The Decorin Sequence SYIRIADTNIT Binds Collagen Type I*

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Decorin belongs to the small leucine-rich repeat proteoglycan family, interacts with fibrillar collagens, and regulates the assembly, structure, and biomechanical properties of connective tissues. The decorin-collagen type I-binding region is located in leucine-rich repeats 5–6. Site-directed mutagenesis of this 54-residue-long collagen-binding sequence identifies Arg-207 and Asp-210 in leucine-rich repeat 6 as crucial for the binding to collagen. The synthetic peptide SYIRIADTNIT, which includes Arg-207 and Asp-210, inhibits the binding of full-length recombinant decorin to collagen in vitro. These collagen-binding amino acids are exposed on the exterior of the \( \beta \)-sheet-loop structure of the leucine-rich repeat. This resembles the location of interacting residues in other leucine-rich repeat proteins.

Decorin is an extracellular matrix proteoglycan involved in collagen type I fibril formation and collagen matrix assembly in a wide range of connective tissues (1–3). Decorin belongs to the small leucine-rich repeat proteoglycan family (SLRPs) (4), which also includes, for example, biglycan (5), fibromodulin (6), lumican (7), and asporin (8, 9). The decorin core protein is composed of 12 tandem leucine-rich repeats (LRRs), each containing an average of 24 amino acid residues. The decorin three-dimensional structure, resolved by x-ray crystallography, reveals an arch-shaped molecule with LRRs composed of parallel \( \beta \)-sheets on the concave surface and short \( \beta \)-strands, 3\( _{10} \) helices, and polyproline II helices on the convex face (10). Close to the N terminus, decorin is substituted with a single chondroitin or dermatan sulfate chain. Decorin also has three potential consenus sites for \( \mathrm{N} \)-glycosylation in the LRR domain (4).

Several SLRPs are involved in the regulation of collagen fibril formation and matrix assembly, as demonstrated in SLRP-deficient mice (11). Decorin deficiency causes skin fragility (12); biglycan-deficient mice suffer from reduced bone mass and osteoarthritis (13); lack of fibromodulin results in weaker tendons and ligaments, causing osteoarthritis (14); and lumican ablation correlates with corneal opacity (15). In all these cases, ultrastructural imaging reveals abnormally developed collagen fibrils.

Decorin presumably affects the lateral association of collagen type I fibrils in vitro as it binds to collagen monomers and delays their accretion to the growing fibrils (1). Collagen fibrils in the skin of decorin-deficient mice are thicker and irregularly shaped, in contrast to smaller, uniform fibrils formed in wild type mice (12). Decorin binds near the C terminus of collagen, close to an intermolecular cross-linking site (16), and seems to have a tutelary function in collagen fibril assembly, which is important for the collagen cross-linking. In addition, decorin also binds collagen type VI (17), transforming growth factor-\( \beta \) (18), fibronectin (19), and epidermal growth factor receptor (20).

The precise decorin-binding site for collagen has not been determined. An early study identified two collagen-binding sites in decorin (21). A later report assigned a collagen-binding site to LRR 5–6 (22) and Glu-180 in decorin LRR 5 (Glu-180 in human decorin corresponds to Glu-181 in the bovine sequence) was proposed to be essential for the interaction (23). Decorin with an E180Q mutation was later demonstrated to bind more strongly to collagen type VI than wild type decorin, and amino acid residues located C-terminal of Gln-153 in decorin were implicated in the collagen binding (24). In addition to the core protein, the dermatan sulfate chain has also been reported to be involved in the collagen interaction (25). This glycosaminoglycan-collagen interaction is weaker than the core protein-collagen interactions (22–24).

It is of interest to characterize this prototype decorin-collagen interaction since other SLRPs, e.g. fibromodulin and lumican, also interact with fibrillar collagens and affect connective tissue properties in similar ways. The concerted action of several SLRPs can determine the structure and function of collagen matrices. In this study, we used site-directed mutagenesis of decorin LRR 5–6 to clarify the location of the collagen-binding site.

