directly binds one or both of these proteoglycans. Both biglycan and decorin were found to specifically co-precipitate with rβig-h3 indicating molecular interactions with the protein (Fig. 1). When the binding experiments were repeated with isolated core proteins from the two proteoglycans, each core protein co-precipitated showing that the βig-h3 binding sites were contained within the core proteins and not in the GAG side chains. Gel and autoradiographic analysis of the co-precipitates confirmed that the precipitated radioactivity was associated with intact proteoglycans or, where appropriate, the isolated cores. Because the specific activities of the two proteoglycans and their cores were matched, comparison between the relative extent of binding could be made. Biglycan was precipitated to a greater extent than decorin suggesting that the former may have a higher affinity for βig-h3. In contrast, the two core proteins were precipitated to an equal extent suggesting that their affinities for βig-h3 were of similar magnitude. Because both cores were precipitated to a greater extent than the intact proteoglycans the results suggest that the interactions were inhibited to some degree by the GAG side chains. However, this could not explain the differences in the extent of βig-h3 binding between the intact proteoglycans, because biglycan has a greater glycosaminoglycan content with two GAG side chains compared with one in decorin. Further immunoprecipitation experiments were performed over a range of proteoglycan concentrations that allowed $K_v$ values to be estimated for the interactions of βig-h3 with decorin and biglycan (Fig. 2). Scatchard analyses estimated the $K_v$ values for βig-h3 binding to decorin and biglycan as $1.02 \times 10^{-7}$ and $5.88 \times 10^{-7}$ M, respectively. This result confirms that biglycan binds βig-h3 more strongly than decorin.

Blocking experiments were performed to confirm the specificity of the interactions and to determine whether the two proteoglycans compete for binding to βig-h3 (Fig. 3, A and B). Preincubation of rβig-h3 with excess unlabeled biglycan completely blocked the subsequent binding of radiolabeled biglycan and decorin. Similarly unlabeled decorin blocked the binding of radiolabeled decorin and biglycan to the rβig-h3. The result indicates that biglycan and decorin either share the same binding site on βig-h3 or that the sites are in close proximity such that the binding of one proteoglycan prevents the subsequent binding of the other. The binding activities were also completely blocked by both biglycan and decorin core proteins, indicating that the GAG side chains of the proteoglycans did not contribute measurably to the interaction of the intact proteoglycans with βig-h3 (Fig. 3, C and D).

**βig-h3 Enhances the Binding of Biglycan and Decorin to Collagen VI**—The co-immunoprecipitation experiments were extended to include collagen VI in its subunit tetrameric form (27). The results are shown in Fig. 4. The co-immunoprecipitation of radiolabeled biglycan in the presence of equimolar mixtures of rβig-h3 and collagen VI was increased 6-fold over rβig-h3 alone (Fig. 4A) and 3-fold over collagen VI alone (Fig. 4B). Similarly co-immunoprecipitation of decorin with the rβig-h3/collagen VI mixture was increased 5-fold over rβig-h3 alone (Fig. 4C) and 3-fold over collagen VI alone (Fig. 4D). The amount of each proteoglycan precipitated with the rβig-h3/collagen VI mixture was similar when either anti-βig-h3 antibody and or anti-collagen VI antibody was used. Thus it appears that βig-h3 enhances the complexing of both proteoglycans to collagen VI and that it participates in a ternary complex in which it interacts directly with both the collagen VI and proteoglycan molecules.

**βig-h3 Rapidly Complexes with Collagen VI Tetramers In Vitro to Form Very Large Aggregates**—Since βig-h3 was found to enhance the interaction of collagen VI with biglycan and decorin, its effect on collagen VI aggregation was investigated in the absence and presence of each proteoglycan. The process of collagen VI aggregation was measured by static light scattering experiments. The direct effect of βig-h3 on aggregation of collagen VI tetrameric subunits is shown in Fig. 5. βig-h3 was shown to cause the rapid aggregation of the pepsi-collagen VI tetramers into very high molecular weight complexes (Fig. 5A). As measured by the light scattering, the process appeared to be essentially complete within 2 min. The degree of aggregation was found to be related to the concentration of rβig-h3 added with the maximum aggregation being observed at equimolar concentrations, and above, of rβig-h3 relative to the collagen. Note that no light scattering signal was obtained for rβig-h3 (not shown) or collagen VI alone, indicating that no detectable aggregation of the individual components was occurring within the time span of the experiment.

