Decorin Binds Fibrinogen in a Zn\textsuperscript{2+}-dependent Interaction

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SUMMARY

We previously have shown that decorin, a member of the small leucine-rich proteoglycan family of extracellular matrix proteoglycans/glycoproteins is a Zn$^{2+}$ metalloprotein at physiological Zn$^{2+}$ concentrations (Yang, V. W-C., LaBrenz, S. R., Rosenberg, L. C., McQuillan, D., and Höök, M., 1999 *J. Biol. Chem.* 274(18), 12454-60). We now report that the decorin proteoglycan binds fibrinogen in the presence of Zn$^{2+}$. The fibrinogen-binding site is located in the N-terminal domain of the decorin core protein and a 45 amino acid peptide representing this domain binds to fibrinogen D fragment with an apparent $K_D$ of $1.7 \times 10^{-6}$ M, as determined from fluorescence polarization data.

Furthermore, we show that Zn$^{2+}$ promotes the self-association of decorin. The N-terminal domain of the core protein also mediates this activity. The results of solid-phase binding assays and gel filtration chromatography suggest that the N-terminal domain of decorin, when present at low micromolar concentrations, forms an oligomer in a Zn$^{2+}$-dependent manner. Thus, Zn$^{2+}$ appears to play a pivotal role in the interactions and biological function of decorin.
INTRODUCTION

Decorin is a member of the small leucine-rich proteoglycan (SLRP) family of related glycoproteins found in mammalian extracellular matrices (ECMs) (2). The core proteins of SLRPs are similar in structural organization and size, ranging from 35 to 42 kDa. The dominant structural feature is a central domain containing 6-10 leucine-rich repeats (LRRs) that is flanked by N-terminal and C-terminal regions with conserved Cys residues (3). The LRR motif has been found in a number of proteins of diverse origin and function and varies in length from 20-29 amino acids (4). Protein crystal structure analysis of the RNAse inhibitor, internalin B, and glycoprotein 1B suggests that each LRR motif forms a β-strand/turn/helix, giving protein segments composed of tandem repeats of LRRs an arch-like shape (5-7). The SLRPs contain 24 amino acid long LRRs and may also assume an arch-shaped structure as proposed by molecular modeling studies of decorin (8).

SLRPs may be subgrouped based on gene organization, amino acid sequence similarity, the number of LRRs in the central domain, and the spacing of Cys residues in the N-terminal and C-terminal segments (3). Members of subgroup I (10 LRRs) include decorin, biglycan and a recently described molecule, asporin (9,10). The former two may contain 1 or 2 dermatan sulfate/chondroitin sulfate chains in the N-terminus, respectively, while the latter appears to occur exclusively as a glycoprotein with a N-terminal stretch of Asp residues. Fibromodulin, lumican, keratocan, and osteoadherin, which may be substituted with keratan sulfate polysaccharides in the LRR domain, as
well as the glycoprotein PRELP, are the members of subgroup II (10 LRRs). Subgroup III (6 LRRs) includes epiphycan and osteoglycin, which may be substituted with dermatan sulfate/chondroitin sulfate and keratan sulfate polysaccharides, respectively, and the glycoprotein opticin (11). The structure of a twelfth member, chondroadherin, differs sufficiently from those of other SLRPs and perhaps should be assigned to a separate subgroup. The spacing of 4 Cys residues in the N-terminal sequence of the members of subgroups I, II and III is Cx₃Cx₆C, Cx₃Cx₉C, and Cx₂Cx₆C, respectively.

One theme in SLRP function appears to be the regulation of extracellular matrix architecture. Decorin, biglycan, fibromodulin and lumican reportedly interact with collagens and influence collagen fibrillogenesis in in vitro assays (12-16). Analyses of transgenic mice generated with targeted inactivation of individual SLRP genes has revealed that the loss of function of each SLRP resulted in a mild phenotype characterized by the abnormal morphology of specific ECMs. Decorin or fibromodulin-deficient mice exhibit collagen fiber defects in tendon, while decorin or lumican-deficient mice have fragile skin, possibly due to abnormal collagen fibers in the dermis (17-19). Lumican-deficient mice also display abnormally thickened collagen fibers in the cornea. Recent studies suggest that the phenotype of mice deficient in both decorin and biglycan is more severe than those observed in cases where decorin or biglycan genes were individually inactivated (20). Taken together, these results indicate a certain degree of functional redundancy among the different SLRP members.

SLRPs may differentially affect cell behavior by modulating growth factor activity or by influencing the interactions of cell surface receptors with matrix components.
Transfection of the decorin cDNA into CHO cells results in the over-expression of the proteoglycan and correlates with reduced proliferation, presumably due to an inhibition of TGF-β signaling (21). Decorin has also been reported to inhibit the growth of certain cancer cell types by a mechanism thought to involve an interaction between decorin and the EGF receptor (22). The ability of SLRPs to affect cell-matrix interactions can involve SLRP-ECM or SLRP-integrin interactions. Decorin has been shown to interfere with the adhesion of mammalian cells to substrates composed of fibronectin and thrombospondin, and in a glycosaminoglycan-dependent manner, decorin inhibits cell migration on fibronectin and collagen matrices (23-26). On the other hand, osteoadherin and chondroadherin were shown to support osteoblast and chondrocyte adhesion through interactions with integrins αvβ3 and α2β1, respectively (27,28).