**EXPERIMENTAL PROCEDURES**

Expression of GST-tagged bacDCNS6 in Bacteria—Bovine decorin cDNA (GenBank™ accession number NM_173906) was used as a template in PCR to amplify cDNA encoding LRR 5–6 of decorin. The primers used for the PCR were: 5\'-AAAG-GATCCACGGATCAAATGC-3' and 5\'-AACCCGGGATATTGTCAGCAATGGC-3'. The amplified cDNA was digested with restriction enzymes BamHI and SalI and ligated into the vector pGEX-4T-3 (Amersham Biosciences). The construct was sequenced to confirm the identity...
and transfected into BL21 *Escherichia coli*, and bacDCN56 protein was expressed according to the manufacturer’s instructions. bacDCN56 was purified under native conditions by glutathione affinity chromatography, as described previously (26), and dialyzed against PBS with 0.2% (v/v) Tween 20 prior to use. The protein concentration was determined with Coomassie protein assay reagent (Pierce).

Expression of His-tagged Fibromodulin in Bacteria—Bovine fibromodulin cDNA (GenBank accession number X16485) was used as a template in PCR to amplify fibromodulin cDNA. The primers used for the PCR were: 5′-CCGAGTCCCCAAATATGAGGAAAGACTCTCAC-3′ and 5′-CTCCGAGGATCTC-GATGAGGCTAGCC-3′. The amplified cDNA was digested with XhoI and BamHI restriction enzymes and ligated into a PET-27b (+) expression vector (Novagen), and the His-tagged protein was expressed in Rosetta cells (Novagen). Fibromodulin was purified using nickel-nitrilotriacetic acid agarose (Qiagen) according to the manufacturer’s instructions. Prior to use, the protein was dialyzed against PBS with 0.2% (v/v) Tween 20.

Site-directed Mutagenesis—bacDCN56 cDNA and bovine decorin cDNA (GenBank accession number NM_173906) were used for site-directed mutagenesis using QuikChange II kit and pBluescript II KS (+/-) cloning vector (both from Stratagene) according to the manufacturer’s instructions. Mutations of bacDCN56, used in bacterial protein expression, were the following: E162Q, E181K, E181Q, T184V, E193Q, Y205F, R207S, D210N. Mutations of bovine decorin, used for mamma- lian cell protein expression, were the following: E181K, R207S, and D210N. Primers used for mutagenesis are listed in supplemental Fig. 1.

Mammalian Expression of Wild Type Decorin and Mutated Decorins—Wild type or mutated decorin cDNA was digested with the restriction enzymes HindIII and BamHI and ligated into the pCEP4 BM40-hisEK expression vector (27), attaching the decorin cDNA to His tag DNA in the N terminus. The constructs were sequenced to confirm identity and transfected into the pCEP4 BM40-hisEK expression vector (27), attaching the decorin cDNA to His tag DNA in the N terminus. The protein was dialyzed against PBS with 0.2% (v/v) Tween 20.

Solution-phase Collagen Binding Assay—Collagen binding assays were performed by the incubation of 15 μg of acetic acid-extracted mouse tail collagen in 100 μl of PBS, pH 7.4, with the addition of different concentrations of radiolabeled decorin, as described previously (17). Neutralization of acid solubilized collagen leads to the formation of collagen fibrils, which bind decorin. After incubation for 5 h at 37 °C, samples were centrifuged for 5 min at 13,000 × g to separate collagen fibrils with bound decorin in the pellet from non-bound decorin in the supernatant. More than 90% of the collagen was recovered as fibrils in the pellet. The supernatant was removed, and the pellet was washed once with 1 ml of PBS. The pellet and the supernatant were then electrophoresed on a 10% SDS-PAGE, and the amount of radiolabeled proteoglycans was determined in a FLA-3000 bioimaging analyzer (Fuji Photo Film Co.). The data were analyzed by non-linear regression using GraphPad Prism 3 software (GraphPad Software, Inc.).

Results

To study the interaction between decorin and collagen type I, we initially expressed decorin LRR 5–6, previously shown to bind collagen (22, 23), as a GST fusion protein in bacteria (bacDCN56). This fusion protein inhibited the binding of mamma- lian-expressed full-length [35S]decorin to collagen in solution, indicating that they bind to the same site on collagen (Fig. 1). This confirms the collagen binding properties of bacDCN56, which was used in the further characterization of the decorin-collagen interaction. To identify the amino acid residues involved in the interaction we performed site-directed mutagenesis. We selected residues in bacDCN56 that were conserved between species, and also exposed on the exterior of the protein (Fig. 2). Thus, the mutations E162Q, E181K, E181Q, Y205F, R207S, and D210N were introduced into bacDCN56 and expressed in E. coli. The binding of the mutated decorin fragments was determined using a solid-phase...
binding assay. bacE162Q, bacE181K, bacE181Q, bacT184V, bacE193Q, and bacY205F showed similar binding properties as the control (non-mutated) bacDCN56. In contrast, bacR207S and bacD210N had lower affinity for collagen (Fig. 3).

