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THE INTERACTION OF DECORIN CORE PROTEIN FRAGMENTS WITH TYPE I COLLAGEN

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To further define the molecular interaction between decorin and type I collagen we generated a 20 kD fragment containing the N-terminal half of the core protein by Endoproteinase Arg C digestion and a 40 kD fragment including all leucine-rich repeats in the central part of decorin core by cleavage with 2-nitro-5-thiocyanobenzoate. The fragments did not influence collagen fibril formation, even at high concentration, and radioactive fragments showed little binding to collagen fibrils. Our observations suggest that neither the N-terminal half nor the central leucine-rich repeats of the decorin core protein can, by itself, interact fully with fibrillar collagen.

Ultrastructural studies clearly show the small proteoglycan decorin bound to collagen fibrils (1,2) although the nature of this interaction is poorly understood. Purified decorin has been shown to bind to collagen type I with high affinity and specificity during *in vitro* fibrillogenesis (3) and in a solid phase assay (4) via an interaction between its core protein and the collagen. The 17-amino acid N-terminal peptide of the core protein and the glycosaminoglycan chain were not required for this binding (5). However, it is not clear what part of the proteoglycan core protein is involved in the interaction. The

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Abbreviations: BSA, bovine serum albumin; C'ase ABC, Chondroitin ABC Lyase; Endo Arg C, Endoproteinase Arg C; GAG, glycosaminoglycan; GuHCl, guanidine hydrochloride; NTCB, 2-nitro-5-thiocyanobenzoate; PBS, phosphate buffered saline; PG, proteoglycan.

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aim of this study was to prepare large peptides from the core protein by chemical and enzymatic methods and assess their ability to bind to collagen.

MATERIALS AND METHODS

Decorin from the tensional region of adult bovine deep flexor tendon, and large proteoglycan from the compressed region of this tendon, were purified by extraction in 4M GuHCl, ion-exchange chromatography, and Sepharose CL-4B chromatography, as described by Evanko and Vogel (6). Radiolabeled decorin was isolated from the medium of bovine tendon fibroblast cultures containing [^{3}H]leucine and [^{35}S]sulfate, as described (3).

Preparation of Endoproteinase Arg C fragment. One mg of decorin in 0.5 ml of 0.1 M NH_4HCO_3 buffer (pH 8.1) was digested with 2.5 U Endoproteinase Arg C (EC. 3.4.22.8, sequencing grade, Boehringer Mannheim), and with 0.125 U Chondroitinase ABC (EC. 4.2.2.4, Miles Scientific) for 20 h at 37 °C. Five µl aliquots were removed at the end of each digestion for SDS/polyacrylamide gel electrophoresis. The digestion was also performed sequentially with C'ase ABC for 1 h and with Endo Arg C for 20 h, in alternating order. For short-time storage the buffer was exchanged into distilled water in a Centricon 10 microconcentrator (Amicon) and frozen.

Preparation of 2-nitro-5-thiocyanobenzoate fragment. The method of Burridge and Bray was used after adaptation (7). Five mg decorin was dissolved in 1 ml 6 M guanidine HCl, 0.2 M Tris acetate, 10 mM dithiothreitol (Sigma), pH 8.0. The disulfide bonds were reduced at 37 °C for 3 h. The buffer was then exchanged into 6 M GuHCl, 0.2 M Tris acetate, 0.1 mM dithiothreitol, pH 8.0, using a Centricon 10. After concentration to 300 µl, 33 µl of freshly prepared 6 M GuHCl, 0.2 M Tris acetate and 0.1 M 2-nitro-5-thiocyanobenzoate (Calbiochem) were added to the reaction mixture and incubated at 37 °C for 48 h. To stop the reaction an equal volume of glacial acetic acid was added. The buffer was exchanged into 50% acetic acid, then into 7 M urea, 10 mM Tris HCl, 0.5% Triton-X 100, pH 7.0, using a Centricon microconcentrator. The peptide was separated from GAG by elution from a 3 ml DEAE-cellulose column (0.5 ml fractions, DE-52, Whatman) with urea buffer containing 0.1 M NaCl, followed by elution with 4 M GuHCl, 50 mM sodium acetate, 0.5% Triton-X 100, pH 6.0. The peptide-containing fractions were combined, the buffer exchanged to 1.75 M urea, 2.6 mM Tris HCl, pH 7 in a microconcentrator, and the sample stored at 4 °C. The same method was used to prepare radiolabeled peptide, starting with [^{3}H]leucine-labeled decorin.