We recently demonstrated that decorin and biglycan are Zn2+ metalloproteins (1). The results of equilibrium dialysis experiments indicated that both decorin and biglycan bind 2 Zn2+ ions with a K_D of approximately $2 \times 10^{-6}$ M. Utilizing Zn2+-chelate chromatography, the Zn2+-binding sites on decorin were localized to the N-terminal sequence of the core protein. Furthermore, a Zn2+-induced change in the circular dichroism spectrum for a peptide mimicking the N-terminal domain suggests that Zn2+ may affect the conformation of this segment of the core protein. These observations lead us to search for a possible functional consequence of the putative structural changes arising from Zn2+ binding to decorin. We now report that Zn2+ influences the binding of decorin to several matrix molecules, including collagens, fibronectin and fibrinogen. Since the interaction of decorin with fibrinogen to our knowledge has not
been previously reported, we decided to characterize the Zn$^{2+}$-enhanced binding of decorin to fibrinogen.

**EXPERIMENTAL PROCEDURES**

*Expression and Purification of Recombinant Decorin Glycoforms*—Recombinant decorin with an N-terminal (His)$_6$-tag was expressed in the mammalian cell-line HT1080 utilizing the vaccinia-virus T7 bacteriophage expression system as previously described (29). A mixture consisting of recombinant decorin proteoglycan and decorin core protein (lacking the chondroitin sulfate polysaccharide) was isolated from the cell culture media using Ni$^{2+}$-chelate chromatography. A HiTrap chelating column (Amersham Pharmacia Biotech) was charged with Ni$^{2+}$ as per the manufacturer’s instructions and equilibrated with 20 mM Tris, 0.5 M NaCl, 30 mM imidazole, 0.2% (w/v) CHAPS, pH 8.0. After loading the cell culture media onto the column, it was washed with 10 column volumes of equilibration buffer to remove unbound material. Decorin was eluted with 3 column volumes of 20 mM Tris, 0.5 M NaCl, 150 mM imidazole, 0.2% (w/v) CHAPS, pH 8.0.

Anion exchange chromatography was utilized to separate decorin proteoglycan from decorin core protein. Following Ni$^{2+}$-chelate chromatography, the decorin preparation was diluted with one volume of 16.8 mM Na$_2$HPO$_4$, 3.2 mM NaH$_2$PO$_4$, 0.2 % (w/v) CHAPS, pH 8.0 and loaded onto a Mono-Q column (Amersham Pharmacia Biotech) equilibrated in 16.8 mM Na$_2$HPO$_4$, 3.2 mM NaH$_2$PO$_4$, 0.3 M NaCl, 0.2 % (w/v) CHAPS, pH 8.0. Decorin core protein was recovered during washing with equilibration buffer, while the proteoglycan eluted in 16.8 mM Na$_2$HPO$_4$, 3.2 mM NaH$_2$PO$_4$, 1.5 M NaCl,
0.2% (w/v) CHAPS, pH 8.0. The purity of these final decorin preparations was determined by SDS-PAGE followed by Brilliant Blue-R staining and by Western blotting using rabbit anti-human decorin antiserum, PR2. In accord with a previous report, decorin proteoglycan appeared as a diffuse band centered at an apparent molecular weight of approximately 90 kDa; whereas the decorin core protein substituted with either 2 or 3 N-linked oligosaccharides was observed with apparent molecular weights of 49 kDa or 53 kDa, respectively, (30).

Purification of MBPDcnNTD and DcnNTD—The maltose binding protein (MBP) and a fusion protein consisting of amino acid residues D₃₁⁻P₇₁ of the murine decorin sequence linked to the C-terminus of MBP (MBPDcnNTD) were expressed and purified from *E. coli* strain TB1 as previously described (1). The purity of these recombinant proteins was examined by SDS-PAGE with Brilliant Blue-R staining.

A 45 amino acid residue decorin peptide (DcnNTD), which includes the murine decorin sequence D₃₁⁻P₇₁ preceded by a GSNG sequence originating from the vector, was expressed as a GST fusion protein, digested with thrombin, and isolated by HPLC under acidic, denaturing conditions as previously described (1). Following the first HPLC step, the DcnNTD preparation was diluted with 3 volumes of 20 mM Hepes, 150 mM NaCl, pH 7.4 (HBS) containing 0.1 mM ZnCl₂, in order to raise the pH and promote Zn²⁺ binding. This preparation was concentrated, loaded onto a C18 column equilibrated in TEAP buffer (aqueous 0.11% (v/v) phosphoric acid, 0.28% (v/v) triethylamine, pH 6.5) and eluted with a gradient of 0 to 85 % acetonitrile in TEAP
buffer. The identity of the final decorin peptide preparation was confirmed by MALDI-TOF mass spectrometry (Tufts University, Boston, MA).

The results of preliminary experiments suggested that a subpopulation of the fusion protein (MBPDcnNTD) and the decorin peptide (DcnNTD) was "inactive" (i.e. incapable of binding to fibrinogen). Because some Zn$^{2+}$ metalloproteins reportedly are susceptible to oxidation during purification from an *E. coli* lysate, we adapted a strategy to reactivate or charge the decorin N-terminal domain with Zn$^{2+}$ ions (31). Following purification, MBPDcnNTD or DcnNTD was pre-incubated with excess DTT and 0.1 mM ZnCl$_2$. The reducing agent was added to break disulfide bonds and make available cysteinyl residues that may participate in Zn$^{2+}$ ion coordination. Subsequently, the reduced protein was dialyzed against HBS, 0.1 mM ZnCl$_2$ to promote Zn$^{2+}$ binding to the decorin N-terminal domain with the removal of the reducing agent.

