jbc The Journal of Biological Chemistry
AFFINITY SITES
ASBMB
American Society for Biochemistry and Molecular Biology

Glycobiology and Extracellular Matrices:

Biologically Active Decorin Is a Monomer in Solution

GLYCOBIOLOGY AND EXTRACELLULAR MATRICES

Silvia Goldoni, Rick T. Owens, David J. McQuillan, Zachary Shriver, Ram Sasisekharan, David E. Birk, Shelly Campbell and Renato V. Iozzo

J. Biol. Chem. 2004, 279:6606-6612.
doi: 10.1074/jbc.M310342200 originally published online December 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M310342200

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 21 of which can be accessed free at
http://www.jbc.org/content/279/8/6606.full.html#ref-list-1

Downloaded from http://www.jbc.org/ by guest on November 3, 2014

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 8, Issue of February 20, pp. 6606–6612, 2004
Printed in U.S.A.
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

Biologically Active Decorin Is a Monomer in Solution*

Received for publication, September 17, 2003, and in revised form, November 24, 2003
Published, JBC Papers in Press, December 3, 2003, DOI 10.1074/jbc.M310342200

Silvia Goldoni‡, Rick T. Owens§, David J. McQuillan§, Zachary Shriver¶, Ram Sasisekharan¶, David E. Birk‡, Shelly Campbell‡, and Renato V. Iozzo‡‖**

From the ‡Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, §LifeCell Corporation, Branchburg, New Jersey 08876, ¶Division of Bioengineering and Environmental Health, Massachusetts Institute of Technology, Boston, Massachusetts 02139, and ‖Cellular Biology and Signaling Program, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

It has been reported that decorin and its protein core can have molecular masses nearly double the size of those previously published, suggesting a dimeric structure. In this study we tested whether biologically active decorin and its glycoprotein core would form dimers in solution. We used homo- and hetero-bifunctional chemical cross-linking reagents, BS^3 and sulfo-SMPB, respectively, as well as glutaraldehyde and found no preferential dimer formation, whether chemical cross-linking was performed in the presence or absence of live cells. Under the same experimental conditions, we easily detected dimers of epidermal growth factor receptor and basic fibroblast growth factor, two glycoproteins known to dimerize. Only at very high cross-linker to decorin molar ratios (2000:1) were trimers and multimers observed, but performing the chemical cross-linking in the presence of a reducing agent abolished these. The elution of decorin protein core in Superose 6 gel chromatography gave masses compatible with monomeric proteins, both before and after denaturation with 2.5 M guanidine HCl. Matrix-assisted laser desorption ionization gave a mass of 44,077 Da for decorin protein core, without any evidence of dimers or oligomers. Extensive oligomerization of the decorin protein core was observed only after dialysis against water and freeze-drying. These oligomers were considered artifacts because they were independent of chemical cross-linking and were resistant to heat denaturation and disulfide-bond reduction. Oligomeric preparations showed markedly reduced biological activity in both phosphorylation and collagen fibrillogenesis assays. Thus, biologically active decorin is a monomer in solution and, as such, is a monovalent ligand for various extracellular matrix proteins, growth factors, and cell surface receptors.

Decorin, the prototypic member of an enlarging family of small leucine-rich proteoglycans (1–5), has attracted considerable attention in the past decade primarily because of its ability to affect several key biological processes. Decorin is known to modulate growth factor activity (6), collagen fibrillogenesis (7–11), receptor tyrosine kinase activity (12, 13), cancer growth (14–18), angiogenesis (19–21), tissue remodeling (22–24), bacterial infection (25), and cardiovascular (26) and periodontal (27) diseases. This multiplicity of functions is due to the unusual structure of the decorin glycoprotein core which harbors 12 leucine-rich repeats flanked by cysteine-rich regions with four at the N terminus and two at the C terminus. Molecular modeling has predicted a horseshoe shape for the decorin core (28), with the inner face made up of parallel β-strands thought to bind directly to various ligands such as collagen type I (29–32), transforming growth factor-β (33), or EGFR[1] (13). This view implies that the biologically active form of decorin is a monomer in solution and thus a monovalent ligand for various extracellular matrix proteins and surface receptors. However, a recent paper (34) has challenged this body of evidence by reporting that the predominant form of decorin in solution is a dimer. According to this report, the inner concave face of decorin, a structural feature that has been proposed to provide sites for protein-protein interaction in the prototype ribonuclease inhibitor (35) as well as other leucine-rich repeat proteins (36), is indeed facilitating the dimerization.

In the present study, we provide several lines of evidence that decorin proteoglycan and its glycoprotein core are primarily, if not exclusively, monomers in solution. First, we purified recombinant decorin under non-denaturing conditions from the secretions of cultured human fibrosarcoma or 293 embryonic kidney cells and verified its biological activity by specific interaction with the EGFR. Second, this decorin preparation was tested in affinity chemical cross-linking reactions using several cross-linking agents of diverse functionality and bridge length, in the presence of live cells and in cell-free systems. In all cases, there was no preferential dimer formation. In contrast, we detected significant dimerization of both EGFR and FGF2, two glycoproteins known to dimerize. Only at very high cross-linker to decorin molar ratios (2000:1) were trimers and multimers observed, but these could be abolished by a reducing agent. Third, the elution of decorin protein core in Superose 6 gel chromatography gave masses compatible with the monomeric proteins, both before and after denaturation with the chaotropic agent GdnHCl. Fourth, MALDI-MS gave a mass of 44,077 Da for decorin protein core, without any evidence for dimers or oligomers. Finally, extensive oligomerization of

* This work was supported in part by National Institutes of Health Grants RO1 CA39481 and RO1 CA47282, Department of the Army Grant DAMD17-00-1-0425 (to R. V. I.), by National Institutes of Health Grants RO1 AR42826 (to D. J. M.) and RO1 EY05129 and RO1 AR44745 (to D. E. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. of Pathology, Anatomy and Cell Biology, Room 249 Jefferson Alumni Hall, Thomas Jefferson University, 1020 Locust St., Philadelphia, PA 19107. E-mail: iozzo@lac.jci.tju.edu.

[1] The abbreviations used are: EGFR, epidermal growth factor receptor; FGF2, basic fibroblast growth factor; sulfo-SMPB, sulfosuccinimidyl-4-(p-maleimidophenyl) butyrate; BS^3, bis(sulfosuccinimidyl) suberate; MAPK, mitogen activated protein kinase; βME, β-mercaptoethanol; TBS, Tris-buffered saline; GdnHCl, guanidine hydrochloride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; BSA, bovine serum albumin; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; PBS, phosphate-buffered saline.

This paper is available on line at http://www.jbc.org

Downloaded from http://www.jbc.org/ by guest on November 3, 2014

decorin protein core was observed after dialysis against water and freeze-drying, and these preparations lost activity in phosphorylation and collagen fibrillogenesis assays. We conclude that biologically active decorin and its glycoprotein core are monomers in solution.