Similar levels of aggregation with rβig-h3 were detected when native collagen VI tetramers were used in place of the pepsi-collagen VI. As with pepsi-collagen VI, rβig-h3 was shown to promote the aggregation of native collagen VI in a concentration-dependent manner and close to maximum aggregation occurred with molar ratios of 1:1 (Fig. 5B). However, native collagen VI had less tendency than pepsi-collagen VI to aggregate with rβig-h3 at ratios below 2:1. The threshold light scattering...
signal for native collagen VI aggregation required 30 nM rβig-h3, whereas some aggregation of pepsin-collagen VI was detected with as little as 2.4 nM rβig-h3. The results suggest that a globular domain(s) of collagen VI had an inhibitory effect on the aggregation process.

Interestingly, preincubation of the individual proteins for 5 min prior to mixing effected the extent of the aggregation (Fig. 6). Allowing collagen VI to pre-equilibrate in the incubation buffer resulted in some inhibition of the extent of aggregation. However, preincubation of the βig-h3 at physiological electrolyte concentrations enhanced the aggregation. rβig-h3 and collagen VI have been shown to self-aggregate slowly when adjusted into physiological buffer from high salt and low pH, respectively (data not shown). Thus the results suggest that homotypic molecular interactions of collagen VI and βig-h3 monomers may influence the size and shape of the aggregate containing the two proteins, i.e. collagen/collagen VI complexing may inhibit the subsequent interaction of βig-h3 with the collagen, whereas βig-h3/βig-h3 interaction may enhance aggregate formation with collagen VI. It is possible that some aggregation of βig-h3 to dimers or oligomers (too small to...
Big-h3 Interactions with Biglycan, Decorin, and Collagen VI

**FIGURE 4.** Ternary complexing of Big-h3 and biglycan or decorin with collagen VI. Aliquots of rBig-h3 (1 μg) and collagen VI (2 μg) were incubated separately, and in combination, for 2 h at 37 °C in TBS (50 μl). 125I-labeled biglycan (A and B) or decorin (C and D) (7 × 10^4 dpm) were added and incubation was continued for 3 h at 37 °C. Bound 125I-labeled proteoglycan was recovered by co-immunoprecipitation with 1 μl of anti-Big-h3 antisera (A and C) or anti-collagen VI antisera (B and D), and measured by direct γ counting. Column 1, rBig-h3; columns 2 and 6, BSA controls; columns 3 and 7, rBig-h3 and collagen VI; column 4, collagen VI (control); column 5, collagen VI; column 8, rBig-h3 (control). The mean ± S.D. of triplicate determinations are shown. Note that both proteoglycans bound much more extensively to the rBig-h3/collagen VI complex than to rBig-h3 or collagen VI alone. The mean ± S.D. of triplicate determinations are shown.

In parallel experiments, Big-h3 was mixed with either native or pepsin-collagen VI in the presence or absence of biglycan or decorin and the complexes were allowed to stabilize. Ovalbumin was also included in each mixture. The complexes were recovered by centrifugation and analyzed by SDS-PAGE. The complexes with pepsin-collagen VI contained substantially less Big-h3 (Fig. 8A) than those with native collagen VI (Fig. 8B), suggesting that the amino-terminal globular domains on the collagen, removed by pepsin treatment, may be important enhancers for binding to Big-h3. For mixtures of three components, each precipitated aggregate contained collagen VI, rBig-h3, and the proteoglycan but lacked any ovalbumin content, confirming that all three molecules were specific components of the complexes. Incubation of the individual molecules alone resulted in little or no precipitation (Fig. 8C). As anticipated, the collagen VI content of the aggregates did not correlate with the static light scattering signal. For instance, in the absence of Big-h3, substantial aggregates of native collagen VI with biglycan and decorin were recovered by centrifugation but these aggregates did not register significant light scattering signals indicating that they were of relatively small particle size. The complexes formed in the presence of rBig-h3 contained similar amounts of precipitable native collagen VI as those formed in the absence of Big-h3. However, rBig-h3

detect by light scattering) enhances its aggregative effect on the collagen.

