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This information is current as  
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*J Immunol* 2005; 175:4715-4723; ;  
doi: 10.4049/jimmunol.175.7.4715  
<http://www.jimmunol.org/content/175/7/4715>

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The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Interactions of the Extracellular Matrix Proteoglycans Decorin and Biglycan with C1q and Collectins<sup>1</sup>

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Decorin and biglycan are closely related abundant extracellular matrix proteoglycans that have been shown to bind to C1q. Given the overall structural similarities between C1q and mannose-binding lectin (MBL), the two key recognition molecules of the classical and the lectin complement pathways, respectively, we have examined functional consequences of the interaction of C1q and MBL with decorin and biglycan. Recombinant forms of human decorin and biglycan bound C1q via both collagen and globular domains and inhibited the classical pathway. Decorin also bound C1 without activating complement. Furthermore, decorin and biglycan bound efficiently to MBL, but only biglycan could inhibit activation of the lectin pathway. Other members of the collectin family, including human surfactant protein D, bovine collectin-43, and conglutinin also showed binding to decorin and biglycan. Decorin and biglycan strongly inhibited C1q binding to human endothelial cells and U937 cells, and biglycan suppressed C1q-induced MCP-1 and IL-8 production by human endothelial cells. In conclusion, decorin and biglycan act as inhibitors of activation of the complement cascade, cellular interactions, and proinflammatory cytokine production mediated by C1q. These two proteoglycans are likely to down-regulate proinflammatory effects mediated by C1q, and possibly also the collectins, at the tissue level. *The Journal of Immunology*, 2005, 175: 4715–4723.

The complement system can be activated via three different pathways: the classical pathway, the lectin pathway, and the alternative pathway. C1q and mannose binding lectin (MBL)<sup>3</sup> serve as ligand recognition molecules of the classical and the lectin pathway of the complement system, respectively. Binding of C1q or MBL to their ligands results in activation of the complement cascade and ultimately to opsonization and possibly lysis of pathogens via the membrane attack complex. Bound IgG and IgM serve as principal ligands for C1q, whereas polysaccharides such as yeast-derived mannan are ligands for MBL. C1q and MBL consist of polymers of structurally related trimeric subunits.

Ligand recognition is mediated by the heterotrimeric C1q globular head (gC1q) domain composed of A, B, and C chains in the case of C1q and by homotrimeric C-type lectin domains in the case of MBL. Trimeric subunits in both C1q and MBL have a characteristic triple-helical collagen-like region (CLR) at the N terminus. Accordingly, C1q and MBL share the ability to bind to receptors on the cell surface including calreticulin (1) and complement receptor 1 (2) via their CLR domain. Thus, it has been shown that C1q exhibits receptor-mediated binding to endothelial cells, leading to the production and secretion of proinflammatory molecules such as MCP-1 and IL-8 (3).

Undesired activation of the complement pathway is regulated via a number of membrane-bound and soluble complement inhibitors. C1 inhibitor, for instance, is a soluble protein that inhibits both the classical and the lectin pathways via its interactions with the serine proteases associated with the C1 and MBL complexes, respectively. Furthermore, several natural C1q-binding proteins, including proteoglycans, when presented in soluble forms, are able to inhibit the functional activity of C1q (4–9).

Decorin and biglycan are structurally related, abundant extracellular matrix (ECM) proteins, which belong to the family of the small leucine repeat proteoglycans. The 44-kDa core protein of decorin contains long repetitive leucine repeats that are thought to play an important role in the collagen-binding capabilities of decorin (4). Depending on the tissue type, the core protein is either attached to a dermatan or a chondroitin glycosaminoglycan chain (5). Decorin plays a role in the arrangement of collagens and in the regulation of TGF- $\beta$  function (6, 7). Bovine and recombinant human decorin as well as recombinant biglycan have been shown to bind C1q (8–10). The affinity of human decorin for C1q was much higher than for collagens, suggesting a biologically important interaction between decorin and C1q (9). Accordingly, bovine decorin was shown to inhibit the classical pathway of complement (8); however, such data concerning human decorin are not available.

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Received for publication September 21, 2004. Accepted for publication August 4, 2005.

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<sup>1</sup>This work has been supported by grants from The Netherlands Organisation for Scientific Research (Nederlandse Organisatie voor Wetenschappelijk Onderzoek Grant 901-12-095), the European Union (Grant QLGI-CT-2001-01039), and by the Dutch Kidney Foundation (Grant C03-6014). M.O. is funded by AETHERNET (Grant QLGL-CT-2002-90937). U.K. is funded by the European Commission, the German National Genome Network, and the Alexander von Humboldt Foundation.

<sup>2</sup>Address correspondence and reprint requests to Dr. Mohamed R. Daha, Department of Nephrology, C3-P, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands. E-mail address: M.R.Daha@lumc.nl

<sup>3</sup>Abbreviations used in this paper: MBL, mannose binding lectin; ECM, extracellular matrix; SP-D, surfactant protein D; CL-43, collectin 43; CLR, collagen-like region; gC1q, C1q globular head; C1qHA, C1q-dependent hemolytic assay; NHS, normal human serum; EA, Ab-opsonized erythrocyte; MASP, mannose-binding lectin-associated serine protease; rhSP-D, recombinant human SP-D; HSA, human serum albumin.

Given the overall structural similarities and functional overlaps between C1q and MBL, we have addressed their interaction with decorin and biglycan and examined whether such interactions modulate the classical and the lectin pathways. In this study, we show that decorin and biglycan bind not only to C1q, but also to members of the collectin family including MBL, surfactant protein D (SP-D), collectin 43 (CL-43), and conglutinin. Decorin strongly inhibits the classical pathway but fails to modulate the lectin pathway. However, biglycan inhibits the classical pathway but also binds MBL and prevents activation of the lectin pathway. At the cellular level, decorin and biglycan are able to prevent binding of C1q to U937 cells as well as to endothelial cells. Furthermore, biglycan prevents the C1q-induced production of MCP-1 and IL-8 by endothelial cells. These results suggest that as inhibitors of C1q, decorin, and biglycan can dampen the classical pathway and minimize proinflammatory cellular responses triggered by C1q. Therefore, decorin and biglycan may have an important role in the resolution of C1q-mediated inflammatory processes in the tissues.

## Materials and Methods

### Purification of human MBL

MBL was purified from human serum, by first precipitating with polyethylene glycol 3350 (7% w/v) (Sigma-Aldrich). The precipitate was resuspended in TBST (pH 7.8) containing 20 mM  $\text{Ca}^{2+}$ . Subsequently, the solution was rotated overnight with mannan-coupled Sepharose beads at 4°C. After washing with TBST- $\text{Ca}^{2+}$  containing 1 M NaCl to remove nonspecifically bound protein, the beads were transferred to a column, and MBL was eluted using TBST containing 10 mM EDTA. Fractions were tested for the presence of MBL by ELISA as described before (11). Peak fractions containing MBL were pooled, concentrated, and dialyzed against PBS. To obtain mannose-binding lectin-associated serine protease (MASP)-free MBL, this preparation was loaded onto a Sepharose 6B fast protein liquid chromatography column using 0.1 M acetic acid containing 0.2 M NaCl and 5 mM EDTA, pH 5, as a running buffer. Fractions were tested for MBL by ELISA and assessed for MASP-2 activity using a C4 consumption assay. Fractions positive for MBL and negative for MASP-2 activity were pooled, dialyzed against PBS, and subsequently stored in aliquots at  $-20^{\circ}\text{C}$ .

