Critical Role of Glutamate in a Central Leucine-rich Repeat of Decorin for Interaction with Type I Collagen*

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The chondroitin/dermatan sulfate proteoglycan decorin is known to interact via its core protein with fibrillar collagens, thereby influencing the kinetics of fibril formation and the final diameter of the fibrils. To define the binding site(s) for type I collagen along the core protein, which is mainly composed of leucine-rich repeat structures, decorin cDNAs were constructed and expressed in human kidney 293 cells. The constructs encoded (i) C-terminally truncated molecules, (ii) core proteins with deletions of selected leucine-rich repeats, or (iii) various point mutations. The deletion of the sixth leucine-rich repeat structure, Met176–Lys201 and the mutation E180K drastically interfered with the binding to recombinant type I collagen fibrils. In contrast, the deletion of the seventh repeat Leu254–Ser292 led at the most to a marginally impaired binding, although the secretion of collagen fibrils, lateral assembly of individual triple helical collagen molecules is delayed (19, 20), and the diameter of the fibrils is decreased (21). That these interactions are also important in vivo can unambiguously be deduced from the phenotype of mice lacking the decorin gene (22). These animals are characterized by fragile skin, and their collagen fibrils have an uneven diameter due to uncontrolled lateral fusion.

There is direct and indirect evidence that collagen-bound decorin is still able to interact with transforming growth factor-β (23), and decorin may even mediate an attachment of type VI collagen to banded collagen fibrils (24). In light of the multitude of potential binding partners of decorin, it would therefore be useful to identify the binding sites for these molecules along the core protein. This would allow an understanding of the assembly of decorin-containing extracellular matrices and of the consequences of a partial proteolytic breakdown of decorin as it happens, for example, in rheumatoid arthritis (25). Decorin belongs to a widely distributed family of proteins that are characterized by about 12 consecutively arranged leucine-rich repeat structures (26, 27), which together form a short β-strand followed by an α-helix. The tertiary structure of one of these proteins has been elucidated by x-ray crystallography at 2.5 Å resolution. The essential features of the molecule are a horseshoe-like structure where the β-sheets form the inner concave surface and the α-helices make up the outer convex face (28). X-ray studies of the complex of ribonuclease inhibitor and its ligand, ribonuclease, indicated that the ligand was in contact with opposing sites of the horseshoe (29) and gave evidence for the conformational flexibility of the leucine-rich repeat structure.

Recently, the re-evaluation of rotary shadowing electron micrographs of a mixture of decorin and of a further proteoglycan made up of leucine-rich repeats, fibromodulin, suggested that decorin, too, is horseshoe-shaped (30). From the results of molecular modeling, it was concluded that the inner concave surface of decorin is of suitable size to accommodate a single triple helix. The possibility of a secondary site located near the C-terminus was also considered (31). Studies performed before on chimeric decorin/biglycan proteins had indicated that a major binding site for type I collagen is located within the two repeats Leu152–Lys201 (32). Similar conclusions were drawn from own studies employing recombinant decorin peptides (33). The data also suggested the presence of a second, less active binding site in the C-terminal half of the core protein. Similarly, in a preliminary report it was concluded that decorin contains at least two functional domains that are involved in the interaction with the collagen-like molecule C1q (34). On theoretical
grounds Scott (30) proposed the sequences Lys\textsuperscript{130}–Arg\textsuperscript{133} and Arg\textsuperscript{272}–His\textsuperscript{275} as binding sites for collagen.

In the present study we describe the type I collagen binding properties of several mutant decorin molecules expressed in and secreted by eukaryotic cells. The results indicate the importance of Met\textsuperscript{176}–Lys\textsuperscript{201}, i.e. of the sixth leucine-rich repeat, for collagen binding and indicate a critical role of glutamate 180.

**EXPERIMENTAL PROCEDURES**

**Expression of Recombinant Decorin in Human 293 Kidney Cells—** Different authors use nonidentical rules for the numbering of individual amino acids and of leucine-rich repeats of decorin core protein. In this communication we number the amino acids from the start methionine and not from the N terminus of the mature decorin core protein, i.e., of the protein obtained after removal of pre- and propeptides. The first leucine-rich repeat is the one comprising Leu\textsuperscript{62}–Asp\textsuperscript{82}, although it can be disputed whether this sequence should be considered as a repeat structure or not.