EXPERIMENTAL PROCEDURES

Materials—All the chemicals unless specified were obtained from Sigma. Dulbecco's modified Eagle's medium, fetal bovine serum, 100× antibiotic-antimycotic solution, and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Mediatech (Herndon, VA). Recombinant human EGF and FGF2 were purchased from Promega (Madison, WI). Bis(sulfosuccinimidyl) suberate (BS^3) and sulfosuccinimidyl-4-(p-maleimidophenyl)butyrate (sulfo-SMPB) were purchased from Pierce. Solutions of cross-linkers were freshly prepared before each experiment. Glutaraldehyde (25% solution) EM grade (Electron Microscopy Sciences, Washington, PA) was stored in aliquots at −80 °C. Nitrocellulose membrane was purchased from Bio-Rad. Antibodies include polyclonal rabbit antibodies against the N-terminal region of decorin (37), against the C-terminal region of the EGF receptor (sc-03, Santa Cruz Biotechnology, Santa Cruz, CA), against FGF2 (sc-79, Santa Cruz Biotechnology), against phospho-p44/42 MAPK (Cell Signaling Technology, Beverly, MA), and a monoclonal antibody against phosphotyrosine (PY20, BD Transduction Laboratories, San Diego, CA). Horseradish peroxidase-conjugated donkey anti-rabbit and sheep anti-mouse antibodies were purchased from Amersham Biosciences. SuperSignal West Pico chemiluminescent substrate was purchased from Pierce.

Expression and Purification of Recombinant Decorin and Biglycan and Gel Filtration Chromatography—Recombinant decorin was produced by using either a recombinant vaccinia virus expression system or a stably transfected 293-EBNA cell line. Generation of the recombinant vaccinia virus encoding the mature form of decorin (38) and biglycan (39) has been described previously. This system was adapted for large-scale expression using a Celligen Plus bioreactor (New Brunswick Scientific, Edison, NJ) packed with Fibra-Cel disks. In brief, HT-1080 cells were seeded in the bioreactor and allowed to grow to saturation. At that time, recombinant virus was added in serum-free media, and the infection was allowed to proceed for 48 h. Serum-free media was then collected, and the recombinant polyhistidine decorin fusion protein was purified by nickel-chelating chromatography and elution with a gradient of 0–250 mM imidazole in 20 mM Tris-HCl, 500 mM NaCl, 0.2% CHAPS, pH 8.0. Fractions containing decorin were pooled, concentrated, and desalted into PBS containing 0.2% CHAPS.

The decorin-expressing 293-EBNA cell line was created by transferring the vaccinia decorin construct into the pCEP4 (Invitrogen) expression vector. After transfection, stable expressing cells were selected with hygromycin. Cells were then grown to saturation in the Celligen Plus bioreactor, and protein production was achieved by switching to serum-free culture media. Conditioned media was collected every 48 h. After concentration of the conditioned media using a Pellicon 2 Tangential Flow system (Millipore, Bedford, MA), recombinant decorin was purified as described above. Both expression systems resulted in the production of protein core and proteoglycan forms of decorin. In some experiments, protein core was separated from proteoglycan after anion-exchange chromatography on Q-Sepharose and elution with a linear gradient of 0.15–2 M NaCl in PBS, 0.2% CHAPS. Protein core samples were analyzed by gel filtration chromatography before and after dialysis against water and freeze-drying. Dried proteins were resuspended in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.0) and chromatographed on Superose 6 HR 10/30 (Amersham Biosciences) in TBS with or without 2.5 M GdnHCl.

Biological Activity of Decorin and Cross-linking Experiments—To assess the biological activity of our decorin and decorin protein core preparations, confluent cultures of A431 squamous carcinoma cells (40) were serum-starved for 16 h and incubated for various times at 37 °C with decorin or its protein core (0.5–1 μM) or EGF (16 nM) in 400 μl total volume of DPBS (2.5 mM Ca^{2+}), 20 mM HEPES, pH 7.5. The cell lysates were subjected to immunoblotting with anti-P-tyrosine, anti-EGFR, and anti-P-MAPK antibodies.

Fibrillogenesis assays were done essentially as described previously (41). Briefly, at 4 °C, stock solutions (1–2 mg ml^{-1}) of pepsin-extracted, bovine dermal type I collagen were neutralized with 10× PBS and brought to the desired concentration with 1× PBS (15 mM sodium phosphate, 0.15 M NaCl, pH 7.2). Decorin protein core was added to aliquots of the mixture at 25–50 μg ml^{-1}. The samples were transferred to the water-jacketed sample holder of a Beckman DU640 spectrophotometer, and assays were performed at 37 °C.

Chemical cross-linking reactions were performed in the presence of A431 cells or in cell-free experimental conditions. In the former case, cells were incubated with decorin for 30 min, and then the cross-linker sulfo-SMPB (1 mM) was added for 20–30 min at 37 °C before being quenched with 90 mM (final concentration) glycine, 9 mM Tris-HCl, pH 8.5, for 5 min. After the cross-linking reaction, 250-μl aliquots were collected and precipitated with 1.5 ml of ethanol/potassium acetate at −20 °C for 18 h. Samples were subjected to a linear gradient SDS-PAGE (3–15% (w/v) gel) with the addition of reducing agent (0.2% βME). In the cell-free experiments, chemical cross-linking was carried out in a 40-μl volume containing either decorin or decorin protein core (100–200 nM) in DPBS (2.5 mM Ca^{2+}) in the presence or absence of 0.2% β ME. 10-μl aliquots of each serially diluted cross-linker (sulfo-SMPB, BS^3, or glutaraldehyde) were added to the mixture and incubated for 30 min at room temperature with gentle shaking. The samples were quenched as above, boiled for 5 min, and subjected to SDS-PAGE (3–15% linear gradient and 8.5% gel for decorin and its protein core, respectively) under reducing conditions. Western immunoblottings to detect decorin were carried out with specific antibodies (37, 38). For additional information, see the text and figure legends.

MALDI-MS—Sinapinic acid (~10 mg ml^{-1}) in 30% (v/v) acetonitrile was used as a matrix solution. About 1 pmol of myoglobin, carbonic anhydrase, or decorin protein core was added to the matrix at 1:10 ratio. After crystallization, the surfaces were washed with water, dried under a stream of nitrogen, and placed into the mass spectrometer. MALDI-MS spectra of the protein preparations were acquired in the linear mode with a Voyager Elite Reflectron time-of-flight instrument (PerSeptive Biosystems) fitted with a 337-nm wavelength nitrogen laser essentially as described before (42). Decorin protein core preparations were analyzed under several different instrument conditions with several different protein concentrations and under conditions that have previously been shown to be amenable to measuring dimer formation for FGF2 (42).