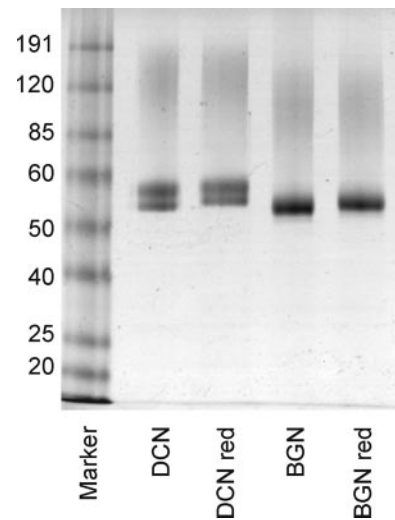
### Preparation of human C1q and its CLR domain

C1q was purified from human plasma as described previously (12). C1q CLR was prepared by digestion of 1 mg of purified C1q with 0.2 mg of pepsin diluted in 0.1 M sodium acetate/0.15 M NaCl (pH 4.5) for 4.5 h in a shaking water bath at 37°C. The solution was neutralized using 1 M Tris (pH 10), dialyzed against PBS/10 mM EDTA, and subsequently loaded onto a human IgG/rabbit IgG-Sepharose column as described previously by Nauta et al. (12) to remove uncleaved C1q as well as gC1q domain. The flow through of the column was shown to contain C1q CLR but not gC1q domain as revealed by specific mAb. The fractions containing the CLR were pooled, dialyzed against PBS, concentrated, and stored in aliquots at  $-20^{\circ}\text{C}$ .

### Other proteins

Recombinant forms of human decorin and biglycan were expressed in mammalian cells using a vaccinia virus/T7 bacteriophage expression system and were isolated under nonreducing conditions as described (9, 10). The purity of recombinant decorin and biglycan was examined on a 10% SDS-PAGE (Fig. 1). Both protein preparations consist of a proteoglycan form as represented by the polydisperse band migrating between 80 and 200 kDa and a core protein form migrating between 50 and 60 kDa. The decorin core protein appears as a doublet and is due to differential asparagine-linked glycosylation (9).

Native human SP-D was purified from pooled amniotic fluid, as described previously (13). A recombinant fragment of human SP-D (rhSP-D) composed of homotrimeric neck region and C-type lectin domains was expressed in *Escherichia coli* and purified as described recently (14). Bovine conglutinin (15) and CL-43 (16) were produced as described. Recombinant forms of the C-terminal globular regions of human C1q A, B, and C chains (ghA, ghB, and ghC, respectively) were expressed in *E. coli* as fusion proteins linked to maltose binding protein and purified as described recently (17). The collagen-free and native heterotrimeric gC1q domain was prepared using human C1q, as described previously (18).



**FIGURE 1.** SDS-PAGE analysis of recombinant decorin (DCN) and biglycan (BGN). Two micrograms of DCN or BGN were run on a 10% polyacrylamide gel under nonreduced conditions (lanes 2 and 4) or reduced conditions with  $\beta$ -mercaptoethanol (lanes 3 and 5).

### Generation of rabbit antisera

Polyclonal antisera against decorin and biglycan were generated in rabbits, using synthetic peptides corresponding to regions near the N terminus of human decorin (GIGPEVPDDRDF-C) and human biglycan (GVLDPDS-VTPTYSAM-C). The peptides were synthesized with an additional cysteine at the C terminus, which was used for coupling to keyhole limpet hemocyanin. The keyhole limpet hemocyanin-peptide conjugates were then used to immunize rabbits following a standard immunization protocol. Both the peptide synthesis and Ab production were performed by Alpha Diagnostic International.

### General ELISA

In general, ELISA experiments were performed using Maxisorb plates (Nunc). For coating, proteins were diluted in coating buffer (100 mM  $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ , pH 9.6) and incubated either overnight at room temperature or for 2 h at 37°C, followed by blocking of nonspecific binding sites with PBS containing 1% w/v BSA for 1 h at 37°C. The secondary Abs were, unless indicated otherwise, diluted in PBS containing 1% w/v BSA/0.05% v/v Tween 20 (PTB) and incubated 1 h at 37°C. Between every incubation step the wells were washed three times with PBS containing 0.05% v/v Tween 20, unless indicated otherwise. Enzyme activity of HRP was detected using ABTS substrate (Sigma-Aldrich).  $A_{414}$  was measured using a microplate biokinetics reader (EL312e; Biotek Instruments).

### Interaction of C1q and MBL with decorin and biglycan

Either decorin (5  $\mu\text{g}/\text{ml}$ ), biglycan (5  $\mu\text{g}/\text{ml}$ ), purified human IgM (3  $\mu\text{g}/\text{ml}$ ), or BSA were coated to microtiter wells. After the blocking step, the wells were incubated with different concentrations of purified human C1q or its CLR diluted in PTB, normal human serum (NHS) diluted in BVB<sup>+</sup> buffer (Veronal-buffered saline, 5% BSA, 1 mM  $\text{CaCl}_2$ , 0.25% Tween 20), or MBL (diluted in BVB buffer with calcium or with 2 mM EDTA but no calcium). Bound C1, C1q, or CLR were detected using a mAb directed against the C1q CLR (mAb 2214) coupled to digoxigenin-3-O-methyl-carbonyl- $\epsilon$ -aminocaproic acid-*N*-hydroxy-succinimide ester (DIG; Boehringer Mannheim). Bound MBL was detected using a mAb against MBL (mAb 3E7; provided by Dr. T. Fujita, Fukushima Medical University, Fukushima, Japan) coupled to DIG. DIG-conjugated mAb were detected using Fab of sheep IgG directed against DIG, coupled to HRP (Boehringer Mannheim).

### C1 binding and complement activation

Decorin, biglycan, IgM, or BSA were coated to wells, followed by incubation with NHS as a complement source, diluted in BVB<sup>++</sup> buffer (BVB<sup>+</sup> + 0.5 mM  $\text{MgCl}_2$ ). Activation of C4 was assessed as described previously (19). Binding of C1 was assessed using a mAb against C1q (mAb 2214) conjugated to DIG. For functional assessment of C1 binding,

wells were incubated with NHS (diluted in BVB<sup>++</sup>) for 2 h at 4°C, followed by incubation with purified C4 and assessment of C4 binding, as described earlier (19).

#### *Inhibition of C1q binding to decorin*

Decorin (5 µg/ml) was coated to microtiter wells, followed by addition of DIG-conjugated C1q in the presence or absence of 5 µg/ml anti-gC1q (mAb 85 or mAb 2204), anti-CLR (mAb 2211), or 100 µg/ml purified mouse Fc tails (provided by Dr. J. Egido (Department of Immunology, Fundación Jiménez Díaz, Autónoma University, Madrid, Spain) and Dr. F. Vivanco (Renal Research Laboratory, Fundación Jiménez Díaz, Autónoma University, Madrid, Spain)) for 1 h at 37°C. After washing, C1q binding was detected using HRP-conjugated Fab of sheep IgG directed against DIG (Boehringer Mannheim).