*Biotinylation of Recombinant Proteins*—The decorin proteoglycan, fusion protein (MBPDcnNTD), or the decorin peptide (DcnNTD) was labeled with biotin utilizing sulfo-NHS-LC-biotin (Pierce) (32). Next, biotin-labeled proteoglycan was dialyzed against HBS containing 0.2% (w/v) CHAPS and 0.1 mM ZnCl$_2$, while MBPDcnNTD and DcnNTD were dialyzed similarly with the omission of CHAPS. Prior to use in experiments, the concentration of recombinant proteins was calculated from the absorbance at 280 nm using the calculated extinction coefficients of 19,862 M$^{-1}$cm$^{-1}$ for decorin proteoglycan, 69,080 M$^{-1}$cm$^{-1}$ for MBPDcnNTD, and 3,280 M$^{-1}$cm$^{-1}$ for DcnNTD (32).
Solid-phase Binding Assays–The binding of decorin to selected proteins was examined using an ELISA-type binding assay. Microtiter plate wells (Immunol 1B, Dynatech Labs) were coated overnight at 4°C with 50 µl of HBS, pH 7.4 containing 1 µg of human fibronectin (kindly provided by Jung Hwa Kim, Center for Extracellular Matrix Biology, Institute of Bioscience and Technology, Houston, TX 77054), human fibrinogen (Enzyme Research Labs), fragment D or fragment E of human fibrinogen (Calbiochem), or chicken ovalbumin (Sigma), or 10 µg of bovine type I collagen (Cohesion), type II collagen from bovine nasal septum (Sigma), type IV collagen from human placenta (Sigma), or type V collagen from human placenta (Sigma). The next day, wells were washed twice with 150 µl of HBS containing 0.5% (v/v) Tween-20 (HBST) and in between each of the following steps in order to remove unbound proteins from the wells. To block residual protein binding sites in the wells after coating, 100 µl of blocking buffer (1% (w/v) ovalbumin in HBS) was added to each well and incubated for 1 hr at ambient temperature. Subsequently, 50 µl of either 0.45 µM biotin-labeled decorin proteoglycan or 0.25 µM biotin-labeled MBPDcnNTD in HBS, 0.4 mM ZnCl₂ was added to each well and allowed to incubate for 4 hr at ambient temperature. For the detection of biotin-labeled proteins retained in the wells, a streptavidin-alkaline phosphatase conjugate (Roche) was diluted 5000-fold in HBS, containing 0.1% (w/v) ovalbumin and 50 µl was dispensed into each well. After a 30 min incubation at ambient temperature, the wells were washed with 150 µl HBST followed by 150 µl HBS. Finally, the alkaline phosphatase reaction was initiated by the addition of 100 µl of a freshly prepared 1 mg/ml p-nitrophenyl phosphate (Sigma) solution in 1.3 M diethanolamine, 1 mM MgCl₂, pH 9.8. After 30 min at 37°C, the absorbance at 405 nm was measured using a
Thermomax microplate reader (Molecular Devices Corp., Menlo Park, CA). The mean value from triplicate wells was plotted utilizing Kaleidagraph (Synergy software, Reading, PA).

Similarly, time-dependent binding experiments were carried out with the following modifications. At the specified times, the blocking solution was washed from triplicate wells with 150 µl of HBST, and either 0.45 µM biotin-labeled decorin proteoglycan, or 0.25 µM, 0.75 µM or 1.8 µM biotin-labeled MBPDcnNTD in 50 µl with HBS, 0.1 mM ZnCl₂ was added. The detection procedure was conducted as previously mentioned.

To monitor the time-dependent binding of fibrinogen to immobilized MBPDcnNTD, microtiter plate wells were coated overnight at 4°C with 1 µg of the fusion protein or MBP in HBS containing 0.1 mM ZnCl₂. The wells were blocked and washed between steps as previously described. At the indicated times, 0.25 µM fibrinogen in HBS was added to each well. Retained fibrinogen was detected by adding anti-fibrinogen polyclonal antibodies (Dako) diluted 1:3000 in HBS, 0.1% (w/v) ovalbumin to each well. After 1 hr, goat anti-rabbit AP-conjugated polyclonal antibodies (Bio-Rad) at a dilution of 1:4000 was added to each well for 30 min. Color development with p-nitrophenyl phosphate and data processing were performed as previously mentioned.

To obtain concentration-dependent binding data and an apparent Kᵤ describing the interaction of each decorin construct with intact fibrinogen or fibrinogen fragment D, a related protocol was followed. Specifically, increasing concentrations of biotin-labeled protein in HBS, 0.1 mM ZnCl₂ were allowed to interact with immobilized molecules for a period of time sufficient to reach equilibrium, as determined from the plateau of each time-dependent binding curve. The mean value of the absorbance at 405 nm was
graphed as a function of protein concentration and analyzed utilizing the program DynaFit (33). Given that MBPDcnNTD recognized fragment D in the presence of Zn\(^{2+}\) (Fig. 3), we analyzed the binding data by fitting it to a single binding site model. The curve obtained from this approach coincided well with the actual data points. Since fibrinogen contains two D regions, we assume that there are 2 identical, independent binding sites for decorin per fibrinogen molecule, resulting in a \(K_D\) of \(M^2\) dimension. The best-fit results from DynaFit were imported into Kaleidagraph and displayed by lines superimposed on the binding curves.

The concentration-dependent binding of biotin-labeled decorin peptide to immobilized fusion protein (MBPDcnNTD) or MBP was similarly assessed. To estimate the number of molecules per oligomer and the \(K_D\) describing decorin self-association, the program DynaFit along with binding models describing the formation of dimers, tetramers or hexamers was employed. The results of fitting to each model appear in the plot of mean absorbance values vs. peptide concentration. We report the \(K_D\) with \(M^3\) dimension since a model for tetramer formation appears to best describe the binding.