Binding assays. Acid soluble collagen was prepared from newborn bovine tendon as described (8). Collagen fibrillogenesis was monitored spectrophotometrically as previously described (9). Binding of radiolabeled molecules to fibrillar collagen was performed according to Brown and Vogel (3). Briefly, soluble collagen and radioactive proteoglycan were combined in PBS (30 mM sodium phosphate, 140 mM NaCl, pH 7.2) in a small centrifuge tube. After overnight incubation at 37 °C the insoluble collagen was pelleted and radioactivity remaining in the supernatant (unbound) and that associated with the fibrils (bound) was determined by counting in a Packard Tri-Carb 1500 liquid

scintillation analyzer. During inhibition assays the PG or peptide was added to soluble collagen and radioactive decorin. Percent inhibition was determined as:
$100 - 100\ [bound_P\ (unbound_C + bound_C) / bound_C\ (unbound_P + bound_P)]$ where P = added PG or peptide and C = control.

For the solid phase binding assays microtiter wells of a MaxiSorp Immuno Plate (Nunc) were coated with either 100 µg of the respective molecule/well in 200 µl 20 mM $NaHCO_3$, 20 mM Na_2CO_3, 0.02% NaN_3, pH 9.2 or 200 µg/well BSA in 200 µl PBS, by overnight incubation at 4 °C. About 1 µg of decorin or peptide was bound to the well after rinsing two times with PBS. Nonspecific binding sites in the wells were blocked by incubation with 200 µl of 10 mg/ml BSA in PBS at room temperature for 1 h. The wells were then rinsed with PBS. The [^{35}S]decorin was diluted in PBS containing 1 mg/ml BSA, and about 6,000 dpm in 50 µl added to each well and incubated at 37 °C for 3 h. Unbound decorin was removed and combined with three succesive rinses (200 µl PBS/well) for radioactivity determination. The bound radiolabeled material was dissolved in 200 µl 2% SDS and counted. Recovery in the bound and unbound pools was > 90%.

RESULTS AND DISCUSSION

Endoproteinase Arg C digestion and the NTCB cleavage method were used because of their specificity. Endo Arg C cleaves at the COOH terminus of each arginine residue. NTCB cleaves at the NH_2 terminus of each cysteine. As there are only a few of these amino acids in the core protein of decorin, the generation of relatively large fragments was anticipated. There was a shift of the alcian blue stained diffuse band when the enzyme was applied to intact decorin (Fig. 1.). If this material was subsequently digested with C'ase ABC, a 20 kD fragment appeared on the gel. The same fragment was generated when the C'ase ABC was applied first followed by the Endo Arg C, or when the two enzymes were added together. This was interpreted to mean that the enzyme digested the C-terminal part of the molecule, generating a 20 kD N-terminal fragment with the GAG chain still attached (Fig. 2).

The peptide generated by NTCB cleavage was separated from GAGs by ion-exchange chromatography (Fig. 3). The major product eluted in 0.1 M NaCl and migrated as a diffuse doublet at ~40 kD. GAGs were retained on the column and eluted in 4M GuHCl. Because the molecular weight of the major product was around 40 kD, we conclude that it included all leucine-rich repeats in the central part of core protein (Fig. 2).

Neither the 20 kD N-terminal Endo Arg C fragment nor the the 40 kD central NTCB fragment influenced collagen fibril formation, even when present at 20 µg peptide/100 µg collagen (Fig. 4). Decorin inhibited the rate of fibrillogenesis when 5 µg or more was added to 100 µg/ml collagen. Based on previous studies, lack

