Fibromodulin Binds Collagen Type I via Glu-353 and Lys-355 in Leucine-rich Repeat 11

Sebastian Kalamajski and Åke Oldberg

From the Department of Experimental Medical Science, University of Lund, SE-221 84 Lund, Sweden

Fibromodulin belongs to the small leucine-rich repeat proteoglycan family, interacts with collagen type I, and controls collagen fibrillogenesis and assembly. Here, we show that a major fibromodulin-binding site for collagen type I is located in leucine-rich repeat 11 in the C terminus of the leucine-rich repeat domain. We identified Glu-353 and Lys-355 in repeat 11 as essential for binding, and the synthetic peptide RLDGNEIKR, including Glu-353 and Lys-355, inhibits the binding of fibromodulin to collagen in vitro. Fibromodulin and lumican compete for the same binding region on collagen, and fibromodulin can inhibit the binding of lumican to collagen type I. However, the peptide RLDGNEIKR does not inhibit the binding of lumican to collagen, suggesting separate but closely situated fibromodulin- and lumican-binding sites in collagen. The collagen-binding Glu-353 and Lys-355 residues in fibromodulin are exposed on the exterior of the β-sheet-loop structure of the leucine-rich repeat, which resembles the location of interacting residues in other leucine-rich repeat proteins, e.g. decorin.

Fibromodulin is an extracellular matrix proteoglycan expressed in dense regular connective tissues, including ligaments, cartilage, and tendons (1). Fibromodulin belongs to the small leucine-rich repeat proteoglycan family (SLRPs) (2, 3), which also includes e.g. biglycan (4), decorin (5), and lumican (6). These SLRPs are involved in the regulation of collagen fibril formation and matrix assembly, as demonstrated in SLRP-deficient mice (7). Decorin-deficient mice have fragile skin (8), fibromodulin deficiency leads to weak tendons and ligaments, causing osteoarthritis (9, 10), and lumican-deficient mice have opaque corneas (11). All these genotypes mediate an abnormal development of collagen matrices.

Fibromodulin and lumican are differentially expressed in developing tendons and possibly act in a coherent manner to regulate collagen matrix assembly (12), as they bind to the same region of collagen, different from the decorin-binding site (13). Fibromodulin binds preferentially in the gap region of assembled collagen fibrils (14) and affects the growth of collagen type I fibrils in vitro, as it binds to collagen monomers and delays their accretion to the growing fibrils (15). Collagen fibrils in tendon of fibromodulin-deficient mice are irregularly shaped with a higher proportion of thin fibrils, in contrast to fibrils formed in wild type mice (9). In addition, fibromodulin also binds collagen type XII (16) and transforming growth factor-β (17). It has also been proposed that fibromodulin and other collagen-binding SLRPs have a function in protecting collagen from collagenase degradation in connective tissues (18). Genomic analysis suggests a role for fibromodulin in metastasis (19), and the proteoglycan is also up-regulated in chronic lymphoid leukemia (20). Fibromodulin is one of just 14 genes progressively changed in expression with age and in progeria (21, 22). Fibromodulin is also implicated in pathways of inflammatory response (23) of cartilage (24) and is regulated by transforming growth factor-β in granulation tissue (25) and during development (26). Fibromodulin has also been reported to be induced by ultraviolet radiation (27). The fibromodulin core protein is composed of 12 tandem leucine-rich repeats (LRRs), each containing an average of 24 amino acid residues. Fibromodulin has nine potential tyrosine sulfations close to the N terminus (28, 29) and five potential consensus sites for N-glycosylation in the LRR domain (30). No function has been ascribed to these carbohydrate chains, and it is known that their absence does not affect the interaction with collagen (31).

The precise fibromodulin-binding site for collagen has not been determined, although it was hypothesized to be present in the C-terminal end of the protein core (31). It is important to characterize the fibromodulin-collagen interaction to distinguish it from other SLRP-collagen interactions. Understanding the influence of the individual SLRPs on the structure and function of collagen matrices could clarify their mechanism of concerted action. We recently demonstrated that a SYIRIADTNIT sequence in decorin binds collagen type I. In this study, we have used protein fragments, site-directed mutagenesis, and synthetic peptides to locate the collagen-binding site in fibromodulin.