Clone D6, which contains the complete coding region of human decorin core protein, has been described previously (35). An EcoRI/HpaI fragment of this clone was ligated into the pUC18 vector (U. S. Biochemicals). A fragment which subsequently was shown to be cloned in the pCDNA3 vector (Invitrogen). The resulting plasmid was used for the expression of recombinant decorin and for the construction of mutated decorin cDNAs. The cDNA for a C-terminally truncated decorin was cloned into the BamHI site of pGem-4Z (Promega), thereby creating a leucine and a stop codon 3' of the Val\textsuperscript{260} codon. Then an EcoRI/AatI fragment of clone D6 was ligated with the AatI/XhoI fragment from pGem-4Z and cloned into pCDNA3 as above. Decorin exhibiting a deletion of the sixth leucine-rich repeat Met\textsuperscript{176}–Lys\textsuperscript{201} was constructed by a two-step polymerase chain reaction procedure. In the first step 5' and 3'-portions of the desired sequence were generated by using the primer pairs 5'-CCAGGAGACTCTGGAATTC-3' (forward) and 5'-GGGCGATGTCAGGACTCTGGAATTC-3' (reverse) and of the sixth leucine-rich repeat Met\textsuperscript{176}–Lys\textsuperscript{201} was constructed by a two-step polymerase chain reaction procedure. In the first step 5' and 3'-portions of the desired sequence were generated by using the primer pairs 5'-CCAGGAGACTCTGGAATTC-3' (forward) and 5'-GGGCGATGTCAGGACTCTGGAATTC-3' (reverse and complement) for K187Q and E180K. Sequencing of the constructs verified the recommendations of the manufacturer. The cells were selected for mycine resistance by adding 750 \( \mu \text{g/ml} \) G418 (Life Technologies, Inc.). Transient transfection of COS cells was performed either by the DEAE-dextran (Sigma) method (37) or with Lipofectin.

**Metabolic Labeling and Proteoglycan Isolation—** Unlabeled reference decorin was purified to about 95% purity from the conditioned medium of cultured human skin fibroblasts as described (10). For the preparation of \[^{35}S\]methionine-labeled proteoglycans, confluent fibroblasts or nearly confluent 293 cells were incubated for up to 3 days in the presence of 20 \( \mu \text{Ci/ml} \) \[^{35}S\]methionine (carrier-free, Amersham-Buchler, Braunschweig, Germany) using 10 \( \mu \text{l/mg} \) \( \text{m}^2 \) culture flask of Eagle's minimum essential medium in which \( \text{MgSO}_4 \) had been replaced by \( \text{MgCl}_2 \), and which was supplemented with nonessential amino acids, penicillin, and 4% (v/v) fetal calf serum. A proteoglycan fraction was obtained from the culture medium by ammonium sulfate precipitation followed by chromatography on DEAE-Trisacryl M (Serva, Heidelberg, Germany) exactly as described (10). Prior to use in binding assays the proteoglycan fraction was dialyzed against phosphate-buffered saline (18 mM sodium phosphate, pH 7.4, 150 mM NaCl) (PBS) in dialysis tubing prewashed with 5% (v/v) bovine serum albumin in PBS.

When indicated, decorin was purified directly from appropriate DEAE fractions by immunoprecipitation using a monospecific polyclonal antiserum against human decorin and immobilizing the immune globulins on protein A-Sepharose (Sigma) as described previously (38). The immune complex was solubilized by a 2-h treatment at 4 °C with 7 M urea in 20 mM Tris/HCl, 0.15 M NaCl, and protease inhibitors. The solution was subjected to ion exchange chromatography on DEAE-Trisacryl, first in the presence and then in the absence of urea, for a stepwise removal of IgG and urea and for renaturation of the proteoglycan. Decorin-containing fractions were dialyzed against PBS as described above.