**βig-h3 Specifically Enhances the Extent of Biglycan Aggregation with Collagen VI**—The βig-h3-collagen VI aggregation experiment was repeated in the presence of 4-fold excess biglycan or decorin (Fig. 7A). First, decorin and biglycan were found to form high molecular weight aggregates with pepsin-collagen VI, in the absence of rβig-h3, within a few minutes of mixing. This result is consistent with the findings of Wiberg et al. (34). The light scattering signals were of similar strength to that obtained between Big-h3 and the collagen. Little observable difference in signal was detected if rβig-h3 was also included in the reaction with decorin and the collagen. However, the light scattering intensity was increased 3-fold if rβig-h3 was included in the biglycan-collagen VI reaction. Thus Big-h3 appeared to be differentially interacting with biglycan to form distinct aggregates with collagen VI, which were more extensive than those formed with rβig-h3, decorin, and collagen VI, and between biglycan and collagen VI only. Similar patterns were evident for the influence of rβig-h3 on the interactions of native collagen VI with the proteoglycans, although, surprisingly, no aggregation was detectable by static light scattering for the native collagen with biglycan or decorin in the absence of rβig-h3 (Fig. 7B).
greatly increased the particular size of the aggregates, especially of the complex formed between the collagen and biglycan, as indicated by the strong light scattering signal.

To determine whether the GAG side chains of the proteoglycans are important for their aggregation with big-h3 and collagen VI, the light scattering experiments were repeated with core proteins isolated from the proteoglycans by enzymatic digestion (Fig. 9, A and B). Static light scattering analysis showed that the core proteins of decorin and biglycan caused only limited aggregation of the pepsin-collagen VI (Fig. 9A). However, when rbig-h3 was added together with each core protein to pepsin-collagen VI, more aggregation was observed but in both instances the degree of aggregation was significantly less than that observed between rbig-h3 and collagen VI only (Fig. 9A). Similar results were obtained when the experiments were repeated with native collagen VI, although as anticipated from results with intact proteoglycans, no detectable aggregation of native collagen VI occurred with either core alone (Fig. 9B). The results indicate that both core proteins partially inhibited the aggregating effect of big-h3 on collagen VI. Unlike intact biglycan, its core did not form characteristically large aggregates with big-h3 and collagen VI. This finding suggests that the GAG side chains of the biglycan are critical for the formation of the very large complexes. When the light scattering experiments were repeated with 8-fold excess of proteoglycans no further increases in the light scattering signals were observed (data not shown), indicating that the contribution of each proteoglycan was already maximal when present in 4-fold excess.

The Globular Domains of Native Collagen VI Influence Ternary Complex Formation with big-h3 and Biglycan—To obtain clues as to which interactions were most important for the enhancement of collagen VI-biglycan aggregation by big-h3, the three components of the interaction were preincubated for 5 min in pairs before the third component was added (Fig. 10). Interestingly, if pepsin-collagen VI and biglycan were allowed to aggregate for 5 min before the addition of rbig-h3 to the
Big-h3 Interactions with Biglycan, Decorin, and Collagen VI

**FIGURE 9.** The isolated core proteins of biglycan and decorin inhibit big-h3-induced collagen VI aggregation. The aggregation experiments shown in Fig. 7 were repeated with the isolated core proteins from biglycan and decorin (480 nM) in place of the intact proteoglycans. A, pepsin-collagen VI; B, native collagen VI. Diamonds and dashed line, rbig-h3; triangles, biglycan core; open squares and dashed line, decorin core; closed squares, rbig-h3 plus biglycan core; open circles, rbig-h3 plus decorin core. The mean ± S.D. of triplicate determinations are shown. Note that both core proteins inhibited the extent of rbig-h3-induced collagen VI aggregation.

**FIGURE 10.** The globular domains of collagen VI have a modulating influence on ternary complex formation with big-h3 and biglycan. The influence of individual intermolecular interactions on ternary complex formation was investigated by incubating two components together for 5 min before the introduction of the third component. Aggregation was monitored by static light scattering. A, pepsin-collagen VI, and B, native collagen VI. Solid triangles, collagen VI (120 nM), rbig-h3 (120 nM) and biglycan (480 nM) mixed simultaneously; squares, collagen VI preincubated with biglycan followed by addition of rbig-h3; circles, collagen VI preincubated with rbig-h3 followed by the addition of biglycan; open triangles, rbig-h3 preincubated with biglycan followed by addition of collagen VI. The mean ± S.D. of triplicate determinations are shown.

reaction mixture, no enhancement of the aggregation was evident. Similar results were obtained if pepsin-collagen VI was preincubated with rbig-h3 prior to the addition of biglycan. In contrast, if rbig-h3 and biglycan were preincubated for 5 min the subsequent addition of pep-

sin-collagen VI caused an increase in signal close to that observed when all three components are directly mixed together. This finding points to the direct interaction of big-h3 with biglycan being a major factor in the enhancement of pepsin-collagen VI-biglycan aggregation by big-h3.