**EXPERIMENTAL PROCEDURES**

Homology Modeling of Fibromodulin—The tertiary structure of fibromodulin was modeled with Swiss-PdbViewer software (32) using the crystal structure of decorin (Protein Data Bank 1xku) as template.
Expression of GST-tagged Fibromodulin Fragments in Bacteria—Bovine fibromodulin cDNA (GenBank™ Accession Number X16485) was used as a template in PCR to amplify cDNAs encoding different regions of fibromodulin. The fragments covered the following primary sequences: FmN-4 (amino acids 19–192), FmN-2 (amino acids 19–144), Fm3–4 (amino acids 138–192), Fm5–7 (amino acids 185–259), Fm8–12 (amino acids 259–375), and Fm11–12 (amino acids 324–375). The primers used for PCR are listed in supplemental Fig. 1. The amplified cDNAs were digested with the restriction enzymes BamHI and SmaI and ligated into the vector pGEX-4T-3 (Amersham Biosciences). The constructs were sequenced to confirm the identity and transfected into BL21 Escherichia coli, and proteins were expressed according to the manufacturer’s instructions. Proteins were purified under native conditions by glutathione affinity chromatography, as described previously (33), and dialyzed against PBS with 0.2% (v/v) Tween 20 prior to use. The protein concentration was determined with Coomassie Blue protein assay reagent (Pierce).

Expression of His-tagged Fibromodulin in Bacteria—Wild type fibromodulin cDNA was used as a template in PCR to amplify fibromodulin cDNA. The primers used for the PCR are listed in supplemental Fig. 1. The amplified cDNA was digested with XhoI and BamHI and ligated into the vector pGEX-4T-3 (Amersham Biosciences). The constructs were confirmed by sequencing and used to transfect human embryonic kidney 293 cells by electroporation, as described previously (35). Cells were grown in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum (Invitrogen). After 24 h, hygromycin (Invivogen) was added to a concentration of 1 mg/ml. After 3 days with medium changes every 3 days. The medium was dialyzed against 300 mM NaCl and 20 mM NaH2PO4 buffer, pH 8.0, and proteoglycans were purified using nickel-nitrilotriacetic acidagarose (Qiagen). Proteoglycans were eluted with 250 mM imidazole in 300 mM NaCl and 20 mM NaH2PO4 buffer (pH 8.0).

Peptide Synthesis—Peptides RLDGNEIKR and RLDGNQIMR were purchased from Schafer-N (Copenhagen, Denmark) and dissolved in PBS with 0.2% (v/v) Tween 20.

Isothermal Titration Calorimetry (ITC)—Calorimetry was performed using a VP-ITC (MicroCal). Samples (fibromodulin fragments and acid-solubilized collagen) were dialyzed against 20 mM phosphate buffer, pH 7.4. Titrations were performed by injections of 10-μl aliquots of 20 μM fibromodulin fragments into ITC sample cell (volume 1.345 ml) containing 2 μM collagen in 20 mM phosphate buffer, pH 7.4, at 25 °C. ITC data were corrected for the heat of dilution of the titrant by subtracting enthalpies of sample injection into protein-free buffer. Data were fitted with Origin software using the non-linear least squares minimization method using one site-binding model (Levenberg-Marquardt algorithm) to calculate the dissociation constant (Kd).

Circular Dichroism (CD)—CD data were collected with a J-810 spectropolarimeter (JASCO). Samples were at concentration of 1 μM in 20 mM phosphate, pH 7.4, buffer, in a 1-mm path length cuvette, at 25 °C. Spectra were recorded from 200 to 250 nm, with a scanning rate of 10 nm/min and an accumulation of three scans.

Solid-phase Collagen Binding Assay—Microtiter 96-well Maxisorp plates (Nunc) were coated for 16 h using 10 μg/ml acetic acid-extracted mouse tail collagen in acetic acid. The wells were washed with PBS and blocked for 1 h with 1 mg/ml bovine serum albumin (BSA) in PBS with 0.2% (v/v) Tween 20. After a single wash using PBS with 0.5% (v/v) Tween 20 (wash buffer), the GST-tagged fibromodulin fragments were added, and after 3 h, the wells were washed five times with the wash buffer. The amounts proteins bound to collagen were determined using 1-chloro-2,4-dinitrobenzene and reduced glutathione, as described previously (36). A similar procedure was followed for assays with His-tagged full-length fibromodulin and mutated fibromodulins, but the detection was done with rabbit anti-His tag antibody ab9108 (Abcam) diluted 1:250 in the wash buffer and with swine anti-rabbit alkaline phosphatase-conjugated antibody (DAKO) diluted 1:500 in the wash buffer as well as with detection with phosphatase substrate p-nitrophenyl phosphate (Sigma). For peptide inhibition assays, the peptide in different concentrations was added to fibromodulin or lumican prior to incubation.