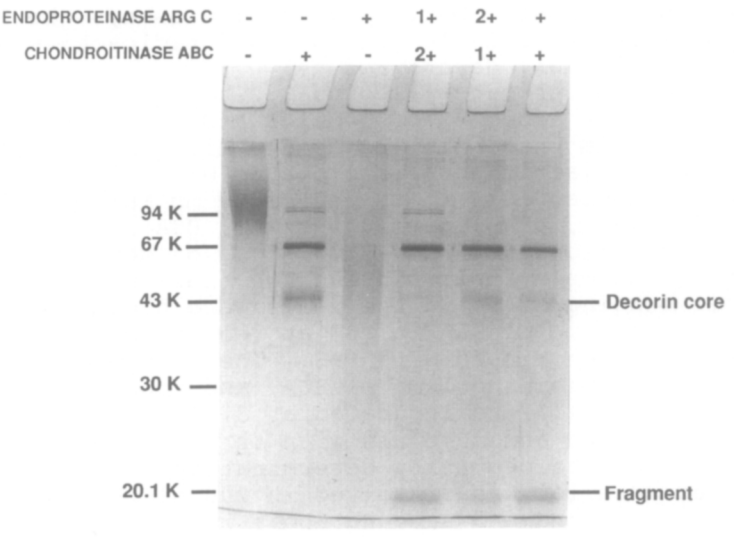

Figure 1. **Electrophoresis of decorin after Endoproteinase Arg C and Chondroitinase ABC digestion.** Decorin (4 μg, lane 1) was digested with 0.01 U C'ase ABC for 1 h (lane 2), or with 0.04 U Endo Arg C for 20 h (lane 3). The digestions were also performed consecutively (+1 = first enzyme; lanes 4,5). The 12% SDS/polyacrylamide gel was stained with Coomassie Blue. A 20 kD fragment was generated by this digestion. MW markers (Pharmacia) are phosphorylase B (94 kD), bovine albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD).

of inhibition indicates that the peptides did not bind to collagen. The peptides had no effect on the inhibition of fibril formation caused by decorin (data not shown), and neither enzymes alone nor peptide buffer alone affected collagen fibrillogenesis. Binding of radioactive NTCB peptide to collagen was only 7 ± 3% compared to binding of 47 ± 5% for a similar amount of the same preparation of intact decorin. The radioactive NTCB peptide migrated as a tight doublet at 40 kD by gel electrophoresis and

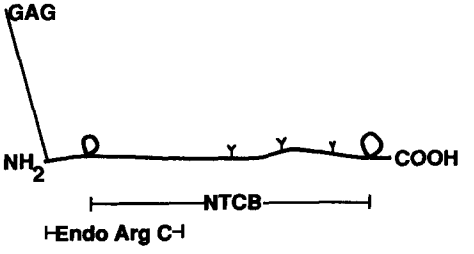

Figure 2. **Representation of decorin and the peptides generated by Endoproteinase Arg C digestion and NTCB cleavage.**