However, different results were obtained when the above experiments were repeated with native collagen VI (Fig. 10B). In contrast to the results using pepsin-collagen VI, preincubation of rbig-h3 with biglycan appeared to reduce the extent of aggregation of subsequently added native collagen VI. Preincubation of native collagen VI with big-h3 closely mimicked the aggregation obtained when all three components were added together simultaneously. However, the most dramatic increase in the light scattering signal was observed when native collagen VI was preincubated with biglycan prior to the addition of rbig-h3. The findings indicate that big-h3 may have a major positive influence on the polymerization of collagen VI in tissue situations particularly if biglycan is already bound to the collagen. This may be important in situations where high levels of big-h3 accumulate in the matrix, e.g., in certain congenital corneal dystrophies. In addition, a globular domain(s) of collagen VI (most likely the amino-terminal domain that is closest to the big-h3 and biglycan binding sites) appears to have a profound influence on big-h3-induced collagen VI aggregation, particularly if biglycan is already attached.

**DISCUSSION**

Recent evidence indicates that SLRPs, particularly decorin and biglycan may be complexed with collagen VI in tissues and that these proteoglycans can influence the rate, size, and shape of collagen VI aggregation in vitro (34, 35). In particular, biglycan was shown to rapidly organize collagen VI monomers into a precise hexagonal network. Decorin was also reported to form similar complexes with the collagen but at a much slower rate. Most of these experiments were performed using pepsin-treated collagen VI, which lacks most of the globular amino- and carboxyl-terminal domains of the molecule. However, native collagen VI was reported to form similar aggregates, at least with biglycan (34). It was speculated that biglycan may be involved in the assembly of similar networks found in tissues such as cornea (28). The binding site for both decorin and biglycan has been located close to the amino-terminal end of the central triple helical region of the collagen VI molecule (33). In a recent study we have shown that big-h3 is covalently bound to collagen VI in some tissues and that the binding site is also close to the amino-terminal end of the triple helix (32). In the present study we have addressed the possibilities that big-h3 might inhibit or enhance biglycan and/or decorin interactions with collagen VI and also may have a positive or negative influence on collagen VI aggregation. For these experiments we have used both pepsin-treated and native collagen VI. The latter was purified from skeletal muscle as the collagen from this tissue was found to be substantially free of big-h3 in contrast to collagen VI sourced from ligament or cornea, which contains covalently bound big-h3 at a ratio of about one big-h3 molecule per tetrameric collagen molecule. To ensure that the native collagen VI was free of non-covalently bound contaminants it was purified by size exclusion chromatography and equilibrium density gradient centrifugation (36).

In co-immunoprecipitation experiments, rbig-h3 was found to directly interact with both biglycan and decorin, with $K_d$ values estimated as $5.88 \times 10^{-8}$ and $1.02 \times 10^{-7}$ M, respectively. These interactions appeared to be of the same order of magnitude but significantly weaker than the non-covalent interaction of big-h3 with collagen VI ($K_d = 1.6 \times 10^{-8}$ M (32)) and those of the proteoglycans with the col-
Big-h3 Interactions with Biglycan, Decorin, and Collagen VI

Big-h3, also known as fibulin-3, is a protein that can differentially modulate the interactions of biglycan and decorin with collagen VI in vitro. The results indicate that big-h3 can influence the rate of formation, size, and shape of such aggregates. Because big-h3 is covalently bound to collagen VI in tissues such as ligament and cornea, it may be important for tailoring the organization of these complexes in a range of tissue situations. The disruption of the normal interactions between big-h3 and collagen VI, and the SLRPs may contribute to the phenotype of big-h3-linked corneal dystrophies that are characterized by the development of abnormal big-h3-containing deposits in the corneal matrix. In contrast, the very low levels of big-h3 found in skeletal muscle make it unlikely that big-h3 contributes to the critical function of collagen VI in this tissue as illustrated by the phenotypic mani-

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2 E. Hansen and M. Gibson, manuscript in preparation.
festations of collagen VI-linked muscular disorders, Bethlem myopathy, and Ullrich dystrophy (30, 31).

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Big-h3 Interactions with Biglycan, Decorin, and Collagen VI