Collagen Fibrillogenesis Assay—The assays were performed as described previously (37). Pepsin-extracted acid-solubilized collagen (Vitrogen) was neutralized and diluted (167 μl) in 150 mM NaCl, 20 mM HEPEs, pH 7.4, with fibromodulin or mutated fibromodulins at 4 °C. Solutions were degassed and then incubated at 37 °C for 10 h in a spectrophotometer, where the absorbance was continuously recorded at 400 nm.
RESULTS

We used bacteria to express the GST-tagged fibromodulin fragments FmN-4, FmN-2, Fm3–4, Fm5–7, Fm8–12, and Fm11–12 to study the interaction between fibromodulin and collagen type I (Fig. 1A). The collagen binding of these fragments was determined by isothermal titration calorimetry. Fm8–12 and Fm11–12 had the same $K_D = 1 \mu M$, and Fm5–7 had a weak binding enthalpy, whereas the other fragments had negligible affinity (Fig. 1, B–G). Fm8–12 and Fm11–12 also interacted with collagen in a solid-phase assay ($K_D = 3 \text{ nm}$), Fm5–7 had low affinity ($K_D = 10 \text{ nm}$, by extrapolation), and the other fragments had no affinity (Fig. 2A). Furthermore, Fm5–7 (E), Fm8–12 (F), or Fm11–12 (G) was injected into 2 $\mu M$ solution of acetic acid-extracted collagen type I. The top graph shows raw data after subtraction of the blank baseline (molar second versus time in minutes). The bottom graph shows normalized integration data (kcal/mole versus molar ratio). The corresponding peaks are aligned vertically.
Fm8–12 and Fm11–12 could inhibit the binding of mammalian-expressed full-length fibromodulin at \( K_d \) to collagen, in contrast to the other non-binding fragments. Fm5–7 had a low inhibitory effect (Fig. 2B).

We performed site-directed mutagenesis of full-length fibromodulin to identify the amino acid residues involved in the fibromodulin interaction with collagen. Residues exposed on the exterior of the \( \beta \)-sheet-loop region of LRR 11 and also conserved in six species were mutated (Fig. 3A). The mutations were R348S, D350N, E353Q, K355M, and R356S. Mutated fibromodulins were expressed in E. coli and purified from the periplasmic compartment to obtain proteins with intrachain disulfide bonds. Collagen binding was determined in a solid-phase binding assays. Wild type fibromodulin had an affinity similar to FmodR348S and FmodR356S (\( K_d = 2.5 \text{ nM} \)), whereas FmodD350N, FmodE353Q, and FmodK355M had no collagen affinity (Fig. 3B). The mutated fibromodulins were subjected to far UV circular dichroism spectroscopy to exclude possible structural changes that could affect the collagen binding. FmodD350N had a marginally blue-shifted CD spectrum (trough at 219 nm), as compared with the spectra of wild type fibromodulin and the other mutants (troughs at around 222 nm) (Fig. 3C). CD spectra of FmodR348S and FmodR356S were similar to wild type fibromodulin (data not shown). To clarify the influence of D350N on collagen binding, we incorporated the mutations in the collagen-binding fibromodulin fragment Fm11–12 and analyzed the collagen affinity. The D350N mutation had no effect on collagen binding of Fm11–12, whereas the other mutations had similar effects as those in full-length fibromodulin (data not shown). This suggests that the mutation D350N causes structural changes in full-length fibromodulin affecting the collagen binding.

We analyzed how the mutated fibromodulin variants influenced the collagen fibrillogenesis in vitro. Incubation of 100 \( \mu \text{g/ml} \) acid-solubilized collagen with wild type fibromodulin at neutral pH and 37 °C led to a hampered collagen fibril formation with low final turbidity, and 2 \( \mu \text{g/ml} \) fibromodulin completely inhibited the collagen fibril formation (Fig. 4A). FmodR348S and FmodR356S restrained the fibrillogenesis as efficiently as wild type fibromodulin (Fig. 4B and F), whereas FmodD350N, FmodE353Q, and FmodK355M had a reduced inhibition of fibril formation (Fig. 4C–E).

A synthetic peptide RLDGNEIKR, which encompasses Glu-353 and Lys-355 in LRR 11, was used in a solid-phase assay to...
inhibit the binding of mammalian-expressed fibromodulin or lumican to collagen. The peptide inhibited the fibromodulin-collagen interaction at $K_i = 1.5 \mu M$, whereas the lumican-collagen interaction was unaffected. In contrast, lumican binding to collagen could be inhibited by fibromodulin (data not shown) as reported previously (13). The control peptide RLDGNQIMR, with E353Q and K355M mutations, did not inhibit the fibromodulin-collagen binding (Fig. 5).