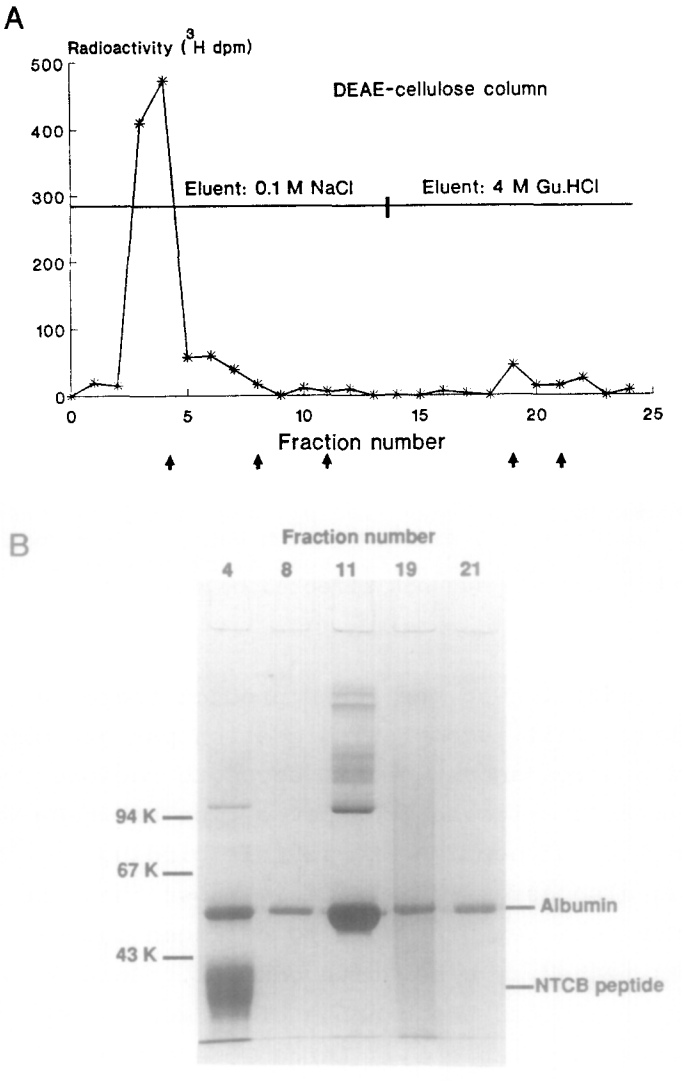

Figure 3. **Fractionation after NTCB cleavage.** (A) Five mg of decorin was mixed with a small amount of [^{3}H]leucine labeled decorin and cleaved by the NTCB method. The products of NTCB cleavage were dissolved in 7 M urea, 10 mM Tris HCl, 0.5% Triton-X 100, pH 7, loaded onto a 3 ml DEAE-cellulose column, and eluted with the same buffer and then with 4 M GuHCl, 50 mM sodium acetate, 0.5% Triton-X 100, pH 6. An aliquot of each 0.5 ml fraction was counted. (B) Twenty μl aliquots of some fractions were precipitated with 200 μl ethanol and analyzed without reduction by 7.5% SDS/polyacrylamide gel electrophoresis. The gel was stained with Coomassie Blue and Alcian Blue. MW markers as in Fig. 1.

fluorography. Although the radioactive Endo Arg C peptide showed somewhat higher binding (16 $\pm$ 2%), this figure is probably inaccurate because Endo Arg C digestion of [^{3}H]decorin was never

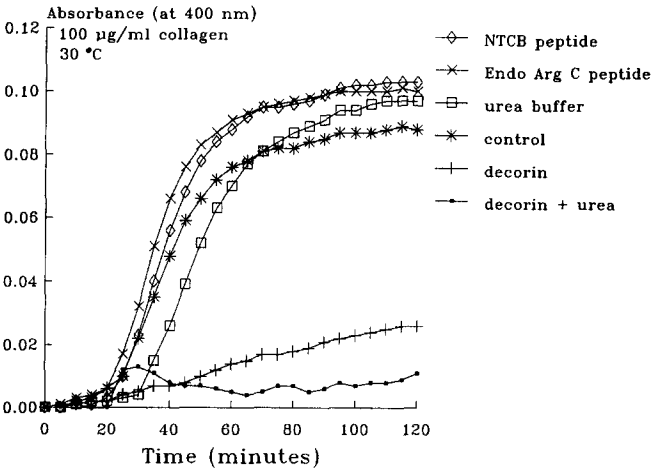

Figure 4. **Effect of peptides of decorin core protein on collagen fibril formation.** Collagen (100 µg) and decorin or peptides (20 µg) were combined in 1 ml PBS (0.14 M NaCl, 0.03 M sodium phosphate, pH 7.2, 30 °C). Collagen fibrillogenesis was inhibited by decorin but not affected by the peptides.

complete (in addition to the 20 kD product there was some intact core protein). This incomplete digestion pattern may have been caused by the large amount of BSA added to radioactive samples to prevent them from sticking to pipette tips and tube walls.

The ability of peptides to inhibit binding of [^{3}H]decorin to collagen was also assessed. As it is known that the large PG of bovine tendon does not bind well to collagen in PBS (3,9), this molecule was tested as a negative control. Both decorin and its NTCB peptide inhibited the binding of intact [^{3}H]decorin to fibrillar collagen (Table 1). Large PG and the Endo Arg C peptide showed less inhibition. Because of the concentration dependent effect of added decorin, a competition for binding between the non-radioactive and radioactive decorin was assumed. However, it is impossible to distinguish between two possible reasons for the inhibition in this type of assay. Peptides could compete with decorin for the binding site on collagen fibrils, or the peptides could bind to radioactive decorin and prevent them from binding to collagen.

A solid phase binding assay was designed to address the question of interaction between the peptides and the labeled decorin. Radioactive decorin was not bound to either decorin or large PG (Fig. 5). The Endo Arg C peptide had the greatest binding capacity. If there is a binding between the Endo Arg C peptide and decorin, perhaps mediated by secondary hydrophobic

Table I.