#### *Binding of decorin and biglycan to immobilized lectins or C1q fragments*

Purified C1q, C1q-derived fragments (CLR, gC1q, ghB), collectins (MBL, SP-D, rhSP-D, conglutinin, and CL-43), or BSA were coated on an ELISA plate at 2 µg/ml. After blocking, decorin or biglycan diluted in PTB containing 2 mM EDTA was added to the wells and incubated for 1 h at 37°C. Binding was detected using rabbit antiserum against decorin or biglycan diluted in PTB, followed by detection with a polyclonal goat anti-rabbit Ab coupled to HRP (Jackson ImmunoResearch Laboratories).

#### *Inhibition of the lectin pathway*

Mannan (100 µg/ml) was coated to an ELISA plate. C1q-depleted plasma (20) was preincubated on ice for 15 min in the presence of decorin, biglycan, or D-mannose and then added to the plate for 1 h at 37°C. As a measure for MBL-pathway activation, C5b-9 deposition in the wells was detected using a mAb against C5b-9 coupled to DIG (AE-11; provided by Dr. T. E. Mollnes, Institute of Immunology, Rikshospitalet University Hospital, Oslo, Norway).

#### *Inhibition of MBL binding*

Mannan (100 µg/ml) was coated to an ELISA plate. After blocking, MBL (1 µg/ml) was added in the presence or absence of different concentrations of either decorin, biglycan, or D-mannose. Bound MBL was detected using a mAb against MBL coupled to DIG (3E7-DIG).

#### *Hemolytic assays*

Inhibition of the classical pathway by decorin or biglycan was assessed using a CH50 assay, which measures the total activity of the classical pathway as well as a C1q-dependent hemolytic assay (C1qHA) as previously described (21). Briefly, in the CH50, Ab-sensitized sheep Ab-opsonized erythrocytes (EA;  $1 \times 10^8$  cells) were incubated with NHS in a fixed dilution for 1 h at 37°C. NHS was preincubated for 15 min on ice with or without decorin, biglycan, or human serum albumin (HSA) before addition of the mixture to the EA. Total classical pathway activity was assessed by measuring EA lysis. In the C1qHA, EA were incubated with a fixed dose of C1q (20 ng/ml) and C1q-depleted plasma (1/100). C1q was preincubated for 15 min on ice with or without decorin, biglycan, or HSA before addition of the mixture to the EA. The C1q-dependent classical pathway activity was assessed by measuring EA lysis. The lytic activity of both CH50 and C1qHA was expressed as a Z value:  $Z = -\ln [1 - ((OD_{414} \text{ lysis by sample}) - (OD_{414} 0\% \text{ lysis})) / ((OD_{414} 100\% \text{ lysis}) - (OD_{414} 0\% \text{ lysis}))]$ . The amount of C1q in the C1qHA and the dilution of the NHS in the CH50 were chosen in such a way that Z value in the absence of inhibitor was ~1 (63% lysis).

#### *Cell culture*

HUVEC and U937 cells were used for flow cytometry and cell-stimulation assays. HUVEC were obtained from fresh umbilical cords after collagenase (Sigma-Aldrich) treatment and were cultured in M199 medium (Invitrogen Life Technologies) containing 10% v/v heat-inactivated FCS (Invitrogen Life Technologies), 100 IU/ml penicillin (Sigma-Aldrich), 100 µg/ml streptomycin (Sigma-Aldrich), 7.5 IU/ml heparin (Sigma-Aldrich), 2 ng/ml EGF (R&D Systems), and 250 pg/ml β-ECGF (BioSource International). Cells were harvested using trypsin and used for flow cytometry analysis. HUVEC were used in the experiments between passages 2 and 4. U937 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin, and 100 µg/ml streptomycin (both from Sigma-Aldrich).

#### *Flow cytometric analysis of C1q binding*

The effect of decorin or biglycan on the ability of C1q to bind to cells was examined using flow cytometry. HUVEC and U937 cells were first washed in low-ionic-strength buffer (0.5× PBS, 155 mM glucose, 1% BSA, and 0.1% sodium azide). Cells were incubated in low-ionic-strength buffer for 30 min on ice. C1q (2.5 µg/ml) was preincubated with different concentrations of decorin or biglycan before being added to the cells. After washing, C1q binding was detected by a polyclonal rabbit anti-C1q Ab (20) followed by PE-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates). Dead cells were excluded from the analysis by the use of propidium iodide (Molecular Probes). The cell-associated fluorescence was measured using a FACSCalibur (BD Biosciences) and results were expressed as the mean fluorescence intensity.

#### *Stimulation of endothelial cells with C1q in the presence of biglycan or decorin*

The effect of decorin and biglycan on the C1q-induced production of MCP-1 and IL-8 was examined using confluent layers of HUVEC on 0.5% gelatin-coated 96-well plates. C1q (20 µg/ml) was coincubated with or without different concentrations of decorin or biglycan at room temperature in AIM-V serum-free medium (Invitrogen Life Technologies) completed with 100 IU/ml penicillin and 100 µg/ml streptomycin (both Sigma-Aldrich). Subsequently, HUVEC were incubated with this mixture for 48 h, then supernatants were harvested and analyzed for IL-8 and MCP-1 levels by a sandwich ELISA, as has been previously described by van den Berg et al. (3).

## **Results**

### *Binding of C1q to decorin and biglycan and inhibition of the classical complement pathway*

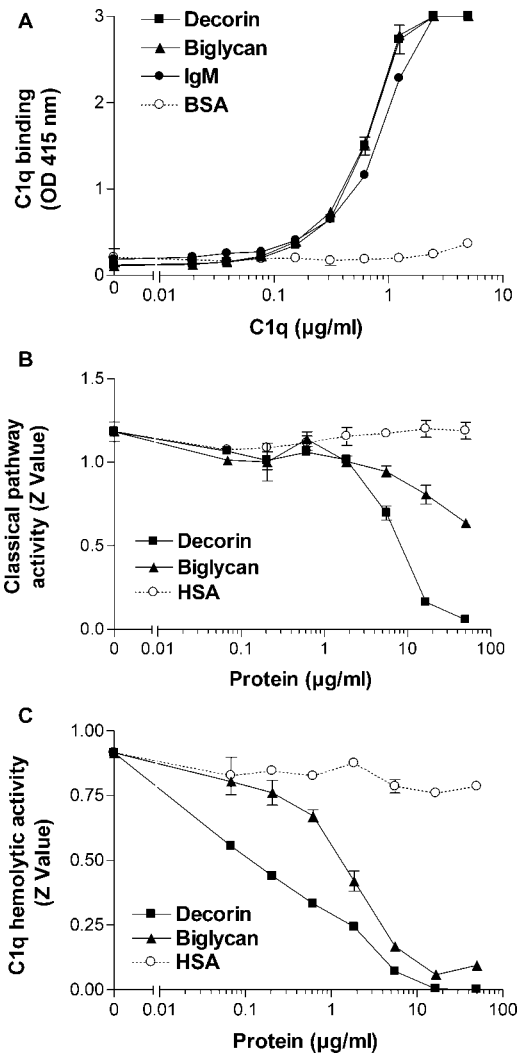
In ELISA, human C1q bound strongly to solid-phase decorin and biglycan in a dose-dependent manner (Fig. 2A). The strength of binding between C1q and proteoglycans was comparable to that between C1q and IgM. No binding was observed to wells coated with BSA (Fig. 2A). Native bovine decorin was shown to bind C1q to a similar extent as recombinant human decorin (results not shown).