RESULTS AND DISCUSSION

Biologically Active Decorin Does Not Form Dimers in Solution with Three Cross-linkers of Diverse Bridge Length and Functional Properties—Our recombinant decorin preparation, which was kept in solution at a concentration of ~1–1.5 mg ml^{-1} (38), contained about 85% decorin proteoglycan, ~13% protein core, and only <2% dimer/trimer, as judged by scanning densitometry on reducing SDS-PAGE (Fig. 1*A*, *lanes 1-3*). To mimic a physiologically relevant situation, we incubated recombinant decorin in the presence of A431 cells for 30 min at 37 °C, followed by a 30-min incubation at 37 °C with sulfo-SMPB, a water soluble, non-cleavable, heterobifunctional cross-linker. Sulfo-SMPB has an extended chain length of 14.5 Å, which limits steric hindrance, and it is reactive toward amino and sulfhydryl groups. The latter property provides hetero-bifunctionality and allows for sequential conjugations with specific groups of proteins, thereby minimizing undesirable polymerization or self-conjugation. Notably, at 1 mM sulfo-SMPB, equivalent to a 1300:1 cross-linker to decorin molar ratio, no preferential dimer formation was observed (Fig. 1*A*, *lane 5*). Only small amounts of trimers and multimers were formed as compared with control (Fig. 1*A*, *lane 4*). Under the same conditions, sulfo-SMPB was capable of cross-linking the EGF-induced EGFR dimers in A431 cells (not shown). The biological activity of decorin was independently proved by its ability to inhibit EGFR phosphorylation while inducing the phosphorylation of the MAPK for prolonged times (not shown), which is in agreement with previous results (12, 40).

In cross-linking studies, it is imperative to ensure that the chemical covalent cross-linking is an accurate reflection of specific protein-protein interactions rather than an artifact of adventitious cross-linking (42). To this end, we performed a detailed analysis of various cross-linkers with different properties and bridge lengths. The spanning arm of a cross-linker is the length between its two reactive groups. The length affects the distance over which the reagent can establish a bond between two primary amines of closely associated proteins. We performed *in vitro* cell-free cross-linking experiments in which we

Downloaded from http://www.jbc.org/ by guest on November 3, 2014

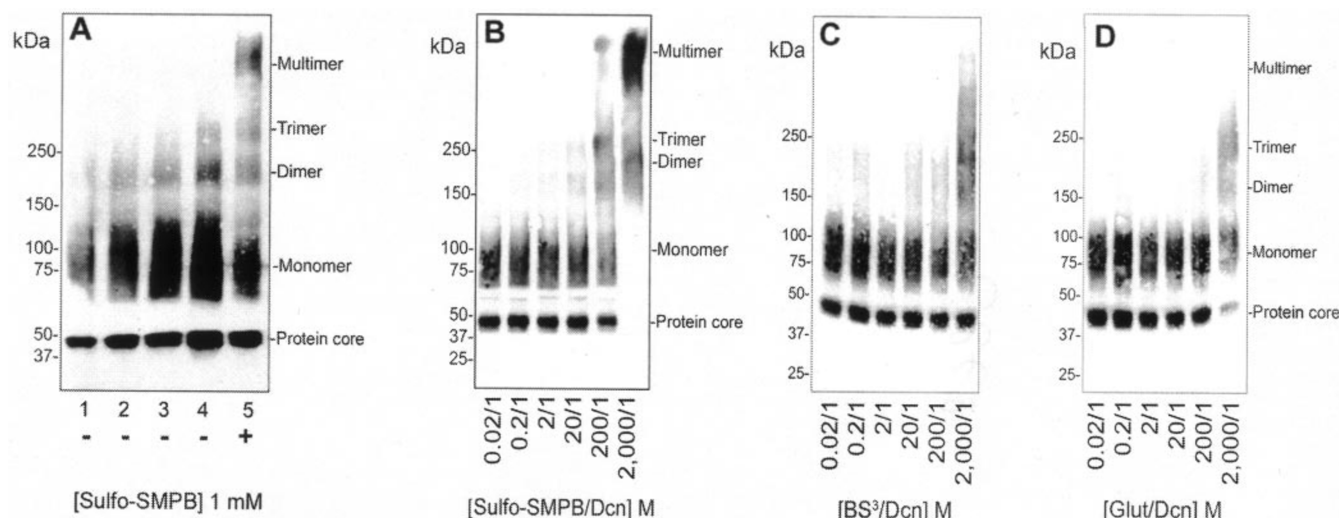

FIG. 1. **Biologically active decorin does not form dimers in solution with three cross-linkers of diverse bridge length and functional properties.** *A*, immunoblotting of increasing concentrations of decorin in a cell-free isotonic buffer of 200, 400, and 600 ng (*lanes 1–3*, respectively) or incubated with A431 cells for 30 min at 37 °C, followed by a 30-min incubation at 37 °C with either buffer (*lane 4*) or 1 mM sulfo-SMPB (*lane 5*). The concentration of decorin (*lanes 4* and *5*) was 0.75 μM, and, thus, the cross-linker to decorin molar ratio was ~1300:1. Proteins were electrophoresed on linear gradient (3–15%) SDS-PAGE under reducing conditions. A polyclonal rabbit anti-decorin antibody (37) was used at 1:4000 dilution, followed by detection via chemiluminescence. *B–D*, immunoblottings of decorin preparations (100 nM each) incubated with increasing concentrations of sulfo-SMPB (*B*), BS^3 (*C*), or glutaraldehyde (*Glut*, *D*) at the indicated molar ratios. The cell-free samples were subjected to chemical cross-linking for 30 min at room temperature, quenched with 90 mM glycine, 9 mM Tris-HCl, pH 8.5, for 5 min, heat denatured at 100 °C for 5 min in the presence of 0.2% βME, and subjected to a linear (3–15%) SDS-PAGE. The migration of molecular mass markers is indicated in the *left margins*, whereas the migration of the protein core, decorin monomers, dimers, trimers, and oligomers is indicated in the *right margins*.

kept the concentration of decorin constant (100 nM) while varying the cross-linker to decorin molar ratios, from 0.02 to 2000:1. In the case of sulfo-SMPB (chain length = 14.5 Å), dimers and multimers were formed only at very high molar ratios (Fig. 1*B*), which probably represented nonspecific polymerization of decorin and its protein core (see below). We then utilized BS^3, a non-cleavable, membrane impermeable, homo-bifunctional cross-linker with a chain length of 11.4 Å and reactivity toward amino groups. In this case, we found very little aggregate formation even at 2000:1 molar ratio of cross-linker to decorin (Fig. 1*C*). Finally, we tested glutaraldehyde, a widely used agent which is also a lysine-specific cross-linker but has a chain length (2.85 Å) about one-quarter that of BS^3. Even at 2000:1 molar ratio of cross-linker to decorin there was no preferential formation of dimers in solution (Fig. 1*D*). Identical results were obtained with and without physiological concentrations (1.5 mM) of Ca^{2+} (not shown). Collectively, these data suggest that decorin does not preferentially exist as a dimer in solution.

Dimers of Decorin Protein Core Are Generated by Non-specific Disulfide Bonding and Are Induced by Freeze-drying—The previous results could have been obfuscated by the fact that dimers of decorin protein core would co-migrate with decorin monomers. To bypass this potential problem, we utilized highly purified preparations of decorin protein core and performed cross-linking experiments in the presence or absence of βME. The latter was used to eliminate the possibility of covalent oligomerization due to disulfide bond formation among the surface-exposed cysteine residues of the native protein core. Trace amounts of decorin protein core dimers were formed only with BS^3 to protein molar ratios of 2000:1 (Fig. 2*A*), but not with glutaraldehyde. Notably, the BS^3-induced dimers could be abolished by performing the cross-linking reaction in the presence of 0.2% βME (Fig. 2*B*).