Inhibition solid-phase binding experiments were also conducted to compare the binding of different decorin constructs to fibrinogen. After coating and blocking the wells as previously described, immobilized fibrinogen or ovalbumin was pre-incubated with increasing concentrations of unlabeled decorin proteoglycan, MBPDcnNTD, DcnNTD or MBP for 4 hrs. Next, biotin-labeled decorin proteoglycan or MBPDcnNTD from a concentrated stock solution was added directly to the wells to reach a final concentration of 0.45 \(\mu\)M or 0.25 \(\mu\)M, respectively, and incubated for 2 hr. This period was chosen because no significant difference in the extent of inhibition was observed
with increased time. The detection steps and data analysis were performed as described.

**Size Exclusion Chromatography**—A Superdex-75 column (Amersham Pharmacia Biotech) with a reported separation range of 3 to 70 kDa was connected to an FPLC system, pre-equilibrated in HBS, and calibrated with blue dextran (2000 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), ribonuclease (13.7 kDa) (Amersham Pharmacia Biotech), a 19 amino acid peptide (1.9 kDa) kindly provided by Sivashankarappa Gurusiddappa, or tryptophan (204 Da) at a flow rate of 0.5 ml/min. The Zn\(^{2+}\)-charged decorin peptide (DcnNTD) at a concentration of 47 µM in HBS, 100 µM ZnCl\(_2\) was applied in a 100 µl volume to the column pre-equilibrated in HBS, 1 µM ZnCl\(_2\). In another experiment, 47 µM DcnNTD was pre-incubated with 10 mM EDTA, 0.1 mM DTT for 3 hr at ambient temperature. Next, the peptide was dialyzed overnight into HBS, 1 mM EDTA, 0.1 mM DTT and run on a column equilibrated in the same buffer. Decorin peptide was detected in the eluate by Western blotting, and asterisks mark these peaks. During a run where buffer lacking the decorin peptide was injected onto the column, additional peaks were present and appear to be due to buffer additives.

**Fluorescence Polarization**—To observe the interaction of the decorin peptide (DcnNTD) with fibrinogen in solution, a fluorescence polarization assay was conducted. To this end, DcnNTD was labeled with fluorescein as described earlier (34). After purification, the molecular weight of the fluorescein-tagged peptide, determined by
MALDI-TOF mass spectrometry (Tufts University), was consistent with that of a singly labeled version of DcnNTD. Next, fluorescein-labeled DcnNTD was resuspended to a final concentration of 20 µM in HBS, 40 µM ZnCl$_2$. Subsequently, fluorescein-labeled DcnNTD was diluted to a final concentration of 10 nM and incubated with increasing concentrations of fragment D or ovalbumin in HBS containing 20 µM ZnCl$_2$ for 2 hr in the dark at ambient temperature. For each sample, the fluorescence polarization signal produced by the excitation of the fluorescein label at 491 nM was measured at a wavelength of 520 nM utilizing an LS50B luminescence spectrometer (Perkin Elmer) with FL-WinLab software (Perkin Elmer). Kaleidagraph was employed to plot binding data as a function of protein concentration and to fit data by nonlinear regression to equation 1,

$$\Delta P = \frac{\Delta P_{\text{max}} \cdot [\text{protein}]}{K_D + [\text{protein}]} \quad \text{(Eq. 1)}$$

where $\Delta P$ refers to the fluorescence polarization signal change, $\Delta P_{\text{max}}$ denotes the maximum fluorescence polarization signal change and $K_D$ corresponds to the equilibrium dissociation constant describing the binding of fibrinogen fragment D to fluorescein-labeled DcnNTD.
RESULTS

Zn$^{2+}$ Enhances Decorin Binding to Matrix Macromolecules

In a previous study, we discovered that Zn$^{2+}$ binding to decorin could alter the conformation of the N-terminal segment of the protein. To explore the possibility that the presence of Zn$^{2+}$ affects the interaction of decorin with other ECM molecules, a solid phase binding assay was conducted in the presence of excess Zn$^{2+}$ or EDTA (Fig. 1). In these studies, we used a recombinant form of the decorin proteoglycan presumably retaining it’s native conformation since it was secreted by a mammalian cell line infected with recombinant vaccinia viruses and isolated without the use of chaotropic or denaturing agents. Decorin was labeled with biotin and incubated in microtiter wells coated with type I, type II, type IV or type V collagen, fibronectin, fibrinogen or ovalbumin. The results of these experiments show that in the presence of the chelating agent EDTA, the amounts of decorin bound to the different ECM proteins were marginally higher than the amounts bound to ovalbumin. However, the addition of Zn$^{2+}$ appears to greatly stimulate the binding of decorin to these ECM proteins, with the possible exception of type II collagen. The inclusion of Zn$^{2+}$ did not result in a substantial increase in decorin binding to the control protein ovalbumin. Since microtiter plate wells coated with fibrinogen bound the highest amount of decorin in the presence of Zn$^{2+}$ and since this interaction previously has not been reported, we chose to further characterize the binding of decorin to fibrinogen as an example of Zn$^{2+}$-dependent decorin binding to ECM molecules.
Localization of the Fibrinogen-Binding Site on Decorin

To identify the domain in decorin that mediates binding to fibrinogen, we examined different components of decorin for their ability to inhibit the binding of biotin-labeled decorin proteoglycan to fibrinogen coated microtiter wells. Initial experiments showed that the intact proteoglycan and the core protein were equally efficient inhibitors in this experiment (data not shown) suggesting that the fibrinogen-binding site is located to the core protein.