**DISCUSSION**

We demonstrate that fibromodulin binds collagen via LRR 11 and the mutations E353Q or K355M impair the collagen binding. The synthetic peptide RLDGNEIKR, encompassing Glu-353 and Lys-355 in LRR 11, inhibits the fibromodulin-collagen interaction in vitro. Also, mutation of Asp-350 impaired collagen binding of full-length fibromodulin, but the CD spectrum of FmodD350N was blue-shifted as compared with the spectrum of wild type fibromodulin and the other mutated fibromodulins. In addition, fibromodulin fragment Fm11–12 with the mutation D350N retained the collagen binding. Presumably, this mutation in full-length fibromodulin produces structural changes that affect the exposure of the collagen-binding site, whereas the smaller fragment retains the collagen affinity due to less influence by the surrounding structures. We conclude that fibromodulin interacts with collagen via neighboring glutamate and lysine residues located in the $\beta$-sheet-loop region of LRR 11. These residues are most likely exposed on the outer surface of the protein. Our results are supported by the study of Font et al. (31), showing that fibromodulin lacking the C-terminal region is not able to coprecipitate with collagen fibrils.

Interestingly, interacting amino acids in other LRR proteins are also located in the $\beta$-sheet-loop region of LRR domains. We have recently identified Asp-210 of decorin as a critical collagen-binding residue (37). In addition, e.g. Asp-175 and Glu-128 of glycoprotein Ib are crucial for complex formation with von Willebrand factor (38), and Asp-435 of ribonuclease inhibitor is important for binding RNase A and angiogenin (39). In these cases, the binding residues are exposed on the outer surface of the $\beta$-sheet-loop of a LRR domain. Similar locations of binding sites can be found in other LRR proteins (40), implying conserved binding patterns.

Fibromodulin inhibits the rate of collagen fibril formation in vitro and lowers the final fibril turbidity (15). Presumably, fibromodulin impairs the fibril growth by binding to collagen, in effect creating thinner collagen fibrils. This inhibition of fibril formation depends on proper intrachain disulfide bonds in fibromodulin (31). Fibromodulin expressed in bacteria and purified from the periplasm inhibits collagen fibrillogenesis, indicating correct fibromodulin intrachain disulfide bond formation. Also, reduction of bacterially expressed fibromodulin abolishes the inhibition of collagen fibril formation and confirms the results by Font et al. (31) (results not shown). The inhibitory effect is prominent at a collagen:fibromodulin molar ratio of 1:7 (collagen molecular mass 300 kDa and fibromodulin molecular mass 42 kDa) (Fig. 4). A similar inhibition was observed using FmodR348S and FmodR356S. FmodE353Q and FmodK355M lost their major influence on collagen fibrillogenesis, although some inhibition remained. This may be due to a weaker collagen-binding site, possibly located in LRRs 5–7. Fm–7 binds weakly to collagen (Fig. 2A) and partially inhibits the fibromodulin-collagen interaction (Fig. 2B).

The peptide RLDGNEIKR inhibits the fibromodulin-collagen type I interaction at $K_i = 1.5 \mu M$. However, the inhibition of fibromodulin binding is partial and implies additional weak cooperating collagen-binding sites in fibromodulin (Fig. 5). This additional binding site may be located in LRRs 5–7.

Fibromodulin and lumican compete for binding to collagen type I, and the two proteins appear to share a common collagen-binding site (13). Interestingly, the peptide RLDGNEIKR does not inhibit lumican binding. Full-length fibromodulin inhibits the binding of lumican to collagen, indicating that the two proteins have overlapping collagen-binding sites. Presumably, the two proteins cannot simultaneously bind collagen for sterical reasons.

The expression of lumican and fibromodulin varies during the development of tendon. Lumican appears to control the early stage assembly of thin fibrils, whereas fibromodulin has a role in the later stages of fibril growth, contributing to the mechanical strength. Most likely, the concerted action of several SLRPs affects the interaction between collagen monomers and fibrils and contributes to the shaping of the collagen matrix structure and function. Binding of SLRPs to collagen mono-
mers and the gap zone of the assembling collagen fibrils may regulate the interchain collagen cross-linking by juxtaposing collagen monomers. This may direct the initial Schiff base formation between lysine and/or hydroxylysine residues and ultimately control the mature interchain cross-linking. Indeed, the collagen cross-linking and assembly may be affected in fibromodulin, lumican, and decorin knock-out mice, as the phenotypes of these mice reveal structurally and functionally altered collagen matrices.

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