Next, we tested whether dimers of decorin protein core could be induced by prior freeze-drying, because removal of the water forces proteins to nonspecifically self-associate. We performed two sets of cross-linking experiments. In the first, protein core preparations in PBS/0.2% CHAPS were directly freeze-dried and then resuspended in the same volume as the controls. In these samples, we found an increased propensity to form dimers at lower (200:1) BS^3 to protein core ratios, and a significantly higher proportion of dimers at BS^3 to protein core molar ratios of 2000:1 (Fig. 2*C*), when compared with control samples that were not previously freeze-dried (Fig. 2*A*). However, all the dimers were non-specifically cross-linked due to disulfide bonding, because they were completely eliminated by performing the chemical cross-linking reaction in the presence of βME (Fig. 2*C*). In the second set of experiments, protein core preparations in PBS/0.2% CHAPS were extensively dialyzed against water, freeze-dried, and then re-suspended in the same volume of reconstituted buffer as the controls. In these samples, we detected dimers that were not prevented by βME (Fig. 2*D*). Moreover, trimers, tetramers, and higher order oligomers of decorin protein core appeared in both reduced and unreduced samples.

As a further control for our cross-linking studies, we investigated FGF2, which is known to oligomerize in solution (42). Under the same experimental conditions, there was a stable dimer formation at very low (0.02:1) cross-linker to FGF2 molar ratio, as expected for a protein with a propensity to self-associate (Fig. 2*E*). Moreover, the dimer increased proportionally to the increase in cross-linker to FGF2 molar ratios, and at 20:1 molar ratio, a trimer appeared. At higher BS^3 to FGF2 molar ratios, the product became a large aggregate that did not penetrate the gel (Fig. 2*E*). Quantification by scanning densitometry revealed a dose-dependent increase in dimer formation (Fig. 2*F*). Because we used the same experimental conditions as those described above for decorin or its protein core, we conclude that the lack of detection of decorin dimers is not a failure of our cross-linking strategy but rather a lack of decorin dimers. Moreover, because the three cross-linkers with different chemical mechanisms and linker geometries yielded the same results, steric or chemical inaccessibility of cross-linking sites is unlikely. This is particularly true because the amino acid targets for all of the three cross-linkers are abundantly located along the decorin protein core. It is noteworthy that in

Downloaded from http://www.jbc.org/ by guest on November 3, 2014

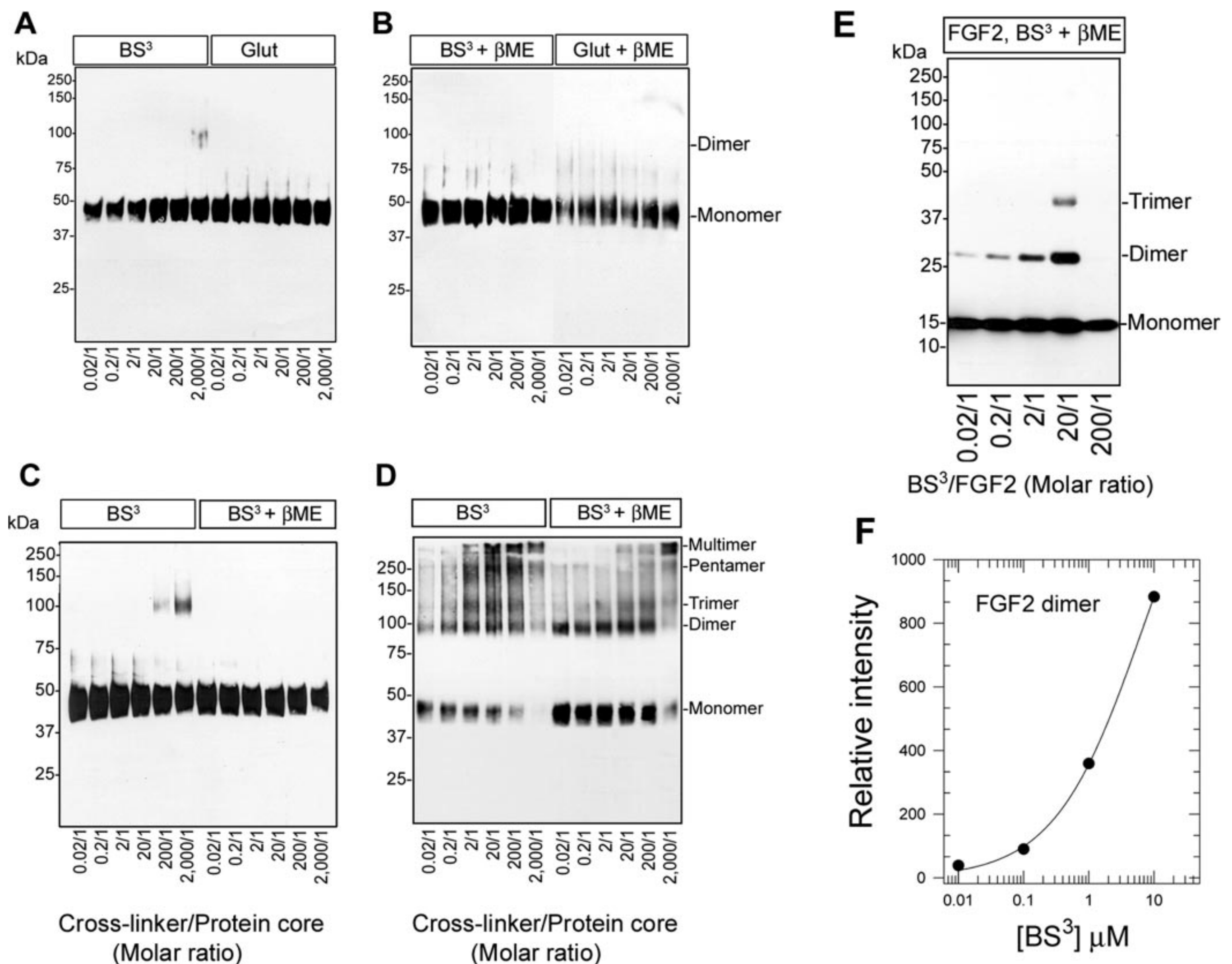

FIG. 2. **Dimers of decorin protein core are generated by nonspecific disulfide bonding and are induced by freeze-drying.** *A*, immunoblotting of decorin protein core (200 nM each) incubated with increasing molar concentrations of BS^3 or glutaraldehyde (*Glut*) at the indicated molar ratios. *B*, same as in *A* but in the presence of 0.2% βME. *C*, same as in *B*, with the exception that the decorin protein core samples were directly freeze-dried in PBS/0.2% CHAPS. *D*, same as in *C* with the exception that the decorin protein core preparations were first dialyzed against water and then freeze-dried. In all cases, the samples were resuspended in the same volume of reconstituted buffer as the controls. Chemical cross-linking and Western immunoblotting were performed as described in Fig. 1 but using an 8.5% SDS-PAGE. The migration of molecular mass markers is indicated in the *left margins*, whereas the migration of the decorin protein core and oligomers is indicated in the *right margin*. *E*, Western immunoblotting of recombinant FGF2 (500 nM) incubated with increasing molar concentrations of BS^3 and 0.2% βME and analyzed on a liner 4–20% gradient SDS-PAGE. *F*, quantification of FGF2 dimer using Scion image analysis software. The values represent the relative intensity (OD) of the blot shown in *E* as a function of increasing BS^3 concentration.

the experimental conditions used in our study, all of the samples were heat-denatured at 100 °C for 5 min under reducing conditions before SDS-PAGE. Thus, the presence of insoluble complexes, which are resistant to heat denaturation/reduction and are independent of chemical cross-linking, indicate that they are artifactual products resulting from dialysis and freeze-drying.