To corroborate the above results, we introduced the D210N, R207S, and E181K mutations in the full-length decorin cDNA and expressed them as His-tagged proteins in 293 cells. The collagen affinities of the mutated decorins were determined in a solution-phase collagen binding assay in which the collagen molecules presumably form fibrils during the incubation at 37 °C. Wild type decorin has a sigmoidal binding curve ($K_D = 60 \text{ nM}$), whereas all mutated decorins have linear binding curves, indicating nonspecific binding (Fig. 4). In a solid-phase assay, wild type decorin also has a sigmoidal binding curve with $K_D = 6 \text{ nM}$, whereas extrapolation of the linear binding curves for mutated decorins gives $K_D = 350 \text{ nM}$ for mamE181K, $K_D = 1 \text{ mM}$ for mamR207S, and $K_D = 1 \text{ mM}$ for mamD210N (Fig. 5).

A synthetic peptide SYIRIADTNIT, which encompasses Arg-207 and Asp-210 in LRR 6, was used to inhibit the binding of decorin and fibromodulin to collagen. In a solid-phase assay, the peptide inhibited the decorin-collagen interaction at $K_I = 4 \text{ mM}$, whereas the collagen-fibromodulin interaction was unaffected. The control peptide SYISIANTNIT, with R207S and D210N mutations, did not inhibit the decorin-collagen binding (Fig. 6).

We also analyzed how the mutations R207S and D210N affected the collagen fibrillogenesis in vitro. The incubation of acid solubilized collagen with decorin at neutral pH and 37 °C...
leads to a slower collagen fibril formation and a lower final turbidity (1). 2 g/ml decorin completely inhibited the formation of fibrils and 1 g/ml inhibited the formation of fibrils by 50% (Fig. 7). The incubation of collagen in the presence of mamD210N at 2 and 1 g/ml affected the fibrillogenesis only marginally. mamR207S at 2 g/ml resulted in 50% inhibition of fibrillogenesis. Also, the peptide SYIRIADTNIT at 150 g/ml lowered the turbidity significantly, whereas the control peptide at the same concentration had no effect.

DISCUSSION

We show that decorin LRR 5–6 domain, encompassing Arg-207 and Asp-210, is of importance for collagen binding. Mutations of these 2 amino acid residues impaired binding to reconstituted collagen fibrils in solution and monomeric collagen adsorbed on plastic surface. Glu-181 (Glu-180 in human decorin corresponds to Glu-181 in the bovine sequence) was previously reported to be essential for the collagen interaction (23) and, interestingly, the E181K and E181Q mutations expressed as GST fusion proteins in bacteria did not impair binding to monomeric collagen-coated surfaces. However, mammalian full-length E181K decorin lost the affinity for collagen. This discrepancy may be due to structural changes induced by the E181K and E181Q mutations rather than direct involvement of Glu-181 in collagen binding. The crystal structure of decorin indicates that the side chain of Glu-181 potentially forms hydrogen bonds with the side chains of Arg-207 and His-159 on the neighboring LRR domains. Glu-181 also forms a hydrogen bond with the peptide bond of the proximal β-sheet (10). Consequently, mutation of this glutamate residue may lead to misfolding of the protein core, and collagen-binding sites could be sterically hindered from interaction in full-length decorin. We used the program CUPSAT (30) to predict changes...
The peptide sequence SYRIADTNIT is located in LRR 6 of decorin and includes the residues Arg-207 and Asp-210. This peptide inhibits the decorin-collagen type I interaction at $K_i = 4 \mu M$. Although this makes the peptide a potent inhibitor of decorin-collagen interaction, the inhibition is not complete. This implies the existence of other, seemingly weaker, collagen-binding sites in decorin and agrees with previous reports (21, 22, 24). Also, binding of mutated decorins to collagen indicates the presence of multiple binding sites in decorin (Figs. 4 and 5). The mutated decorins have linear binding curves, as opposed to sigmoidal binding curve of wild type decorin, which indicates weak or nonspecific binding. Since it is conceivable that the glycosaminoglycan chain could also contribute to collagen binding, we assessed the binding of chondroitinase ABC-treated decorin in the solid-phase assay and compared it with decorin proteoglycan. Both decorins had similar collagen binding, suggesting minor contribution by the glycosaminoglycan chain in collagen binding (data not shown).