The ability of decorin and biglycan to inhibit the functional activity of the classical pathway was examined using hemolytic assays. The effect of human decorin and biglycan on the total activity of the classical pathway was examined in a CH50 assay, using total human serum as a complement source. Decorin inhibited the hemolytic activity of the classical pathway ( $IC_{50} \sim 5 \mu\text{g/ml}$ ) in a dose-dependent manner (Fig. 2B). Although biglycan also inhibited the classical pathway, it was found to be ~10 times less effective than decorin. In agreement with a direct interaction of decorin with C1q, decorin was able to dose-dependently inhibit the hemolytic activity of C1q in a C1qHA, with an  $IC_{50}$  of ~0.1 µg/ml (Fig. 2C). In addition, biglycan was able to inhibit C1q hemolytic activity but less effectively than decorin ( $IC_{50} \sim 1 \mu\text{g/ml}$ ). The control protein HSA did not have any effect on complement-induced lysis of EA.

### *Solid-phase decorin binds C1 but fails to activate the classical pathway*

Because immobilized ligands of C1q are known to activate the classical pathway, we examined whether immobilized decorin and biglycan had similar properties. When coated to microtiter wells and incubated with different serum concentrations as a complement source, both decorin and biglycan failed to activate C4. In contrast, IgM, as a positive control, activated complement, leading to strong deposition of C4 (Fig. 3A). BSA, which was used as a negative control protein, did not activate C4.

Because decorin and biglycan did not activate the classical pathway despite binding C1q, we sought to examine whether decorin and biglycan were able to bind C1 (C1q in association with C1r and C1s). Solid-phase decorin and IgM, but not biglycan, bound

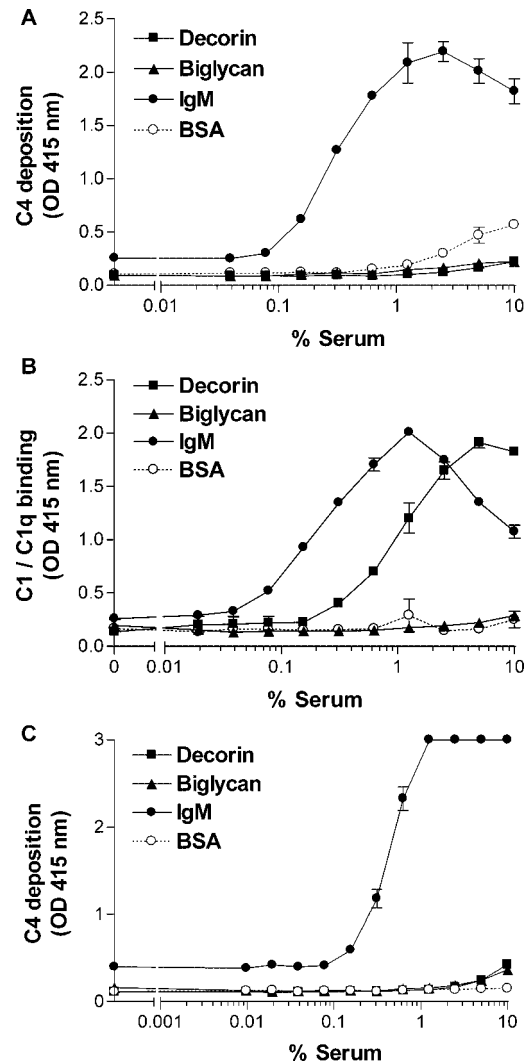


**FIGURE 2.** Decorin and biglycan bind C1q and inhibit the classical complement pathway. *A*, Microtiter wells were coated with decorin, biglycan, IgM, or BSA and subsequently incubated with different concentrations of C1q. Binding was detected using a C1q-specific mAb. *B*, NHS was premixed with different concentrations of decorin, biglycan, or HSA, followed by incubation with EA and measurement of hemolysis. *C*, C1q (20 ng/ml) was preincubated with different concentrations of decorin, biglycan, or HSA and then mixed with C1q-depleted serum and EA.

C1 in a dose-dependent manner (Fig. 3*B*). When C1 was allowed to bind human IgM, and then incubated with exogenous C4, a strong activation of C4 was observed (Fig. 3*C*). However, no activation of exogenous C4 was observed after binding of C1 to decorin. Furthermore, neither biglycan nor BSA did induce activation of exogenous C4.

#### Characterization of the interaction between decorin and C1q

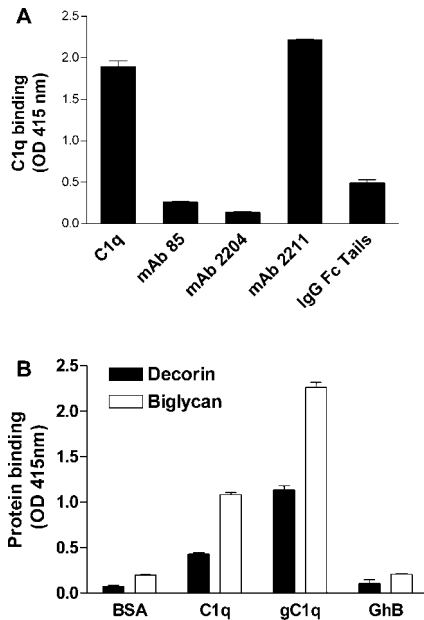
To identify the regions/domains within C1q that interacted with decorin, a competitive ELISA was performed where ligands and Abs directed against gC1q or CLR domains of C1q were allowed to compete for binding of C1q to immobilized decorin. Coincubation of C1q with two mAb directed against the gC1q domain (mAb 2204 and mAb 85) completely abolished C1q binding to decorin (Fig. 4*A*). Furthermore, purified Fc portions of mouse IgG that bind to the gC1q domain efficiently competed with the binding of C1q to decorin. As a negative control for inhibition, we used an Ab (mAb 2211) that recognizes the CLR portion of C1q, which did not



**FIGURE 3.** Decorin binds C1 but does not activate the classical pathway. *A*, Decorin, biglycan, IgM, or BSA were coated onto microtiter wells and incubated with NHS for 1 h at 37°C. C4 deposition was measured using a C4-specific mAb. *B*, Decorin, biglycan, IgM, or BSA were coated and then incubated with NHS in a Ca<sup>2+</sup>-containing buffer for 2 h at 4°C. C1 binding was detected using mAb 2214. *C*, Decorin, biglycan, IgM, or BSA were coated and incubated with NHS for 2 h at 4°C. The wells were washed with buffer containing Ca<sup>2+</sup> and then incubated with human C4 for 1 h at 37°C, followed by C4 detection.

inhibit the C1q-decorin interaction. Furthermore, the interaction of decorin and biglycan with the gC1q domain was studied by comparing the ability of decorin or biglycan to directly bind to immobilized intact C1q, gC1q, and the recombinant form of ghB (Fig. 4*B*). Biglycan and decorin showed strong binding to the immobilized native gC1q domain, significantly better than to intact C1q. However, both proteoglycans did not bind to the recombinant ghB. In a similar binding experiment, neither decorin nor biglycan showed any detectable binding to immobilized recombinant modules ghA, ghB, or ghC, suggesting a requirement for a heterotrimeric structure of the gC1q domain for interaction with proteoglycans (results not shown).