A potential problem with our recombinant decorin protein core is the presence of a His_6 tag at the N terminus (38), which could potentially interfere with dimer formation in solution. To address this issue, we digested the His_6 tag with Factor Xa, which was later removed by binding to benzamidine-Sepharose. The cleavage of the His_6 tag and bridge sequence was confirmed by SDS-PAGE and N-terminal sequencing. The latter, obtained with an Applied Biosystem Procise Sequencer, gave the predicted first nine amino acid residues of human decorin plus an initial Gly belonging to the bridge sequence (GMLEDEASGI). When this preparation was used in experiments identical to those described above, we found no dimers or oligomers using BS^3 to protein ratios between 0.02:1 and 2000:1 and a constant concentration (~300 nM) of cleaved decorin protein core (not shown).

As an additional control, we tested biglycan protein core because it was shown previously to oligomerize in solution, a process further enhanced by the presence of Zn^{2+} (43). It has been reported that bovine articular cartilage biglycan, at concentrations >50 μg ml^{-1} and in the presence of 5 mM Zn^{2+}, existed predominantly as a hexamer with a *z*-average molecular weight of $\sim 6 \times 10^5$ (43). The biglycan protein core formed even larger multimers, with a *z*-average molecular weight of $\sim 6 \times 10^6$, suggesting that the glycosaminoglycan chains could limit self-association (43). However, by using the same cross-linking conditions as those described above, we failed to detect any dimerization or oligomerization of biglycan protein core in the presence or absence of Zn^{2+} (not shown). Notably, in the original purification of biglycan (43), the proteins were used at a relatively high concentration (~5 mg ml^{-1}) and subjected to a final step of ethanol precipitation and drying under a vacuum

Downloaded from http://www.jbc.org/ by guest on November 3, 2014

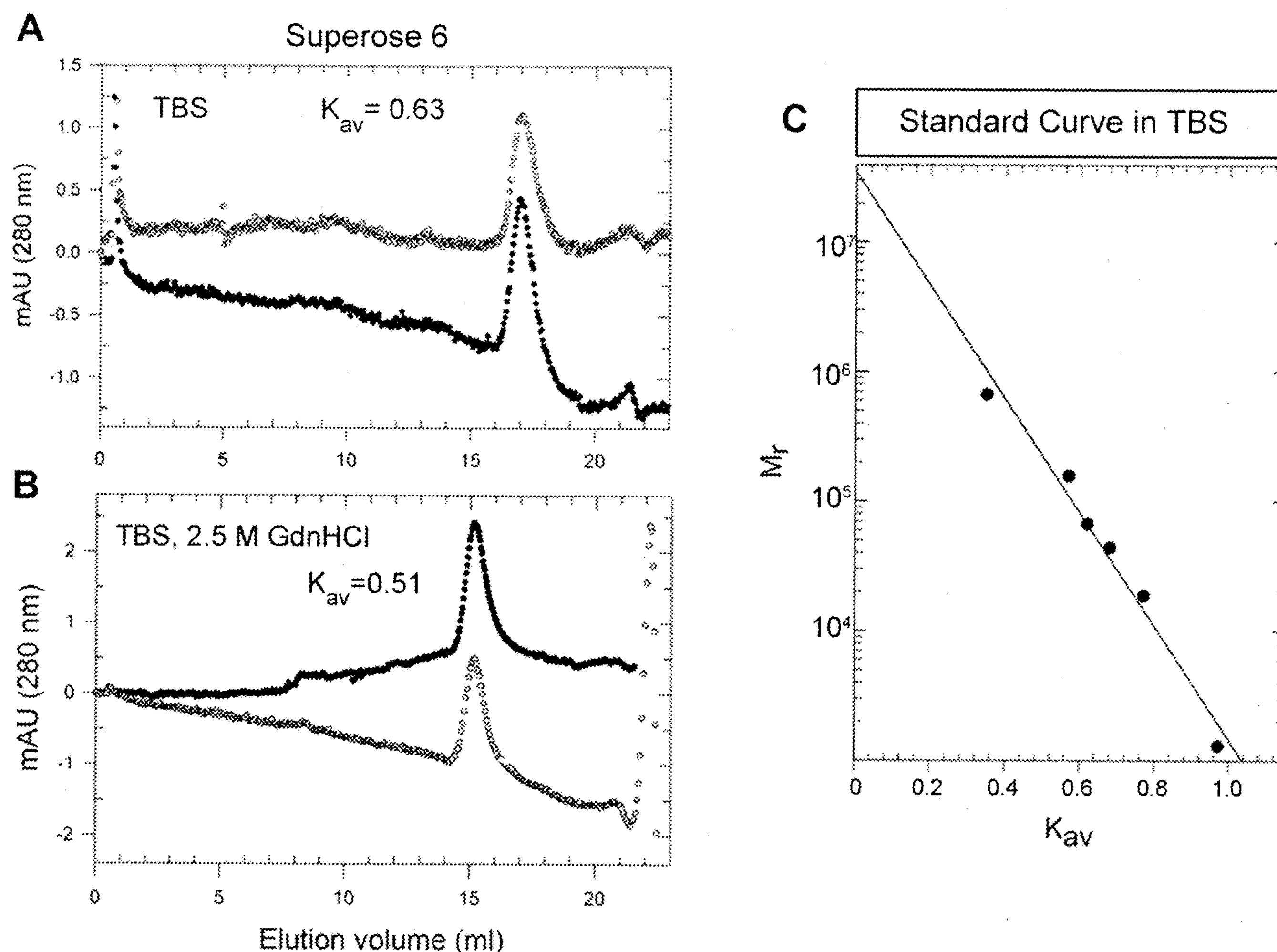

Fig. 3. **Effects of GdnHCl on the elution behavior of decorin protein core from Superose 6 chromatography.** *A*, elution profiles of decorin protein core samples run in TBS before (●) or after (○) freeze-drying. *B*, elution profiles of decorin protein core run in TBS, 2.5 M GdnHCl before (●) or after (○) freeze-drying. *C*, semilogarithmic plot of K_{av} *versus* log M_r for various standard proteins including thyroglobulin (667 kDa), IgG (158 kDa), BSA (66 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.3 kDa) run in the same Superose 6 column equilibrated with TBS.