The binding of decorin to fibrinogen shows a strong Zn$^{2+}$-dependence. Previously, we localized the Zn$^{2+}$-binding site to the N-terminal segment of the decorin core protein. Therefore, we explored the possibility that this domain also contains the fibrinogen-binding site (Fig. 2A). Initially, we examined a recombinant form of the N-terminal domain of decorin expressed in *E. coli* and presented as a maltose-binding protein fusion (MBPDeNNTD) for the ability to inhibit the binding of biotin-labeled decorin proteoglycan to adsorbed fibrinogen. Indeed, MBPDeNNTD inhibits the binding of biotin-labeled decorin proteoglycan in a concentration-dependent manner. Essentially complete inhibition was seen at concentrations of MBPDeNNTD greater than or equal to 2 µM, with half-maximal inhibition occurring at 0.31 µM of the recombinant fusion protein. This inhibitory activity is comparable to that observed with the unlabeled proteoglycan, where half-maximal inhibition is achieved with 0.58 µM decorin. In contrast, MBP alone did not interfere with decorin binding to fibrinogen, suggesting that this inhibitory activity is specific to the N-terminal domain of decorin.
To confirm that the fibrinogen binding activity is due to the decorin-derived component of MBPDcnNTD, we examined the inhibitory activity of the decorin N-terminal domain (DcnNTD) in the absence of a fusion partner (Fig. 2B). We expressed the 45 amino acid residue DcnNTD as a recombinant glutathione S-transferase (GST) fusion protein with a thrombin cleavage site located in a linker peptide between GST and DcnNTD. After thrombin digestion of GST-DcnNTD, DcnNTD was initially purified under reducing conditions and subsequently isolated in the presence of Zn\textsuperscript{2+}. This Zn\textsuperscript{2+}-charged peptide was then examined for the ability to inhibit the binding of biotin-labeled proteoglycan to fibrinogen. We observed dose-dependent, complete inhibition of decorin proteoglycan binding to fibrinogen by the isolated decorin peptide, with half-maximal inhibition observed above 1 µM DcnNTD and complete inhibition achieved at 5 µM DcnNTD. From these experiments, we conclude that the fibrinogen-binding site on decorin is located in the N-terminal domain of the core protein. In fact, we subsequently showed in direct binding assays that DcnNTD binds to fibrinogen (see below).

**The Decorin-Binding Site on Fibrinogen**

To localize the decorin-binding site on fibrinogen, we first conducted a solid-phase binding assay to determine whether biotin-labeled decorin proteoglycan or MBPDcnNTD recognizes the plasmin generated fibrinogen fragments D or E (Fig. 3). We found that in the presence of Zn\textsuperscript{2+}, both decorin proteins bound to microtiter wells coated with intact fibrinogen or fragment D but not to the E fragment or the control protein ovalbumin. In the presence of EDTA, the binding of decorin to all four proteins
was minimal. Hence, in a Zn\(^{2+}\)-dependent fashion, decorin appears to specifically recognize the globular D regions of fibrinogen, mainly consisting of amino acid residues 111-197 of the alpha chain, 134-461 of the beta chain and 88-406 of the gamma chain (35,36).

**Characterization of the Decorin-Fibrinogen Interaction**

To gain insight on the mechanism of decorin-fibrinogen recognition, we initially wanted to determine the K\(_D\) for the interaction of decorin proteoglycan with fibrinogen (Fig. 4A). In the presence of Zn\(^{2+}\), fibrinogen or ovalbumin coated microtiter wells were incubated with increasing concentrations of the biotin-labeled proteoglycan until equilibrium was reached. Minimal amounts of decorin were retained in the ovalbumin containing wells. However, decorin bound fibrinogen in a concentration-dependent, saturable manner. Assuming that there are two identical, independent decorin-binding sites on fibrinogen, we analyzed these data utilizing the program DynaFit and obtained an apparent K\(_D\) of 6.8 X 10\(^{-7}\) M\(^2\).

In a previous experiment, we showed that the decorin peptide, DcnNTD, completely inhibits decorin proteoglycan binding to fibrinogen. To directly observe the interaction of DcnNTD with fibrinogen, increasing concentrations of biotin-labeled decorin peptide were allowed to equilibrate with fibrinogen or ovalbumin coated wells in the presence of Zn\(^{2+}\) (Fig. 4B). Low levels of DcnNTD were detected in the ovalbumin containing wells. In contrast, the decorin peptide binding to fibrinogen was concentration-dependent and saturable with an apparent K\(_D\) of 3.0 X 10\(^{-7}\) M\(^2\).
We also demonstrated that MBPDcnNTD recognizes both fibrinogen and fragment D in a Zn$^{2+}$-dependent fashion. In this experiment, the concentration-dependent binding of biotin-labeled MBPDcnNTD to adsorbed fibrinogen, fragment D or ovalbumin was observed under equilibrium conditions with Zn$^{2+}$ present (Fig. 4C). MBPDcnNTD was minimally retained by wells coated with ovalbumin. However, MBPDcnNTD incrementally bound to both fibrinogen and fragment D in accord with increasing MBPDcnNTD concentration until a plateau was reached. Based on the analysis of these data utilizing DynaFit, an apparent $K_D$ of 1.2 X 10$^{-7}$ M$^2$ describes MBPDcnNTD binding to fibrinogen and an apparent $K_D$ of 9.0 X 10$^{-8}$ M characterizes MBPDcnNTD recognition of fragment D.

To further characterize the binding of the decorin N-terminal domain to fibrinogen fragment D, we conducted fluorescence polarization experiments. A fluorescein-labeled version of the decorin peptide was mixed with increasing concentrations of fragment D or ovalbumin in the presence of Zn$^{2+}$ (Fig. 5). Little or no change in the fluorescence polarization signal was observed for the peptide in the presence of ovalbumin. Instead, we observed an increase in the fluorescence polarization signal of the fluorescein-peptide conjugate with increasing concentrations of fragment D until a maximum was approached. An estimated $K_D$ of 1.7*10$^{-6}$ M describes the interaction of the decorin N-terminal domain with fibrinogen fragment D in solution.

