Journal of Cellular Biochemistry 67:75–83 (1997)

Decorin Inhibits Cell Attachment to Thrombospondin-1 by Binding to a KKTR-Dependent Cell Adhesive Site Present Within the N-Terminal Domain of Thrombospondin-1

Blandine Merle,[1]* **Luc Malaval,**[1] **Jack Lawler,**[2] **Pierre Delmas,**[1] **and Philippe Clezardin**[1]

[1]INSERM Research Unit 403, Hôpital Edouard Herriot, Lyon Cedex, France
[2]Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215

Abstract Skin decorin (DCN) is an antiadhesive dermatan sulfate-rich proteoglycan that interacts with thrombospondin-1 (TSP) and inhibits fibroblast adhesion to TSP [Winnemöller et al., 1992]. Molecular mechanisms by which DCN interacts with TSP and inhibits cell adhesion to TSP are unknown. In the present study, we showed that skin DCN and bone DCN (chondroitin sulfate-rich proteoglycan) were quantitatively identical with respect to their ability to interact with TSP. Using a series of fusion proteins corresponding to the different structural domains of TSP, binding of [^{125}I]DCN to TSP was found to be dependent of the N-terminal domain and, to a lesser extent, of the type 1 repeats and the C-terminal domain of TSP. In addition, heparan sulfate drastically inhibited [^{125}I]DCN binding to solid-phase adsorbed TSP (80% inhibition), suggesting that DCN could bind to the N-terminal domain of TSP through interaction with heparin-binding sequences. To address this question, a series of synthetic peptides, overlapping heparin-binding sequences ARKGSGRR (residues 22–29), KKTR (residues 80–83) and RLRIAKGGVNDN (residues 178–189), were synthesized and tested for their ability to interact with DCN. [^{125}I]DCN interacted only with peptides VDAVRTEKGFLLLASLRQMKKTRGT and KKTRGTLLALERKDHS containing the heparin-binding consensus sequence KKTR. These peptides contained glycosaminoglycan-dependent and -independent binding sites because [^{125}I]DCN binding to VDAVRTEKGFLLLASLRQMKKTRGT and KKTRGTLLALERKDHS was partially reduced upon removal of the glycosaminoglycan chain (65% and 46% inhibition, respectively). [^{125}I]DCN poorly bound to subpeptide MKKTRG and did not bind at all to subpeptides VDAVRTEKGFLLLASLRQ and TLLALERKDHS, suggesting that heparin-binding sequence MKKTRG constituted a DCN binding site when flanked with peptides VDAVRTEKGFLLLASLRQ and TLLALERKDHS. The sequence VDAVRTEKGFLLLASLRQMKKTRGTLLALERKDHS constitutes a cell adhesive active site in the N-terminal domain of TSP [Clezardin et al., 1997], and DCN inhibited the attachment of fibroblastic and osteoblastic cells to peptides VDAVRTEKGFLLLASLRQMKKTRGT and KKTRGTLLALERKDHS by about 50 and 80%, respectively. Although fibroblastic cells also attached to type 3 repeats and the C-terminal domain of TSP, DCN only inhibited cell attachment to the C-terminal domain. Overall, these data indicate that modulation by steric exclusion of cell adhesion to a KKTR-dependent cell adhesive site present within the N-terminal domain of TSP could explain the antiadhesive properties of DCN. J. Cell. Biochem. 67:75–83, 1997.

Key words: decorin; thrombospondin-1; cell attachment

INTRODUCTION

The extracellular matrix is a complex set of collagens, noncollagenous proteins, and proteoglycans that, by interacting with cell surface receptors, modulates the migration [Huttenlocher et al., 1995], proliferation [Dalton et al., 1995], and differentiation [Juliano et al., 1993] of many different cells types. These macromolecules can be subdivided into adhesive (e.g., collagens, fibronectin, thrombospondin-1 [TSP], vitronectin) and antiadhesive (proteoglycans, SPARC, tenascin) molecules [for review, see Sage et al., 1991; Chiquet-Ehrismann, 1995]. Although antiadhesive properties of SPARC and tenascin could be related to receptor-mediated events [Murphy-Ullrich, 1991; Yost et al., 1993],

Abbreviations: TSP, thrombospondin-1; DCN, decorin; GAG, glycosaminoglycan.

Contract grant sponsor: INSERM; contract grant sponsor: National Institutes of Health, contract grant number HL-28749.