(43). Thus, biglycan aggregates could have likely been produced by a high concentration of the sample and subsequent removal of salts and water.

Decorin Protein Core Behaves as a Monomer when Chromatographed on Superose 6 or Analyzed by MALDI-MS—In the next set of experiments, we wanted to test whether our preparations of decorin protein core behaved as a monomer when chromatographed on a Superose 6 column and whether their elution behavior in the presence or absence of the chaotropic agent GdnHCl would change *vis à vis* that of standard globular proteins. Notably, both the freeze-dried and native protein cores eluted as a single peak in TBS with K_{av} = 0.63 (Fig. 3*A*), which is just after the elution position of BSA (K_{av} = 0.62, Fig. 3*C*). This gives an estimated M_r of ~55 kDa for the decorin core, a value that supports a monomeric protein. When the samples were denatured with 2.5 M GdnHCl, either directly or after freeze-drying, the elution of both samples of decorin protein core was slightly retarded (K_{av} = 0.51, Fig. 3*B*). Notably, however, BSA (66 kDa) and the IgG heavy chain (55 kDa) had a K_{av} of 0.529 and 0.535, respectively, when chromatographed on the same column and buffer (TBS, 2.5 M GdnHCl). Thus, the results are not consistent with a dimeric nature of the decorin protein core because the protein nearly co-elutes with BSA in the presence or absence of GdnHCl.

In the samples that were dialyzed against water and then freeze-dried, a large peak (>5 × 10^6 kDa) was detected in the void volume (not shown). This material could be eliminated by centrifugation prior to Superose 6 chromatography, further indicating that removal of salts and freeze-drying lead to the formation of higher order oligomers of decorin protein core, in agreement with the data shown above (Fig. 2*D*).

We then tested similar preparations of biologically active decorin protein core with MALDI-MS, a powerful technique that can measure directly whether a given polypeptide can self-associate. Indeed, after bringing the samples to dryness, the decorin protein core was still diluted *vis à vis* the large excess of matrix (1:10, protein to matrix ratio), thereby allowing the material to be analyzed at a concentration comparable with those found *in vivo*. Consistent with the fact that decorin is heavily glycosylated, the signal for decorin resulted in a broader *m/z* range (Fig. 4*C*) than either myoglobin (Fig. 4*A*) or carbonic anhydrase (Fig. 4*B*), with an $[M]^+$ signal centered at 44,077. If one subtracts 1.6 kDa, given by the His_6 tag and bridge sequence at its N terminus, the resulting value of 42.4 kDa is nearly identical to the molecular mass of ~42.3 kDa that can be calculated for the decorin protein core (~36.3 kDa for the polypeptide and ~6 kDa for the three *N*-linked oligosaccharides). Importantly, under no situation, *i.e.* using several protein concentrations between 0.2 and 1.2 μM, was any oligomer (including dimer) observed, even under conditions that

Downloaded from http://www.jbc.org/ by guest on November 3, 2014

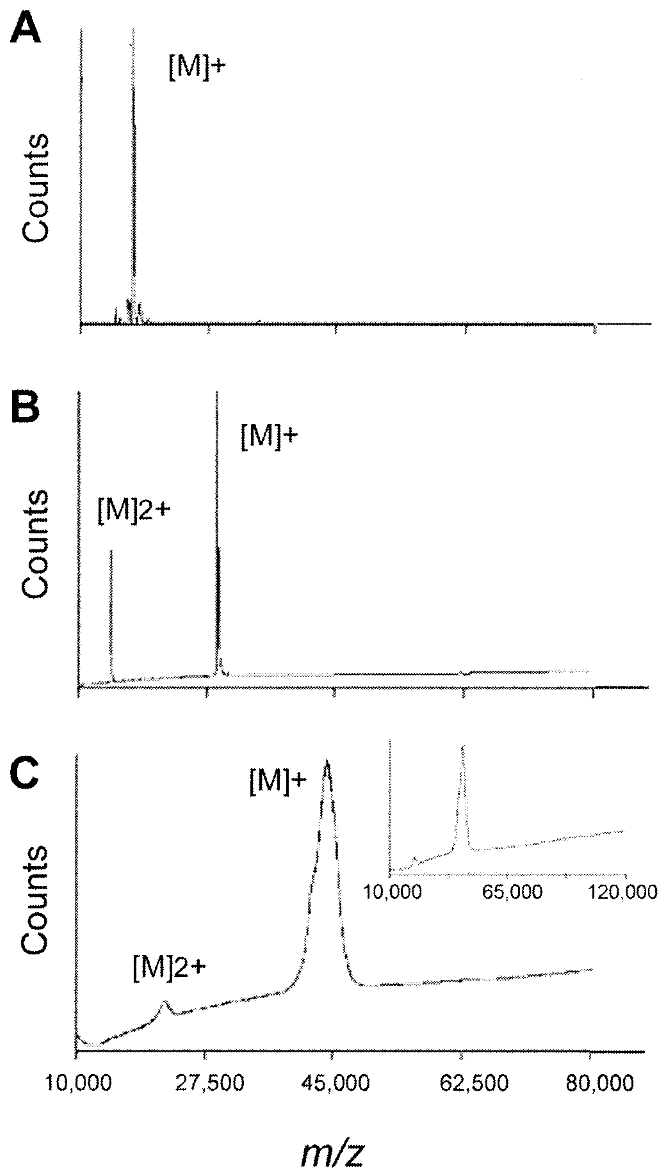

FIG. 4. **MALDI-MS analysis of decorin protein core.** *A* and *B*, representative mass spectra of myoglobin (theoretical *m/z* [+1 ion] of 16,957) and carbonic anhydrase (theoretical *m/z* [+1 ion] of 28,870), respectively. The measured values, after calibration, of the $[M]^{1+}$ ion were 16,953 and 28,856 for myoglobin and carbonic anhydrase, respectively. *C*, representative mass spectra of decorin protein core. The $[M]^{1+}$ signal is centered at 44,077.