To further characterize the interaction of decorin with fibrinogen, we examined the time-dependent binding of decorin proteoglycan to immobilized fibrinogen in the presence of Zn$^{2+}$ (Fig. 6A). Biotin-labeled proteoglycan at a concentration of 0.45 µM was incubated with microtiter plate wells coated with fibrinogen for increasing lengths of
time and required approximately 4 hrs to reach saturation. Although the binding was slow, decorin does appear to specifically recognize fibrinogen since minimal amounts of the proteoglycan were retained in the ovalbumin containing wells.

Next, the time-dependent binding of MBPDcnNTD to fibrinogen or ovalbumin coated wells was explored utilizing several concentrations of the recombinant fusion protein (Fig. 6B). Alternatively, the time required for MBPDcnNTD to saturate the decorin-binding sites depended on the concentration of the fusion protein. For example, a maximum level of binding was observed about 1.5 hr after the addition of 0.25 µM MBPDcnNTD to fibrinogen containing wells. However, at concentrations of 0.75 µM and 1.8 µM MBPDcnNTD, the decorin binding-sites became saturated after only 30 min. Binding to ovalbumin over time was minimal at all three concentrations of MBPDcnNTD tested.

In an ELISA-type experiment, we also monitored the binding of 0.25 µM fibrinogen to wells coated with either MBPDcnNTD or MBP over time (Fig. 6C). Fibrinogen slowly accumulated on the MBP coated surface in what we think is a nonspecific interaction. On the other hand, fibrinogen binding to MBPDcnNTD reached a plateau already after approximately 30 min. The results of these time-dependent binding experiments lead us to hypothesize that decorin-fibrinogen interaction involves more than one step.

**Zn\textsuperscript{2+} Promotes the Self-association of Decorin**

Earlier studies by Liu et al. suggested that biglycan proteoglycan forms dimers in the presence of EDTA and hexamers in the presence of Zn\textsuperscript{2+} (37). Previously, we reported
that both biglycan and decorin proteoglycans are Zn$^{2+}$ metalloproteins capable of binding 2 Zn$^{2+}$ ions with similar affinity at the N-terminal region of the core protein (1). Initially, we examined the possibility that the 45 amino acid decorin peptide, DcnNTD, self-associates in a Zn$^{2+}$-dependent fashion by gel filtration chromatography (Fig. 7). The peptide at a concentration of 47 µM in the presence of either Zn$^{2+}$ or EDTA with DTT was applied to a Superdex-75 gel filtration column with a reported separation range of 3 to 70 kDa. These chromatograms show that the decorin peptide elutes in a significantly lower volume in the presence of Zn$^{2+}$ than in the presence of EDTA with DTT. The Zn$^{2+}$-charged peptide elutes in a broad peak located at 8.5 ml, the same volume required to elute albumin (Table I). However, in the presence of EDTA with DTT, DcnNTD (4.88 kDa) elutes in 13.5 ml, which is intermediate of the elution volume of ribonuclease (13.7 kDa) and for a 19 amino acid peptide standard (1.9 kDa). These results demonstrate that Zn$^{2+}$ promotes the self-association of the decorin peptide.

To quantitatively evaluate the Zn$^{2+}$-dependent self-association of the decorin N-terminal peptide, increasing concentrations of biotin-labeled DcnNTD were incubated with MBPDcnNTD, MBP or ovalbumin adsorbed to microtiter plate wells in the presence of Zn$^{2+}$ (Fig. 8). The decorin N-terminal domain appears to specifically recognize itself when presented as a fusion to MBP, as the peptide was only minimally retained in MBP or ovalbumin coated wells. The amount of decorin peptide bound to the MBPDcnNTD coated wells increased with concentration, reached a maximum at approximately 10 µM peptide and could not be increased by the addition of more peptide. To estimate the $K_D$ for the decorin N-terminal domain binding to MBPDcnNTD as well as the number of molecules per oligomer, the data were analyzed utilizing the program DynaFit to test
models for the formation of dimers, tetramers, or hexamers. The curve that appears most coincident with the binding data is that describing tetramer formation with an apparent $K_D$ of $6.5 \times 10^{-7}$ M$^3$. These results suggest that the decorin N-terminal domain self-associates to form an oligomer containing a defined number of monomeric components.
DISCUSSION

We show here that Zn\textsuperscript{2+} promotes the interaction of decorin proteoglycan with fibrinogen. Fibrinogen circulates in blood as a soluble 340 kDa glycoprotein at a concentration of approximately 9 µM (38). During the final stage of the clotting cascade, thrombin cleaves fibrinopeptides from the central E region of fibrinogen to yield fibrin that rapidly assembles into a provisional matrix. When vascular damage causes the extravasation of blood, fibrinogen comes in contact with extracellular matrix components, including decorin exhibiting a broad tissue distribution (39,40). The possibility that decorin and fibrinogen may co-localize in the extracellular milieu during wound repair coupled with our interest in the role of Zn\textsuperscript{2+} as a regulator of decorin structure and function lead us to further characterize the decorin-fibrinogen interaction.