Inhibition of [^{3}H]Decorin Binding to Collagen

Inhibitor	Bound dpm	Unbound dpm	% of Inhibition
control	1817±107	2165±76	0 by definition
Decorin			
0.2 μg/ml	1832±44	2167±116	0±2
2.0 μg/ml	1314±299	2493±103	25±12
20.0 μg/ml	956±81	3115±56	49±3
NTCB peptide			
urea buffer	1739±90	2573±58	12±3
0.2 μg/ml	1691±23	2601±97	3±2*
2.0 μg/ml	1399±72	3209±66	22±3*
20.0 μg/ml	1158±79	3447±47	34±3*
Endo Arg C pept.			
0.2 μg/ml	1757±51	2038±98	0±1
2.0 μg/ml	1727±30	2341±176	8±4
20.0 μg/ml	1663±158	2418±74	12±5
Large PG			
0.2 μg/ml	1762±89	2182±121	3±5
2.0 μg/ml	1758±12	2130±66	2±2
20.0 μg/ml	1526±84	2357±91	14±4

*Values obtained after subtraction of the value of inhibition caused by an equivalent amount of urea buffer alone. Results represent the mean ± S.D. of three samples.

interactions, it may be an explanation for the slight inhibition of [^{3}H]decorin binding to collagen caused by this peptide. The NTCB peptide showed a weaker interaction with radioactive

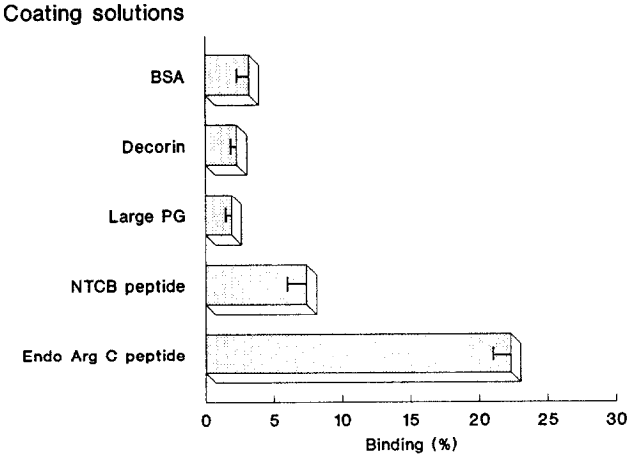

Figure 5. **Solid phase binding assay.** [^{35}S]decorin (~ 6,000 dpm in 50 μl PBS containing 1 mg/ml BSA) was added to microtiter wells previously coated with the indicated materials and incubated at 37 °C for 3 h. After washing, the amount of bound radioactivity was quantified. Results are expressed as the mean ± S.D. of quadruplicate determinations.

decorin. Thus the ability of the NTCB peptide to inhibit decorin binding to collagen is probably caused by an interaction between the peptide and the binding site on collagen. These observations mean that the N-terminal half of the core protein probably does not participate in binding decorin to collagen. It has been shown that after reduction decorin binding to collagen is diminished (3,10). The NTCB peptide's diminished binding is consistent with this. As the Endo Arg C fragment containing the N-terminal disulfide loop of decorin proved to be not important for the binding, the C-terminal loop may be important to maintain the appropriate structure for binding.

Another possible explanation for these observations is that decorin-collagen binding is the result of several weak interactions between the proteoglycan core protein and collagen fibrils, rather than an interaction between a short fragment of the proteoglycan molecule and collagen. In this case, any change in the core protein structure would affect binding.

ACKNOWLEDGMENT

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REFERENCES

1. Scott, J.E. (1988) Biochem. J. 252, 313-323.
2. Pringle, G.A. and Dodd, C.M. (1990) J. Histochem. Cytochem. 38, 1405-1411.
3. Brown, D.C. and Vogel, K.G. (1989) Matrix 9, 468-478.
4. Hedbom, E. and Heinegard, D. (1989) J. Biol. Chem. 264, 6898-6905.
5. Vogel, K.G., Koob, T.J., and Fisher, L.W. (1987) Biochem. Biophys. Res. Comm. 148, 658-663.
6. Evanko, S.P. and Vogel, K.G. (1990) Matrix 10, 420-436.
7. Burridge, K. and Bray, D. (1975) J. Mol. Biol. 99, 1-14.
8. Chandrakasan, G., Torchia, D.A. and Piez, K.A. (1976) J. Biol. Chem. 251, 6062-6067.
9. Vogel, K.G., Paulsson, M. and Heinegard, D. (1984) Biochem. J. 223, 587-597.
10. Scott, P.G., Winterbottom, N., Dodd, C.M., Edwards, E. and Pearson, C.H. (1986) Biochem. Biophys. Res. Comm. 138, 1348-1354.