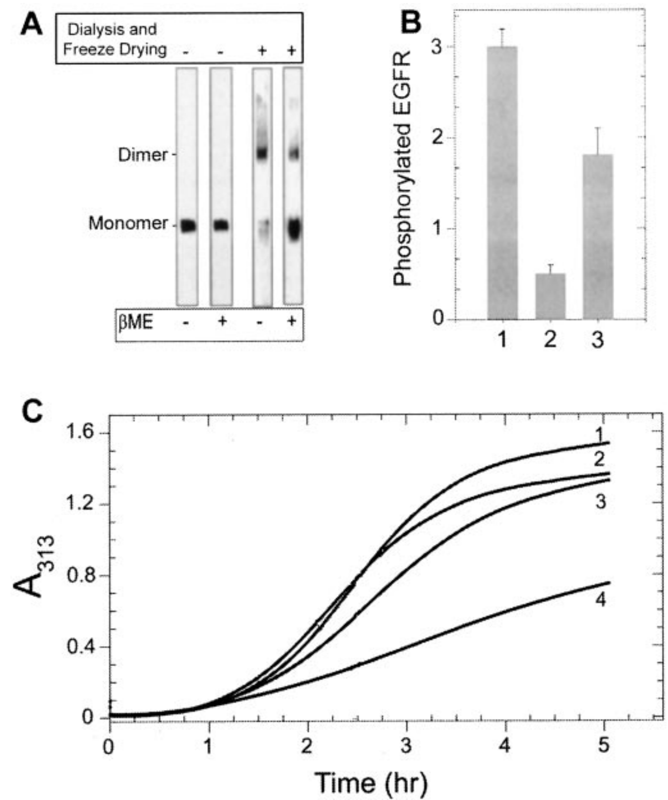

FIG. 5. **Dimeric decorin protein core shows reduced biological activity.** *A*, immunoblotting of decorin protein core (200 nM), which was either untreated or dialyzed against water and freeze-dried. Before SDS-PAGE, the samples were incubated for 30 min in the absence or presence of 0.2% βME, as indicated. *B*, quantification by scanning densitometry of the degree of tyrosyl phosphorylation of the EGFR in serum-starved A431 cells incubated for 30 min with buffer alone (*bar 1*), native (*bar 2*), or dialyzed/freeze-dried (×3) decorin protein core (~1.2 μM). The values represent the mean ± S.D. of three independent experiments obtained by immunoblotting of total cell lysates (~50 μg/lane) reacted with monoclonal antibody PY20 against phosphotyrosine, stripped, and re-probed with a rabbit polyclonal antibody against the C terminus of human EGFR. *C*, effects of decorin protein core on type I collagen fibrillogenesis. Untreated monomeric decorin protein core (*curve 4*) dramatically inhibits the kinetics of fibrillogenesis and reduces final turbidity, measured as A_{313}, compared with assays with type I collagen without protein core (*curve 2*). In contrast, assays with two different preparations of dialyzed/freeze-dried protein core (*curves 1* and *3*) were comparable with assays with no protein. This representative turbidity-time curve was consistent with those run at collagen concentrations of 1–2 mg ml^{-1} and protein core concentrations of 25–50 μg ml^{-1} at 37 °C.

have previously been shown to be amenable to measuring dimer formation for FGF2 (42).

Dimeric and Oligomeric Decorin Show Reduced Biological Activity—Based on the results presented above, we reasoned that if the dimeric and oligomeric protein cores were nonspecific aggregates, then these products should have a reduced biological activity. To test this hypothesis, we utilized decorin protein core (the same batch of protein core shown in Fig. 2) that was either untreated or dialyzed against water and freeze-dried. In the latter case, the predominant species was a dimer, which represented up to 70% of the entire preparation (Fig. 5*A*); this proportion was slightly reduced in the presence of βME. We then tested this preparation concurrently with native decorin protein core utilizing a highly sensitive assay, that is, EGFR phosphorylation in quiescent A431 squamous carcinoma cells. In three independent experiments, the dialyzed/freeze-dried decorin protein core showed a significantly reduced activity *vis á vis* the native decorin protein core (Fig. 5*B*, compare *bar 3* with *bar 2*). Because we used a significant amount of decorin protein core (~1.2 μM) in these experiments, it is likely that the inhibitory activity upon EGFR phosphorylation was due to the residual monomeric form of decorin protein core, which still constituted ~30% of the total protein supplied to the cells.

The effect of multimerization of decorin protein core on its biological activity was further analyzed using a collagen fibrillogenesis assay. Monomeric decorin protein core retarded the rate of fibrillogenesis and markedly reduced the final turbidity (Fig. 5*C*, *curve 4*) compared with type I collagen without protein core (Fig. 5*C*, *curve 2*). In contrast, when two protein core preparations that were dialyzed and freeze-dried were utilized in these turbidity-time assays, there was little, if any, effect on either the kinetics of fibril formation or final turbidity (Fig. 5*C*, *curves 1* and *3*). Fig. 5*C* is a representative assay, and the results were comparable with assays with collagen concentrations between 1 and 2 mg ml^{-1} and protein core concentrations between 25 and 50 μg ml^{-1}. These data indicate that multim-

Downloaded from http://www.jbc.org/ by guest on November 3, 2014

erization of the decorin protein core significantly reduces its biological activity in collagen fibrillogenesis assays.

The results of this study clearly point out that decorin and its glycoprotein core behave as monomeric proteins using several independent approaches. Also, biglycan protein core was found not to oligomerize. In contrast, dimers of EGFR and FGF2 could be easily detected under our experimental conditions, thereby validating the cross-linking experiments. The major difference in experimental design between the present work and that of Scott *et al.* (34) is that these authors have extensively dialyzed their samples against water and subsequently freeze-dried them. Moreover, the samples were resuspended at a relatively high concentration (~10 μM) and subsequently diluted to nM range (~60 nM). Because no decrease in average mass was found, it was concluded that the dimer was stable at very high dilutions and did not have a measurable dissociation constant under the conditions used in their experimental protocols. In our system, we obviously worked with monomers because our original preparations were similarly concentrated (11.9 μM for the decorin, 7.4 μM for the decorin protein core, and 10.6 μM for the cleaved protein core) and subsequently diluted to 100–300 nM. Thus, these preparations were sufficiently concentrated to allow proper protein-protein interaction. It is noteworthy that even higher numbers of M_r multimers were found with biglycan preparations, which were also subjected to removal of salt and water (43).

Our study provides strong evidence that decorin monomers are the biologically active forms and that decorin dimers are an *in vitro* artifact generated by removing water and salts, which causes nonspecific self-association during the freeze-drying step. In our experimental conditions, decorin proteoglycan and glycoprotein core preparations were always kept in solution under non-denaturing conditions, and they showed biological activity as monomers. Moreover, the reduced biological activity of the dimeric protein core preparations suggests that this conformation was not suited to interact with cell surface receptors and trigger a biological signal. Decorin and other leucine-rich repeat proteoglycans have been demonstrated to retard the kinetics of collagen fibrillogenesis and reduce final turbidity in collagen fibrillogenesis assays (10, 44, 45). The decorin protein core produced without exposure to denaturants was shown to inhibit the rate of fibrillogenesis and the final turbidity (46). Likewise, our data using the untreated decorin protein core showed identical effects. In contrast, when decorin protein core was multimerized by dialysis and freeze-drying, there was little effect on collagen fibrillogenesis. The fact that our monomeric preparations were fully active does not support the proposal that decorin is preferentially a dimer in solution and does not warrant re-evaluation of a large body of published literature on the biology of decorin.

Acknowledgments—We thank C. C. Clark, M. Nugent, and J. Hoek for valuable discussion and advice, C. C. Reed for help with the computer programs, and L. Fisher for the generous gift of antibodies.