In a direct binding ELISA, CLR domain was able to bind decorin, although nearly 15 times less efficiently than intact C1q (Fig. 5*A*). C1q, but not CLR domain, bound IgM (Fig. 5*B*), confirming that the CLR preparation did not contain portions of gC1q domain. Furthermore, mAb directed against the gC1q domain



**FIGURE 4.** Decorin and biglycan bind the gC1q domain of human C1q. *A*, DIG-conjugated C1q was preincubated in the presence of gC1q-specific mAb (mAb 2204 and mAb 85), a noninhibitory mAb directed against the CLR domain (mAb 2211), or mouse Fc tails. After incubation on a decorin-coated ELISA plate for 1 h at 37°C, C1q binding was detected using anti-DIG Abs. *B*, C1q, native gC1q, ghB, or BSA were coated on a plate (2 μg/ml) followed by incubation with either 10 μg/ml decorin or 1 μg/ml biglycan. Binding of decorin and biglycan was detected using a rabbit antiserum raised against decorin and biglycan, respectively.

(mAb 85 and mAb 2204) did not bind the CLR preparation, whereas a mAb specific to the CLR domain (mAb 2214) showed strong binding (data not shown). Both decorin (Fig. 5C) and biglycan (Fig. 5D) bound immobilized CLR domain in a dose-dependent manner. Detection with an Ab against the CLR domain of C1q confirmed that both C1q as well as CLR were able to bind well to an ELISA plate (Fig. 5E).

#### *Decorin and biglycan bind to C1q and members of the collectin family*

In view of the binding of decorin and biglycan to C1q, we further investigated their interaction with MBL and other collectins, considering similar overall structure and presence of triple-helical collagen regions. In a direct binding ELISA using a calcium-free buffer, both decorin and biglycan bound to immobilized C1q, native SP-D and rhSP-D, and the bovine conglutinin and CL-43 in a dose-dependent manner (Fig. 6, A and B). Decorin and biglycan showed the strongest binding to conglutinin, whereas only biglycan could detectably bind to immobilized MBL. This binding could not be improved by incubation in the presence of calcium (not shown). These data suggest that decorin and biglycan recognize a broad range of collagen-containing innate immune molecules without requiring calcium.

#### *MBL binds to decorin in a Ca<sup>2+</sup>-dependent way but does not inhibit the lectin pathway*

The observation that biglycan can interact with MBL in Ca<sup>2+</sup>-free conditions, prompted us to investigate 1) whether biglycan and decorin can interact with native MBL via its Ca<sup>2+</sup>-dependent C-type lectin domain, 2) whether, by binding MBL, decorin and biglycan can modulate the lectin pathway, and 3) whether this modulation involves interference in the ligand binding of the C-type

lectin domain. Purified MBL was incubated with immobilized decorin or biglycan in the absence or presence of Ca<sup>2+</sup>, and binding was assessed using a mAb against MBL. Decorin and biglycan clearly bound MBL, but only in the presence of calcium (Fig. 7A), suggesting the involvement of the calcium-dependent C-type lectin domains of MBL in this interaction.

To assess the effect of the MBL-proteoglycan interactions on the activation of the lectin pathway, mannan-coated plates were incubated with C1q-depleted plasma as a complement source, followed by assessment of complement activation and generation of the C5b-9 complex as assessed with a mAb directed against C5b-9. Decorin was not able to inhibit the lectin pathway-mediated formation of C5b-9 (Fig. 7B). In contrast, biglycan nearly completely inhibited activation of complement via the MBL pathway with increasing concentration (IC<sub>50</sub> ~40 μg/ml). Also D-mannose, a known inhibitor of the lectin pathway, clearly inhibited complement activation (IC<sub>50</sub> ~5 μM).

To determine whether decorin and biglycan could modulate lectin pathway activation by inhibiting the binding of MBL to its ligand, MBL in the presence or absence of different concentrations decorin, biglycan, or D-mannose was incubated on a mannan-coated plate. Subsequently, binding of MBL was detected. Neither biglycan nor decorin could prevent the binding of MBL to its ligand, suggesting that inhibition of the lectin pathway by biglycan was not at the level of ligand binding (Fig. 7C). In contrast, D-mannose as a ligand for the C-type lectin domain could inhibit the binding of MBL to mannan completely.

#### *Decorin and biglycan inhibit the binding of C1q to HUVEC and U937 cells*

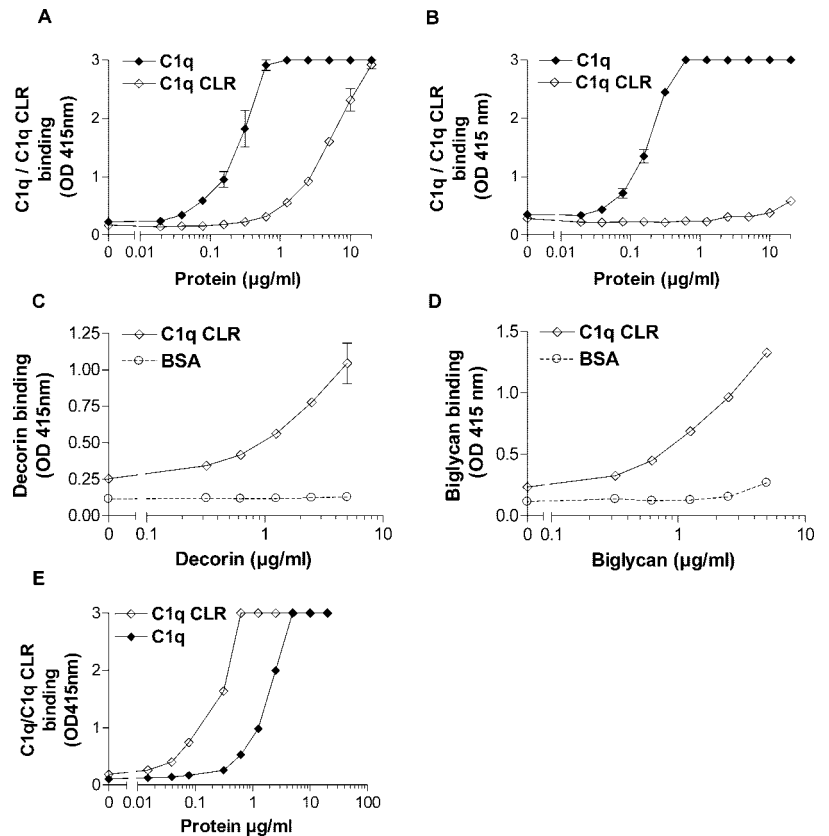
Because C1q is known to modulate various immune cells through its interaction with C1q receptors (3), we sought to establish whether decorin or biglycan would interfere with C1q-cell interactions. Flow cytometry revealed that C1q was able to bind to both HUVEC and U937 cells. Decorin and biglycan strongly inhibited the binding of C1q to HUVEC (Fig. 8A). Decorin prevented the C1q binding to HUVEC in a dose-dependent manner with an IC<sub>50</sub> of ~0.1 μg/ml (Fig. 8B). Decorin was also able to inhibit the binding of C1q to U937 cells with an IC<sub>50</sub> between 0.1 and 1 μg/ml (Fig. 8C).