For preparation of \[^{35}S\]methionine-labeled proteoglycans, medium was changed to methionine-free Waymouth MAB 87/3 medium supplemented with 4% of dialyzed fetal calf serum. After 1 h of preincubation, labeling medium was added (7 ml/75-cm\(^2\) culture flask), which contained 100 \( \mu \text{Ci of}[^{35}S]\)methionine (specific radioactivity, 1.07 \( \text{mCi/\mu mol} \); Amersham-Buchler), and incubation continued for up to 6 h. In pulse-chase experiments with \[^{35}S\]sulfate or \[^{35}S\]methionine, the chase medium was obtained from the culture medium in dialysis tubing prewashed with 5% (v/v) bovine serum albumin in PBS.

In the present study we describe the type I collagen binding properties of several mutant decorin molecules expressed in and secreted by eukaryotic cells. The results indicate the importance of Met\textsuperscript{176}–Lys\textsuperscript{201}, i.e. of the sixth leucine-rich repeat, for collagen binding and indicate a critical role of glutamate 180.

**RESULTS**

**Expression of Recombinant Decorin Species—** Various decorin cDNAs were constructed, all of which contained the glycosaminoglycan chain attachment site but encoded for ei-
chondroitinase ABC lyase treatment was omitted. However, it remained into the culture medium, and this was observed when the cells secrete a small fraction of glycosaminoglycan-free core protein (Fig. 2). In contrast to normal skin fibroblasts, 293 cells did not secrete a single core protein band with a somewhat faster mobility than the wild-type core protein carrying three asparagine-bound oligosaccharides, leading to the doublet core protein bands seen. This is suggestive but not proof for the attachment of three N-glycosidically linked oligosaccharides.

In this context it is interesting to note that decorin K187Q and decorin K200Q yielded two protein bands of somewhat different mobility upon treatment with chondroitin ABC lyase, which could indicate that subtle changes in the sequence of the core protein may influence the processing of the oligosaccharides.

The electrophoretic mobility of [35S]sulfate-labeled proteoglycans is also shown in Fig. 2. Truncation of the core protein or elimination of individual leucine-rich repeats did not result in major differences in the electrophoretic mobility of the broad band of the intact proteoglycan. However, as in the case of [35S]methionine-labeled material, considerable differences were noted between the various decorin constructs in the quantities of [35S]sulfate-labeled proteoglycan secreted into the culture medium. In 293 cells transfected with the full-length decorin cDNA, about 50% of all secreted [35S]sulfate-labeled macromolecules could be precipitated with antibodies against decorin. Similar data were obtained for decorin species carrying point mutations. In five independent experiments, for each preparation of decorin with a deletion of Met176–Lys203 and Leu202–Ser222, the percentage or the isotope recovered from the proteoglycan fraction as decorin varied between 25 and 42% and 4 and 10%, respectively. Furthermore, in addition to the lowered proportion, there was also a reduction in the total quantity of incorporated radiosulfate. Truncation of the C-terminally located residues Asp311–Lys329 also was accompanied by a lowered proportion of this recombinant proteoglycan in the culture medium (10–15%). This may indicate that some of the mutant proteoglycans could not be transported normally from the endoplasmic reticulum to the plasma membrane. Decreased secretion of decorin carrying deletions of either Met176–Lys203 or Leu202–Ser222, however, was not observed in pulse-chase experiments with [35S]sulfate (data not shown). Identical pulse-chase experiments using [35S]methionine could not be performed in 293 cells, because despite the preincubation with methionine-free medium, it took at least 3 h to equilibrate the methionyl tRNA pool with the radioactive amino acid. The data...
obtained after a 3-h pulse, however, indicated that decorin with deletions of leucine-rich repeats was continuously being chased into the medium pool during 19 h, whereas the full-length decorin pulse was completely secreted during the first 4 h of chase. These observations can be explained by the hypothesis that decorin with deletions is transported through the rough endoplasmic reticulum more slowly after protein synthesis, but after it is modified into a proteoglycan in the Golgi network all forms are secreted out of the cell at the same approximate rate. Secreted proteoglycans remained stably in solution and could be reproducibly used for binding studies.