REFERENCES

1. Iozzo, R. V. (1997) *Crit. Rev. Biochem. Mol. Biol.* **32,** 141–174
2. Hocking, A. M., Shinomura, T., and McQuillan, D. J. (1998) *Matrix Biol.* **17,** 1–19
3. Iozzo, R. V. (1999) *J. Biol. Chem.* **274,** 18843–18846
4. Neame, P. J., and Kay, C. J. (2000) in *Proteoglycans: Structure, Biology and Molecular Interactions* (Iozzo, R. V., ed) pp. 201–236, Marcel Dekker, Inc., New York, NY
5. Reed, C. C., and Iozzo, R. V. (2003) *Glycoconj. J.* **19,** 249–255
6. Yamaguchi, Y., Mann, D. M., and Ruoslahti, E. (1990) *Nature* **346,** 281–284
7. Toole, B. P., and Lowther, D. A. (1968) *Biochem. J.* **109,** 857–866
8. Toole, B. P. (1969) *Nature* **222,** 872–873
9. Vogel, K. G., Paulsson, M., and Heinegård, D. (1984) *Biochem. J.* **223,** 587–597
10. Rada, J. A., Cornuet, P. K., and Hassell, J. R. (1993) *Exp. Eye Res.* **56,** 635–648
11. Danielson, K. G., Baribault, H., Holmes, D. F., Graham, H., Kadler, K. E., and Iozzo, R. V. (1997) *J. Cell Biol.* **136,** 729–743
12. Iozzo, R. V., Moscatello, D., McQuillan, D. J., and Eichstetter, I. (1999) *J. Biol. Chem.* **274,** 4489–4492
13. Santra, M., Reed, C. C., and Iozzo, R. V. (2002) *J. Biol. Chem.* **277,** 35671–35681
14. Santra, M., Mann, D. M., Mercer, E. W., Skorski, T., Calabretta, B., and Iozzo, R. V. (1997) *J. Clin. Invest.* **100,** 149–157
15. Santra, M., Eichstetter, I., and Iozzo, R. V. (2000) *J. Biol. Chem.* **275,** 35153–35161
16. Iozzo, R. V., Chakrani, F., Perrotti, D., McQuillan, D. J., Skorski, T., Calabretta, B., and Eichstetter, I. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3092–3097
17. Reed, C. C., Gauldie, J., and Iozzo, R. V. (2002) *Oncogene* **21,** 3688–3695
18. Troup, S., Njue, C., Kliewer, E. V., Parisien, M., Roskelley, C., Chakravarti, S., Roughley, P. J., Murphy, L. C., and Watson, P. H. (2003) *Clin. Cancer Res.* **9,** 207–214
19. Schönherr, E., O'Connell, B. C., Schittny, J., Robenek, H., Fastermann, D., Fisher, L. W., Plenz, G., Vischer, P., Young, M. F., and Kresse, H. (1999) *Eur. J. Cell Biol.* **78,** 44–55
20. de Lange Davies, C., Melder, R. J., Munn, L. L., Mouta-Carreira, C., Jain, R. K., and Boucher, Y. (2001) *Microvasc. Res.* **62,** 26–42
21. Grant, D. S., Yenisey, C., Rose, R. W., Tootell, M., Santra, M., and Iozzo, R. V. (2002) *Oncogene* **21,** 4765–4777
22. Funderburgh, J. L., Hevelone, N. D., Roth, M. R., Funderburgh, M. L., Rodrigues, M. R., Nirankari, V. S., and Conrad, G. W. (1998) *Investig. Ophthalmol. Vis. Sci.* **39,** 1957–1964
23. Huttenlocher, A., Werb, Z., Tremble, P., Huhtala, P., Rosenberg, L., and Damsky, C. H. (1996) *Matrix Biol.* **15,** 239–250
24. Xu, G., Guimond, M.-J., Chakraborty, C., and Lala, P. K. (2002) *Biol. Reprod.* **67,** 681–689
25. Guo, B., Norris, S., Rosenberg, L. C., and Höök, M. (1995) *Infect. Immun.* **63,** 3467–3472
26. Kinsella, M. G., Fischer, J. W., Mason, D. P., and Wight, T. N. (2000) *J. Biol. Chem.* **275,** 13924–13932
27. Häkkinen, L., Strassburger, S., Kahari, V. M., Scott, P. G., Eichstetter, I., Iozzo, R. V., and Larjava, H. (2000) *Lab. Invest.* **80,** 1869–1880
28. Weber, I. T., Harrison, R. W., and Iozzo, R. V. (1996) *J. Biol. Chem.* **271,** 31767–31770
29. Svensson, L., Heinegård, D., and Oldberg, Å. (1995) *J. Biol. Chem.* **270,** 20712–20716
30. Schönherr, E., Hausser, H., Beavan, L., and Kresse, H. (1995) *J. Biol. Chem.* **270,** 8877–8883
31. Kresse, H., Liszio, C., Schönherr, E., and Fisher, L. W. (1997) *J. Biol. Chem.* **272,** 18404–18410
32. Keene, D. R., San Antonio, J. D., Mayne, R., McQuillan, D. J., Sarris, G., Santoro, S. A., and Iozzo, R. V. (2000) *J. Biol. Chem.* **275,** 21801–21804
33. Schönherr, E., Broszat, M., Brandan, E., Bruckner, P., and Kresse, H. (1998) *Arch. Biochem. Biophys.* **35,** 241–248
34. Scott, P. G., Grossmann, J. G., Dodd, C. M., Sheehan, J. K., and Bishop, P. N. (2003) *J. Biol. Chem.* **278,** 18353–18359
35. Kobe, B., and Deisenhofer, J. (1995) *Nature* **374,** 183–186
36. Kobe, B., and Kajava, A. V. (2001) *Curr. Opin. Struct. Biol.* **11,** 725–732
37. Fisher, L. W., Stubbs, J. T., III, and Young, M. F. (1995) *Acta Orthop. Scand.* **66,** 61–65
38. Ramamurthy, P., Hocking, A. M., and McQuillan, D. J. (1996) *J. Biol. Chem.* **271,** 19578–19584
39. Hocking, A. M., Strugnell, R. A., Ramamurthy, P., and McQuillan, D. J. (1996) *J. Biol. Chem.* **271,** 19571–19577
40. Csordás, G., Santra, M., Reed, C. C., Eichstetter, I., McQuillan, D. J., Gross, D., Nugent, M. A., Hajnóczky, G., and Iozzo, R. V. (2000) *J. Biol. Chem.* **275,** 32879–32887
41. Birk, D. E., and Silver, F. H. (1984) *Arch. Biochem. Biophys.* **235,** 178–185
42. Davis, J. C., Venkataraman, G., Shriver, Z., Raj, P. A., and Sasisekharan, R. (1999) *Biochem. J.* **341,** 613–620
43. Liu, J., Laue, T. M., Choi, H. U., Tang, L. H., and Rosenberg, L. (1994) *J. Biol. Chem.* **269,** 28366–28373
44. Birk, D. E., and Lande, M. A. (1981) *Biochim. Biophys. Acta* **670,** 362–369
45. Vogel, K. G., and Trotter, J. A. (1987) *Collagen Relat. Res.* **7,** 105–114
46. Neame, P. J., Kay, C. J., McQuillan, D. J., Beales, M. P., and Hassell, J. R. (2000) *Cell Mol. Life Sci.* **57,** 859–863

Downloaded from http://www.jbc.org/ by guest on November 3, 2014