#### *Biglycan inhibits C1q-induced MCP-1 and IL-8 production*

Interaction of C1q with endothelial cells has been shown to result in production of inflammatory cytokines and chemokines, such as MCP-1 and IL-8. Therefore, we examined the effect of decorin and biglycan on C1q-induced MCP-1 and IL-8 production by HUVEC. Stimulation for 48 h with C1q alone resulted in strongly increased MCP-1 (~110 ng/ml) and IL-8 (~5 ng/ml) production (Fig. 9) compared with cells cultured in the presence of medium alone (~50 ng/ml and <0.1 ng/ml, respectively). The presence of decorin together with C1q resulted in slight inhibition of MCP-1 production (Fig. 9A) and had no effect on the C1q-induced IL-8 production (Fig. 9B). It was noted that decorin by itself slightly increased MCP-1 and IL-8 production by HUVEC, which effect almost nullified the inhibitory effects of decorin on C1q-induced MCP-1 and IL-8 production. In contrast, addition of biglycan led to a dose-dependent decrease of C1q-induced MCP-1 production and completely abrogated the effect of C1q on IL-8 production (Fig. 9, C and D). Biglycan by itself had no effect on MCP-1 and IL-8 production.

## **Discussion**

Components of the ECM are considered important for structural integrity, cell signaling, and survival within tissue organization.



**FIGURE 5.** Decorin and biglycan bind the CLR domain of human C1q. Various concentrations of C1q or its CLR preparation were allowed to bind to immobilized decorin (A) or IgM (B). C1q and CLR binding was probed using mAb 2214. Different concentrations of decorin (C) or biglycan (D) were allowed to bind immobilized CLR or BSA. Binding of decorin and biglycan was probed using a rabbit anti-serum raised against decorin or biglycan, and then detected using goat anti-rabbit HRP conjugate. E. Microtiter wells were coated with different concentrations of C1q or CLR, and binding was detected using mAb 2214.

The ECM proteins can also play an active role in the innate immune response, as recently described for mindin (also called spondin 2), which binds bacteria and functions as opsonin for murine macrophages (22). Other ECM proteins have also been shown to regulate the complement system. Earlier, decorin, an ECM proteoglycan, was shown to bind C1q and inhibit the classical pathway of complement (8).

The ECM proteoglycans decorin and biglycan possess ~55% similarity on the amino acid level. However, the secondary structure of both proteins as well as the spatial and temporal expression is different (6). Considering this, we wanted to investigate whether the recombinant forms of both proteins have similar effects on the classical pathway.

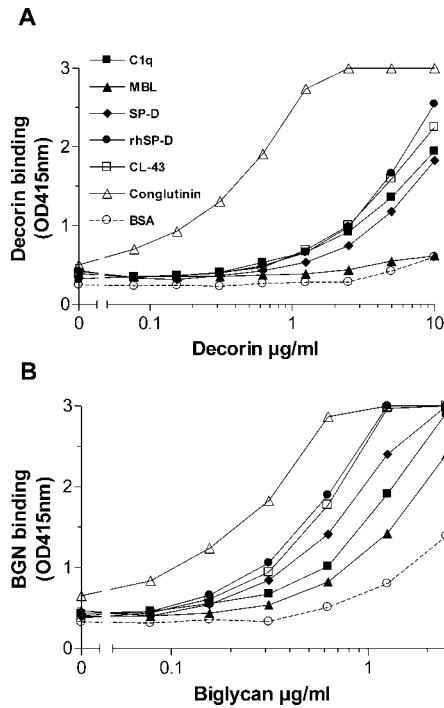
Human decorin and biglycan possess a higher affinity for immobilized C1q than for their well-known ligands, including collagens I, II, III, V, and VI (10), which suggests a possibly physiologically important interaction. In addition, we now show that the binding of C1q to immobilized decorin and biglycan is similar to the binding of C1q to its natural ligand IgM. Furthermore, human decorin, as described for bovine decorin, is capable of completely inhibiting the C1q-dependent lysis of Ab-opsonized erythrocytes. Interestingly, a similar effect was observed for biglycan.

Immobilized human decorin can bind intact C1, whereas biglycan is not able to bind C1. Furthermore, in contrast to other ligands that bind C1 such as IgM, IgG, and pentraxins, decorin completely fails to activate the classical pathway, although the protein was presented in a multimeric fashion by immobilization on plastic, a condition that is likely to facilitate complement activation. The ability of decorin to bind C1 is also reflected by its ability to inhibit the complement-mediated lysis of Ab-opsonized erythrocytes in the presence of whole serum as a complement source, where C1q is present in a calcium-rich environment and, therefore, predominantly present in the C1 form. Biglycan can also inhibit comple-

ment-mediated erythrocyte lysis via the classical pathway, but at a much higher  $IC_{50}$  than decorin, which is consistent with its undetectable binding to C1.

Because the level of decorin in the circulation is low (~0.9 ng/ml) (23), it seems unlikely that decorin plays a major role as inhibitor of the classical pathway in the circulation. However, at the cellular level, decorin is estimated to be present in the ECM in concentrations between 5 and 12.5 µg/ml (24). Thus, under conditions of tissue damage or remodeling of the ECM, decorin may play a role in inhibiting the classical pathway. However, biglycan is not able to bind C1 and has a much lower inhibitory effect on complement-mediated lysis of opsonized erythrocytes, which appears to suggest that under normal physiological conditions, biglycan may not be as relevant as decorin in the regulation of the classical pathway. However, infiltrating cells like macrophages have been shown to secrete biglycan in a model of renal inflammation, upon their stimulation with inflammatory cytokines (25). Hence, in inflammatory conditions, the additional biglycan could have an effect on the classical pathway of complement.