The domains that mediate decorin-fibrinogen binding were deduced from our analysis of the results of solid phase and fluorescence polarization binding assays. We determined that recombinant decorin proteoglycan, expressed by mammalian cells using vaccinia virus as well as recombinant forms of the N-terminal domain produced in \textit{E. coli} exhibit equivalent apparent K_D values for binding to fibrinogen coated wells. Thus, additional decorin core protein segments beyond the N-terminal domain do not appear to be required for stable binding to fibrinogen or fragment D. MBPDcnNTD bound to fragment D coated wells with an apparent K_D of 9.0 X 10\textsuperscript{-8} M. However, fragment D binding to DcnNTD in solution approached saturation with an estimated K_D of 1.7 X 10\textsuperscript{-6} M. These divergent observations could be a consequence of the complexity of the molecular interactions under study. For instance, the possibility that
several multimeric forms of the decorin peptide are present and that they may differ in affinity for the D domain of fibrinogen has not been incorporated into our analyses. In addition, the conformation of the fibrinogen D domain reportedly may be altered by immobilization (41). Hence, the apparent $K_D$ values depend on the experimental conditions of the assay. Nevertheless, we show here that the N-terminal $\text{Zn}^{2+}$-binding domain of decorin interacts with the D region of fibrinogen with a reasonable affinity.

Decorin and biglycan are self-associating $\text{Zn}^{2+}$ metalloproteins. A previous report showed that biglycan proteoglycan is hexameric in the presence of $\text{Zn}^{2+}$ and dimeric in the presence of EDTA (37). Subsequently, we discovered that both decorin and biglycan bind 2 $\text{Zn}^{2+}$ ions with the N-terminal segment of the core protein (1). We now propose that the N-terminal domain of decorin core protein mediates the $\text{Zn}^{2+}$-dependent formation of oligomers. Our gel filtration chromatography experiments show that $\text{Zn}^{2+}$-charged DcnNTD elutes as a broad peak, possibly indicating the coexistence of several oligomeric forms in the presence of $\text{Zn}^{2+}$. Similarly, we observed several overlapping peaks during gel filtration chromatography of MBPDCnNTD in the presence of $\text{Zn}^{2+}$ (data not shown). We also noticed that the peak elution volume of DcnNTD shifts to slightly higher volume as we decrease the concentration of peptide loaded, suggesting a dissociation of oligomers (data not shown). Moreover, DcnNTD oligomers are effectively dissociated by incubation with excess EDTA. Liu and colleagues showed that the biglycan core protein, lacking the 2 chondroitin sulfate polysaccharides normally attached to the N-terminal domain, formed aggregates in the presence of $\text{Zn}^{2+}$ (37). We did not detect DcnNTD or MBPDCnNTD in the column void volume and observed saturation binding of DcnNTD to MBPDCnNTD coated wells. Taken together, these
results indicate that the N-terminal domain can form oligomeric structures with defined compositions.

The mechanism(s) whereby Zn$^{2+}$ promotes the binding of decorin to a variety of other matrix components including type I, type IV, and type V collagen, fibronectin and fibrinogen remains to be determined. In some cases, perhaps increased decorin binding in the presence of Zn$^{2+}$ is attributable to the formation of oligomeric forms that bind to the adsorbed matrix proteins; while reduced apparent binding in the presence of EDTA may reflect bound monomeric forms. Alternatively, Zn$^{2+}$ may play a role in the folding and structure the decorin N-terminal domain. In this case, the conformation of the N-terminal domain formed in the presence of Zn$^{2+}$ exhibits a higher affinity for some matrix molecules, such as fibrinogen, than the conformation of the N-terminal domain in the presence of EDTA. Our study illustrates the significance of Zn$^{2+}$ as a modulator of decorin structure and function. This relationship should be considered in future efforts to elucidate the biological function(s) of decorin and perhaps other SLRPs.

The biological consequence(s) of a putative decorin-fibrinogen interaction is unclear at this time. To determine whether these molecules can interact in a physiological setting, we passed human plasma over a decorin matrix composed of MBPDcnNTD covalently attached to Sepharose 4B. SDS-PAGE and Western blots showed that both fibrinogen and fibronectin were retained by the decorin matrix during extensive washing and eluted with EDTA (data not shown). Retention of fibronectin by the decorin N-terminal domain is interesting since a previous study postulated a second fibronectin-binding site on decorin, in addition to the previously reported NKISK site of LRR 1 (42). Fibrinogen, in the form of fibrin, and fibronectin represent dominant protein components
of the provisional matrix formed during the initial stage of wound repair (43). When present at sites of wound repair, decorin might participate in organizing the structure of the provisional matrix, thus one could speculate that decorin plays a role in the wound healing process.
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** FOOTNOTES **

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FIG. 1. **Screen for Zn$^{2+}$-enhanced binding of decorin to extracellular matrix components.** Microtiter plate wells were coated with 1 µg of fibronectin, fibrinogen, or ovalbumin (negative control), or 10 µg of type I, II, IV, or V collagen overnight at 4°C in 20 mM Hepes, 0.15 M NaCl, pH 7.4 (HBS). Biotin-labeled decorin proteoglycan was added to the wells at a final concentration of 0.45 µM in HBS supplemented with either 0.4 mM ZnCl$_2$ or EDTA for a 4 hr incubation. After washing the wells to remove unbound proteoglycan, bound decorin was detected utilizing a streptavidin-AP conjugate and p-nitrophenyl phosphate as a substrate. Similar data were obtained in replicate experiments.