We also investigated how mutated decorins and synthetic peptides affect collagen fibrillogenesis. It has been demonstrated that decorin inhibits the rate of collagen fibril formation in vitro and reduces the turbidity of the formed fibrils (1). It is believed that decorin binds collagen and interferes with the lateral growth of fibrils. The reduction in turbidity is primarily due to the formation of thinner collagen fibrils in the presence of decorin. Our results show that decorin dramatically inhibits fibrillogenesis at a collagen:decorin molar ratio of 1:8 (collagen molecular mass 300 kDa and decorin molecular mass 100 kDa) (Fig. 7). mamR207S also significantly inhibits fibrillogenesis at a molar ratio of 1:8, although not as efficiently as decorin. This may be explained by the collagen binding mediated by Asp-210 in mamR207S. Also, mamD210N retains some inhibition of fibril formation, which indicates additional collagen-binding sites, not involving Asp-210 and Arg-207. Interestingly, the synthetic peptide SYRIADTNIT also showed inhibition of collagen fibril formation when present at high concentrations. A collagen:peptide molar ratio of 1:360 (collagen molecular mass 300 kDa and peptide molecular mass 1.3 kDa) resulted in 25% inhibition of fibril formation. The control peptide SYISIANTNIT did not interfere with the fibril formation. Apparently, the synthetic peptide SYRIADTNIT has the capacity to bind collagen and interfere with the lateral growth of fibrils.

The collagen-binding site of decorin is homologous to sequences in asporin and biglycan, which are the closest relatives of decorin and belong to the same SLRP subfamily. The decorin sequence SYRIADTNIT is partially conserved in the asporin sequence HIRIAEAKLT and in the biglycan sequence NYLRISEAKLT. The sequences are located in LRR 6, and their collagen binding properties are unknown.

REFERENCES
5. Fisher, L. W., Termine, J. D., and Young, M. F. (1989) J. Biol. Chem. 264,
Synthetic peptide SYIRIADTNIT at 10 µg/ml (●) and 150 µg/ml (▲). Control peptide SYISIANTNIT at 150 µg/ml (X). Control with collagen alone (■). Turbidity at 400 nm was measured continuously at 4 min intervals and plotted versus elapsed time.

SUPPLEMENTAL FIGURES

Supplemental Figure 1. Primers used for site-directed mutagenesis of bacDCN56 and bovine full-length decorin.

Supplemental Figure 2. Sequence alignment of decorin LRR 5-6 in 15 species.
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<tr>
<th>Mutation</th>
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| E162Q    | 5′-CCGCCTGGGATCCAAACCAGATCACCACCCAAAGTGCG-3′
          | 5′-CGCACTTTTGTTGATCTTGTTGGGATCCACGCGG-3′           |
| E181K    | 5′-CCAGATGATCGTCTGAAAACCTTGGCACCAACCCCG-3′
          | 5′-CGGGTTGGTGCAAGTTGTTACGACGATCATCTGG-3′           |
| E181Q    | 5′-CCAGATGATCGTCTGAACCTTGGCACCAACCCCG-3′
          | 5′-CGGGTTGGTGCAAGTTGTTACGACGATCATCTGG-3′           |
| T184V    | 5′-GTCGTAGAAACCTTGCGGCAACACCACCCCTGAGAG-3′
          | 5′-CTCTTCAGCGGGTTGGCGCAAGTTCTACGAC-3′              |
| E193Q    | 5′-GAAGAGCTCAGGGCATCAAAATGGAGCCTTTTCAGG-3′
          | 5′-CCTGAAGGCTCATTGTTCATTGCTGTAGCTCTTC-3′           |
| Y205F    | 5′-GAATGAAGAAGCTCTCCTCCTCATCAGGCATTGCTGAAGAG-3′
<pre><code>      | 5′-GTCAGCAATGCGGATGAGGGAGGAGCCTTCTCATTTC-3′        |
</code></pre>
<p>| R207S    | 5′-GAAGCTCTCCTCACATCAGCATGCTGACACAAAT-3′            |
| 5′-ATTTGTGTCAGCAATGCTGATGTAGAGAGGCATTTC-3′         |
| D210N    | 5′-CCTACATCCGCATTGCTAACAAATAATACCCGGG-3′            |
| 5′-CCCCGGGTATATTGTGGTACGCAATGCGGATGTAGG-3′         |</p>
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