The mechanism of binding of C1q to decorin is a complicated issue. The ability of decorin to bind to several different collagens would favor binding via the CLR domain of C1q. However, C1q binding to bovine decorin has been described to be mediated via both the gC1q as well as the CLR of C1q (8), hence bovine decorin was proposed to bind at the hinge region between the gC1q and CLR domains. Consistent with this, Abs directed against the gC1q domain are able to completely inhibit the binding of C1q to immobilized decorin. Furthermore, the mouse IgG-Fc tails, as a natural ligand for the gC1q domain, appear to prevent C1q from binding to immobilized decorin. Curiously, individually expressed modules of the gC1q domain (ghA, ghB, and ghC) failed to interact with decorin, although they do bind various C1q ligands differentially, indicating their functional activity and proper folding

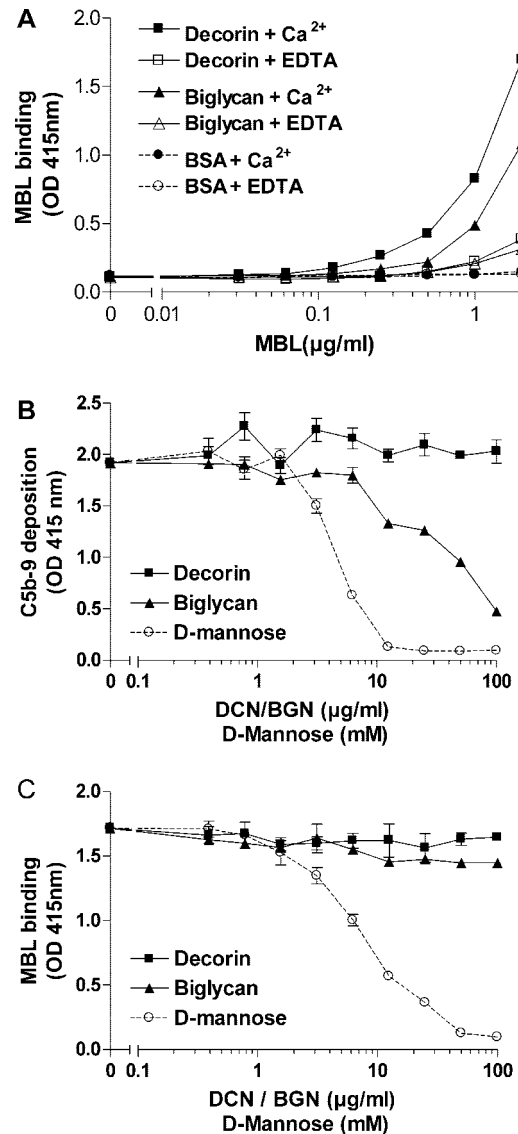


**FIGURE 6.** Decorin and biglycan interact with the members of the collectin family, including MBL, SP-D, conglutinin, and CL-43. Microtiter wells were coated with 2  $\mu\text{g/ml}$  C1q, MBL, native SP-D, rhSP-D, bovine conglutinin, CL-43, or BSA. Wells were incubated with decorin (A) or biglycan (B), both in the presence of 2 mM EDTA. Proteoglycan binding was detected using specific rabbit antisera.

(17). However, the native gC1q domain, prepared after collagenase digestion of native C1q, is able to strongly interact with decorin and biglycan, indicating that the C1q-proteoglycan interaction might require a combined heterotrimeric structure of the gC1q domain and the individual chains may contribute to proteoglycan binding.

We also noticed a direct interaction of C1q CLR domain with decorin and biglycan, consistent with the collagen-binding properties of these proteoglycans. However, relative to the binding to intact C1q, the interaction of decorin and biglycan was far less than as compared with the binding of C1q and the gC1q domain to both proteoglycans. Furthermore, recent data indicate that interactions with isolated C1q CLR could be a result of the preparation, altering the physical-chemical properties of the molecule, and the binding characteristics of isolated CLR are different from those of intact C1q (26). Moreover, our experiments with inhibitory Abs against the gC1q domain clearly indicate that the primary interaction of proteoglycans with intact C1q involves the gC1q domain.

C1q has a number of characteristics in common with members of the collectin family. These molecules are all characterized by a multimeric structure consisting of trimeric subunits, as well as by similar collagenous domains containing Gly-X-Y repeats. Ligand recognition takes place via gC1q domain for C1q and via C-type lectin domain for collectins (27). Recently, SP-D has been shown to bind decorin (28), and this interaction involves C-type lectin domain binding to decorin-attached glycosaminoglycan chain, whereas the decorin core protein can bind via SP-D collagen region (28). In the present study, we validated the interaction of decorin and biglycan with native SP-D and rhSP-D, which represents the trimeric C-type lectin domains. We observed that immobilized CL-43, conglutinin, rhSP-D, and SP-D can bind decorin and biglycan in a dose-dependent and calcium-independent manner, suggesting a protein-protein interaction. Furthermore, in

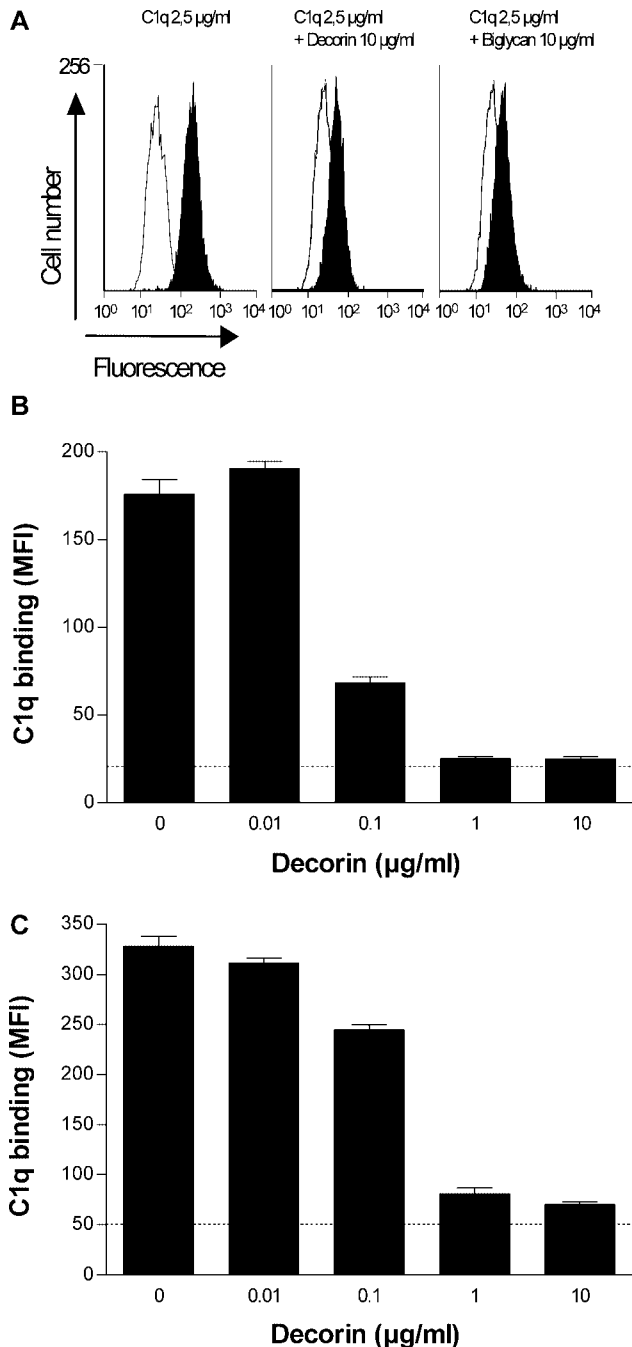


**FIGURE 7.** Decorin and biglycan bind MBL, but only biglycan inhibits the lectin pathway. A, Microtiter wells were coated with 5  $\mu\text{g/ml}$  decorin, biglycan, or BSA and incubated with different concentrations of purified MBL in the presence or absence of  $\text{Ca}^{2+}$ . MBL binding was detected using an MBL-specific mAb. B, C1q-depleted serum was preincubated with different concentrations of decorin, biglycan, or D-mannose followed by incubation on a mannan-coated ELISA plate for 1 h at 37°C. C5b-9 deposition was detected using a specific mAb. C, MBL was preincubated in the presence of different concentrations of decorin, biglycan, or D-mannose and then allowed to bind to mannan-coated microtiter wells. Binding of MBL was detected using an MBL-specific mAb.