FIG. 2. **Location of the fibrinogen-binding site on decorin.** A, wells containing adsorbed fibrinogen or ovalbumin (○) were pre-incubated with increasing concentrations of unlabeled decorin proteoglycan, DcnPg, (■), MBPDCnNTD (●), or MBP (□) in HBS, 100 µM ZnCl$_2$, pH 7.4. B, microtiter plate wells coated with fibrinogen (●) or ovalbumin (○) were pre-incubated with increasing concentrations of unlabeled decorin peptide (DcnNTD) in HBS, 100 µM ZnCl$_2$, pH 7.4. In both A and B, biotin-labeled decorin proteoglycan was added to a final concentration of 0.45 µM. The binding of biotinylated molecules was detected as previously described. These data are representative of replicate experiments.
FIG. 3. **Location of the decorin-binding domain of fibrinogen.** Intact fibrinogen, fragment D, fragment E, or ovalbumin was coated on microtiter plate wells and allowed to incubate with either 0.45 µM biotin-labeled decorin proteoglycan, DcnPg, or 0.25 µM biotin-labeled MBPDcnNTD in HBS, pH 7.4 containing either 0.4 mM ZnCl₂ or EDTA. Biotin-labeled protein was detected as previously described. These data are congruent with replicate experiments.

FIG. 4. **Concentration-dependent binding of decorin to immobilized fibrinogen.** A, increasing concentrations of biotin-labeled decorin proteoglycan were incubated with microtiter plate wells coated with fibrinogen (●) or ovalbumin (○). B, increasing concentrations of a biotin-labeled decorin peptide (DcnNTD) were incubated with adsorbed fibrinogen (●) or ovalbumin (○). C, increasing concentrations of biotin-labeled MBPDcnNTD were incubated with adsorbed fibrinogen (●), fragment D (□), or ovalbumin (○). Biotin-labeled proteins were detected as previously described. Values of nonspecific binding to the negative control, ovalbumin, were subtracted from the binding data prior to analysis. Superimposed on each data set is the fit generated utilizing the program DynaFit (33) to estimate K_D values for the interactions taking place in HBS, 100 µM ZnCl₂, pH 7.4. Similar results were observed in replicate experiments.

FIG. 5. **Concentration-dependent interaction of fibrinogen fragment D with DcnNTD in solution.** Increasing concentrations of fibrinogen fragment D (●) or ovalbumin (○) were allowed to incubate with the Zn^{2+}-charged, fluorescein-labeled
decorin peptide in HBS, 20 µM ZnCl₂, pH 7.4. The best fit generated utilizing the program Kaleidagraph overlays the D fragment-peptide binding data.

FIG. 6. **Time-dependent binding experiments.** A, biotin-labeled decorin proteoglycan at a concentration of 0.45 µM was incubated in microtiter plate wells coated with fibrinogen (●) or ovalbumin (○) for increasing lengths of time in HBS, 100 µM ZnCl₂, pH 7.4. B, biotin-labeled MBPDCnNTD at a concentration of 0.25 µM (●), 0.75 µM (■), or 1.8 µM (□) was incubated with microtiter wells coated with fibrinogen or ovalbumin (○) for different lengths of time in HBS, 100 µM ZnCl₂, pH 7.4. In both A and B, biotin-labeled protein was detected as previously described. C, fibrinogen at a concentration of 0.25 µM was incubated in microtiter wells coated with either MBPDCnNTD (●) or MBP (○) for various lengths of time in HBS, 20 µM ZnCl₂, pH 7.4. Bound fibrinogen was detected utilizing anti-fibrinogen polyclonal antibodies followed by goat anti-rabbit AP-conjugated polyclonal antibodies. Antibody-fibrinogen complexes bound to the immobilized molecules were detected using p-nitrophenyl phosphate as a substrate. These data are representative of replicate experiments.

FIG. 7. **Gel filtration chromatography of DcnNTD in the presence of either Zn²⁺ or EDTA with DTT.** A, Zn²⁺-charged decorin peptide (DcnNTD) at an initial concentration of 47 µM was loaded onto a Superdex-75 column and eluted in HBS, 1 µM ZnCl₂, pH 7.4. B, the Zn²⁺-charged decorin peptide was pre-incubated with 10 mM EDTA, 0.1 mM DTT, pH 7.4 and dialyzed overnight against the running buffer consisting of HBS, 1 mM EDTA, 0.1 mM DTT, pH 7.4 prior to chromatography. C, gel filtration column calibration
standards were loaded and run individually or pair-wise as per the manufacturer’s instructions. The characteristics of each standard are reported in Table I.

FIG. 8. **Concentration-dependent binding of DcnNTD to MBPDcnNTD.** Increasing concentrations of the biotin-labeled decorin peptide (DcnNTD) in HBS, 100 μM ZnCl₂, pH 7.4 were incubated for 2 hr with microtiter plate wells coated with MBPDcnNTD (●), MBP (■), or ovalbumin (○). Retained biotin-labeled peptide was detected as described earlier. The curves superimposed on the data represent the best-fit results utilizing the program DynaFit with a dimer (- - - - - -), tetramer (———) or hexamer (- - - - - - -) model. Replicate experiments yielded similar data.
Table I

*Characteristics of the gel filtration calibration standards.*

| Standard                | Peak (Fig. 7C) | Molecular weight (kDa) | Radius (Å) | Elution volume (ml) |
|-------------------------|----------------|------------------------|------------|---------------------|
| Blue dextran            | 1              | 2000                   | ND         | 7.5                 |
| Albumin                 | 2              | 67                     | 35.5       | 8.5                 |
| Ovalbumin               | 3              | 43                     | 30.5       | 9                   |
| Chymotrypsinogen A      | 4              | 25                     | 20.9       | 11                  |
| Ribonuclease            | 5              | 13.7                   | 16.4       | 12.5                |
| Peptide (19 amino acids)| 6              | 1.9                    | ND         | 14                  |
Figure 1.
Figure 2B.
Figure 3.
Figure 4A.
Figure 4B.
Figure 5.
Figure 6B.
Figure 6C.
Figure 8.
Decorin binds fibrinogen in a Zn2+-dependent interaction
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