Binding to Reconstituted Type I Collagen Fibrils—Binding of decorin to reconstituted type I collagen fibrils was measured by allowing a crude, native proteoglycan preparation to interact with the fibrils and then quantitating decorin in the unbound and bound fractions. In most cases, the results were verified using immunopurified decorin in the binding assay. The latter method has the disadvantage that the immune complex had to be disrupted by urea and that a renaturation step was required. In the first assay native decorin is used, but other proteoglycans as for example biglycan are also present in the preparation and may compete for binding (40).

In a first set of experiments the binding of wild-type decorin from fibroblast medium and that of recombinant full-length decorin from 293 cells transfected with the full-length decorin cDNA (rDCN) were obtained after a labeling period of 72 h with $[^{35}S]$sulfate by immunoprecipitation of the conditioned medium.

![](image1.png)

**FIG. 3.** Binding of decorin from normal human skin fibroblasts and from transfected 293 cells to reconstituted type I collagen fibrils. Wild-type decorin (wt DCN) from fibroblasts and decorin from 293 cells transfected with the full-length decorin cDNA (rDCN) were obtained after a labeling period of 72 h with $[^{35}S]$sulfate by immunoprecipitation of the conditioned medium.

For the most interesting constructs the dose dependence of collagen binding was investigated in greater detail (Fig. 5). It can clearly be seen that decorin with a deletion of Met$^{176}$–Lys$^{201}$ as well as decorin E180K have an impaired capability for collagen binding at all doses tested. Nonradioactive, wild-type decorin and full-length decorin, K187Q, were replaced during site-directed mutagenesis. A comparison of the binding properties of the different decorin constructs is given in Table I. The data corroborate the observation that in contrast to the deletion of Met$^{176}$–Lys$^{201}$, the deletion of Leu$^{202}$–Ser$^{222}$ only slightly affects collagen binding. Truncation of the C terminus (deletion Asp$^{261}$–Lys$^{359}$) also lead to an only moderately impaired collagen binding. Interestingly, the replacement of lysine residue 200 by glutamine resulted in an even better collagen binding, a finding that was confirmed in three independent series of experiments. On the other hand, replacement of glutamate 180 by lysine resulted in the production of a proteoglycan whose interaction with type I collagen was strongly reduced, although not to the same extent as upon deletion of the entire leucine-rich repeat structure.

For the most interesting constructs the dose dependence of collagen binding was investigated in greater detail (Fig. 5). It can clearly be seen that decorin with a deletion of Met$^{176}$–Lys$^{201}$ as well as decorin E180K have an impaired capability for collagen binding at all doses tested. Nonradioactive, wild-type decorin from fibroblasts reduced the binding of all of the radioactive species shown in Fig. 5, as well as all other decorin constructs. Double-reciprocal plots indicated that this inhibition was competitive in nature. However, in agreement with previous findings (33, 32), the relatively high quantity of bound decorin at the lowest doses of decorin exhibiting a deletion of
Met\textsuperscript{176}–Lys\textsuperscript{201} and of decorin E180K suggested the presence of a second binding site that is not affected in these mutants. From a Scatchard plot (Fig. 6) of these two constructs, it can be concluded that it is specifically the number of binding sites that is reduced in the mutant.

Because the peptide Lys\textsuperscript{130}–Arg\textsuperscript{133} was considered as a binding site for type I collagen (30), peptide Val\textsuperscript{129}–Leu\textsuperscript{134} was tested as competitor of decorin binding. No effect was observed at all concentrations tested (1.5–150 μM) (data not shown).

DISCUSSION

The results of this study provide a further example of the possibility of producing recombinant decorin being N-glycosylated and linked with a chondroitin/dermatan sulfate chain (13, 32, 41–45). For the first time, however, deletions of individual leucine-rich repeats, point mutations, and truncation of C-terminal sequences were introduced.