the absence of calcium, only biglycan showed an interaction with immobilized MBL, indicating that biglycan may bind to the CLR domain of MBL. However in the presence of  $\text{Ca}^{2+}$ , purified MBL binds efficiently to immobilized decorin and biglycan, suggesting that the C-type lectin domains of MBL could also bind to carbohydrates present on decorin and biglycan, as is the case for SP-D (28).

The ability of decorin to bind to MBL is not reflected in its ability to inhibit the lectin pathway of complement. Most likely, the interaction of decorin with the C-type lectin domain of MBL in the fluid phase is insufficient to inhibit complement activation significantly because addition of decorin has no effect on the binding of MBL to a immobilized ligand. In contrast, the interaction of decorin with C1 does result in the inhibition of complement. As

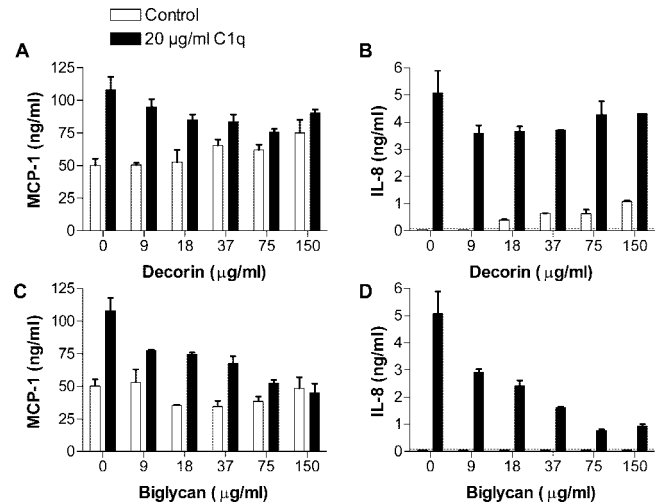




**FIGURE 8.** Decorin and biglycan inhibit the interaction of C1q with HUVEC and U937 cells. *A*, Purified C1q was preincubated with decorin or biglycan and then incubated with HUVEC. C1q binding was detected by flow cytometry using a polyclonal anti-C1q polyclonal Ab and followed by goat anti-rabbit-PE. Data are expressed as mean fluorescence intensity. *B* and *C*, C1q was preincubated with different concentrations of decorin in half isotonic buffer and then incubated with either HUVEC or U937 cells. C1q binding was detected using a C1q-specific polyclonal Ab followed by goat anti-rabbit-PE.

opposed to decorin, biglycan is capable of inhibiting activation of the lectin pathway, although it does not seem to inhibit the MBL-ligand interaction. Therefore, it is likely that the inhibitory effect of biglycan on the lectin pathway activation involves the interaction of biglycan with the CLR domain of MBL, possibly interfering in binding and/or activation of MASP-2.

The C1q molecule is known to bind to immune cells via cell membrane receptors and to induce the production of inflammatory



**FIGURE 9.** Biglycan inhibits the C1q-induced production of IL-8 and MCP-1 by HUVEC. HUVEC were cultured with (■) or without (□) 20 μg/ml C1q in the presence or absence of different concentrations of either decorin (*A* and *B*) or biglycan (*C* and *D*). After 48 h, the concentration of MCP-1 and IL-8 in the supernatant was determined by ELISA.

cytokines and chemokines, such as MCP-1 and IL-8, by endothelial cells (3). We found that decorin and biglycan strongly inhibit the binding of C1q to cells, presumably interfering in receptor-mediated interactions. In addition, biglycan clearly had an inhibitory effect on the C1q-induced MCP-1 and IL-8 production by endothelial cells. Therefore, decorin and biglycan not only function as inhibitors of the classical pathway of complement activation, but may have an additional role in down-regulating the proinflammatory effects of C1q on cells, by inhibition of the production of cytokines. In contrast, it has been observed that biglycan, via interaction with TLR-2 and -4 on macrophages, can induce the expression of inflammatory mediators like TNF- $\alpha$  and MIP-2 (25). Furthermore, in biglycan-deficient mice, the absence of biglycan has been associated with a survival benefit in a mouse model of sepsis (25). Together, these results indicate that biglycan is able to modulate inflammation in several ways, and its final effect may be strongly dependent on the context, the site, and the phase of the inflammatory process.

It has been described previously that infusion of decorin can ameliorate fibrosis in an experimental model of kidney disease, induced by anti-Thy1 Abs, which was explained by an inhibitory effect on TGF- $\beta$  function (29). However, because this is a complement-dependent model, it is possible that the ameliorating effects of decorin might be partially explained by its ability to prevent the activation of the classical complement pathway. Accordingly, complement inhibition may contribute to a reduction of the degree of damage inflicted to tissues, ultimately resulting in less fibrosis. Moreover, in an experimental model of unilateral ureteral obstruction, mice deficient in decorin showed exaggerated apoptosis, mononuclear cell infiltration, tubular atrophy, and matrix deposition, as compared with wild-type mice (30). Whether increased tissue damage in decorin-deficient mice is associated with activation of the classical complement pathway, is worth investigating.

In summary, we have shown that the human proteoglycans decorin and biglycan interfere differentially with various aspects of complement-mediated inflammatory responses, including activation of the complement cascade via classical and lectin pathways, and endothelial cell activation. Furthermore, interaction of these

proteoglycans with collectins suggests a potential role in the modulation of collectin function. Our results indicate that decorin might function at the tissue level as a modulator of complement-dependent inflammation. It appears that local expression of decorin and biglycan may limit tissue damage after injury.

## Acknowledgments

We thank Dr. T. Fujita (Fukushima Medical University), Dr. C. E. Hack (Sanquin Research, Amsterdam, The Netherlands), Dr. T. E. Mollnes (Institute of Immunology, Rikshospitalet University Hospital), Dr. F. Vivanco (Renal Research Laboratory, Fundación Jiménez Díaz, Autónoma University), and Dr. J. Egido (Department of Immunology, Fundación Jiménez Díaz, Autónoma University) for supplying valuable reagents.

## Disclosures

The authors have no financial conflict of interest.

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