The main result of the present study was the observation that the sixth leucine-rich repeat Met\textsuperscript{176}–Lys\textsuperscript{201} and specifically glutamate 180 within this repeat are of special importance for the interaction with reconstituted type I collagen fibrils. The deletion of just any whole repeat itself is not a necessary condition to interfere with collagen binding because decorin with a deletion of the seventh repeat Leu\textsuperscript{202}–Ser\textsuperscript{222} exhibited, at most, marginally impaired binding properties. Taking into account the horseshoe model of the tertiary structure of decorin and the proposal that collagen triple helices are in contact with the inner concave surface of the proteoglycan (31), it seems likely that there is sufficient flexibility within the core protein to compensate for the loss of at least certain single repeat structures.

Decorin is not the only chondroitin/dermatan sulfate proteoglycan that binds to fibrillar collagens. The homologous proteoglycan, biglycan, has been shown to interact with type I collagen, too, although the dissociation constants obtained from Scatchard plots were higher for glycanated biglycan than for glycanated decorin (40). The sixth leucine-rich repeat of biglycan is homologous with the sixth repeat of decorin, and there is, as in decorin, a glutamate residue at the fifth position of this repeat (46). Thus, the different affinities of decorin and biglycan for type I collagen are unlikely to result from structural differences within this single repeat. It had been observed, however, that glycosaminoglycan-free biglycan exhibited a
much higher affinity for reconstituted collagen fibrils than the glycated species (40), and it is interesting to consider that the different affinities of decorin and biglycan for collagen are a reflection of the different number of glycosaminoglycan chains. 

Unfortunately, we have not been able to obtain sufficient quantities of pure recombinant proteins under nondenaturing conditions to investigate the tertiary structure by CD spectroscopy. Different CD spectra for decorin purified under denaturing conditions and native decorin had been reported recently (43). In the horseshoe model of decorin the carbohydrate group of glutamate 180 is oriented toward the water face at the inner concavity, and it seems likely that also in this case the overall structure of the core protein is stabilized by hydrophobic interactions between the adjacent β-sheets and by interactions between the α-helices at the outer convex surface (31).

The mechanism of collagen fibril assembly in the absence or the presence of decorin is by no means fully understood. It is assumed that decorin inhibits the lateral association of collagen monomers into oligomers, and during the early phases of fibril formation a 1:1 stoichiometry between the two macromolecules may be assumed (19). When collagen fibrils grow laterally this molar ratio changes, and there is less than one proteoglycan per D-period of the fibril (47). Reconstituted collagen fibrils as used in the present study bind maximally one decorin per 20 monomers (48). Thus, it appears that during fibrillogenesis in vivo there is a continuous association and dissociation of decorin, whereas in the in vitro assays the collagen molecules at the surface of the fibril can stably bind the proteoglycan. Whether or not the binding sites and the binding properties are fully identical during these different stages of interactions remains to be investigated.

The interpretation of the data of binding studies between type I collagen and decorin is further complicated by the proposal made on experimental and theoretical grounds (31, 33, 34, 48) that decorin possesses at least two type I collagen binding sites. Molecular modeling of decorin indicated that its concave face could accommodate a single triple helix. The reference sequence KGEAPGERGSE has been considered to be present at the contact site between both macromolecules (31) because of the proposal that this sequence is a part of the α2 chain (I) at the d-band of the D-period (49), i.e., at the site of decorin binding. Because decorin also interacts with other fibrillar collagens and the complement component C1q (15, 16, 20, 34, 50), we searched the Swissprot database for sequence homology. Some of the relevant data for N-terminal regions with sequence homology are given in Fig. 7. With respect to the reference sequence only the position of basic amino acid residues was fully conserved. This could indicate that the same site in decorin core protein interacts with the different collagens and C1q, respectively, and it also implicates the importance of acidic residues for collagen binding. From the results of the present study, glutamate 180 should be considered as such a critical residue. A second collagen binding site has been located in the C-terminal portion of decorin (31, 33). The structural prerequisites for this interaction are not yet known. Studies to address this problem would best be done in dynamic fibrillogenesis assays and may require the addition of additional decorin constructs.

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FIG. 7. Examples of sequences with homology to a putative reference sequence of an α2 (I) chain at the d-band in the gap zone (31).
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