*Correspondence to: B. Merle, INSERM Research Unit 403, Pavillon F, Hôpital Edouard Herriot, Place d'Arsonval, Lyon Cedex, France.
E-mail: b-merle@cismsum.univ-lyon1-fr

Received 7 April 1997; accepted 11 June 1997

molecular mechanisms by which proteoglycans inhibit cell adhesion are largely unknown. Among these proteoglycans, decorin (DCN) is a small leucine-rich proteoglycan expressed in a wide range of connective tissues, including bone and skin tissues [for review, see Kresse et al., 1993]. It is composed of a 40-kDa protein-core substituted with a single tissue-type specific glycosaminoglycan (GAG) chain. In bone, DCN bears chondroitin-sulfate chains [Franzén et al., 1984], while in skin it exists as a dermatan-sulfate proteoglycan [Choi et al., 1989]. Although DCN has a tissue-type specific GAG chain, most of the functional studies are performed with skin DCN. In this respect, skin DCN modulates fibrillogenesis in vitro by interacting with type I collagen [Kresse et al., 1993]. Skin DCN also modulates cell adhesion to fibronectin and TSP, but not to type I collagen [Winnemöller et al., 1991; Bidanset et al., 1992; Winnemöller et al., 1992]. Inhibition of cell adhesion to fibronectin by skin DCN occurs through interactions of the dermatan sulfate chain and the protein core of DCN with the fibronectin cell adhesive domain [Winnemöller et al., 1991; Bidanset et al., 1992]. Skin DCN also interacts with TSP through its dermatan sulfate chain and protein-core [Winnemöller et al., 1992]. However, the molecular mechanisms by which skin DCN inhibits cell adhesion to TSP are unknown. Finally, skin DCN induces growth suppression of Chinese hamster ovary cells by virtue of its ability to block transforming growth factor-β (TGF-β) activity [Yamaguchi et al., 1990]. As opposed to skin DCN, DCN isolated from bovine bone extracts rather increases the bioactivity of transforming growth factor-β in MC3T3-E1 osteoblastic cells [Takeuchi et al., 1994]. Thus, the function of DCN may vary considerably, depending on the proteoglycan source, suggesting that the interaction of bone DCN with extracellular matrix proteins known to interact with skin DCN (TSP, fibronectin, collagens) warrants examination.

With the aim of identifying molecular mechanisms by which DCN exerts its antiadhesive properties, we show, in the present study, that skin and bone DCN interact to a similar extent with TSP, and that DCN inhibits adhesion of fibroblastic and osteoblastic cells to TSP through specific interaction with a KKTR-dependent cell adhesive site present within the N-terminal domain of TSP. A modulation by steric exclusion of cell adhesion to a KKTR-dependent cell adhesive site present within the N-terminal domain of TSP could therefore explain the antiadhesive properties of DCN.

METHODS

Proteins and Peptides

Decorin (DCN) was extracted from bovine long bones [Termine et al., 1981] and purified by gel filtration, followed by ion-exchange chromatography. The purity was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and amino acid analysis. Identification of DCN was further confirmed by western-blotting of the native and chondroitinase-treated protein followed by immunodetection with a monoclonal antibody against bovine DCN (DS1, kindly provided by Dr A.R. Poole, Montreal). Bovine fetal skin DCN was generously provided by Drs. H.U. Choi and L.C. Rosenberg (Montefiore Medical Center, Bronx, NY) [Choi et al., 1989]. Human platelet TSP was obtained from SERBIO (Gennevilliers, France) or purified by Mono-Q anion-exchange chromatography as previously described [Clezardin et al., 1984]. Glutathione S-transferase (GST) fusion proteins encoding for the N-terminal domain (amino acids 1–90), type 1 (amino acids 385–522), type 2 (amino acids 559–669), and type 3 repeats (amino acids 784–932), and the C-terminal domain (amino acids 877–1152) of TSP were prepared as previously described [Adams et al., 1994]. The synthesis and characterization of peptides overlapping cell adhesive sequences of the N-terminal domain of TSP were described elsewhere [Clezardin et al., 1997]. Each synthetic peptide was coupled to bovine serum albumin (BSA), using the carbodiimide or the bis-diazobenzidine method. TSP and DCN were iodinated by the chloramin T method [Hunter et al., 1983]. Specific activity was within the range $2–3 \times 10^7$ cpm/μg.

DCN was digested with chondroitin ABC lyase (0.2 mU enzyme/μg DCN; EC 4.2.2.4, from *Proteus vulgaris*, ICN) in Tris buffer (10 mM Tris, pH 7.4) for 60 min at 37°C to remove GAG chains.

Blot-Overlay Assay

A bovine bone protein extract was gel-filtered on a Sepharose CL-6B column, and eluted proteins were collected then subjected to electrophoresis on a 5:3 Tris–Tricine polyacrylamide

gel, according to Schägger et al. [1987]. The gel was equilibrated in 50 mM boric acid, 0.1% (w/v) SDS, pH 8, for 30 min at room temperature, and western-blotted onto nitrocellulose (Millipore) in 50 mM boric acid, 50 mM Tris–HCl buffer, 4 hrs at room temperature. After saturation in Tris buffer [50 mM Tris–HCl, 2 mM $CaCl_2$, 0.03% (w/v) sodium azide, pH 8] containing 4% (w/v) BSA, nitrocellulose strips were incubated at 4°C with [^{125}I]TSP (1×10^6 cpm/ml) diluted in Tris buffer containing 4% (w/v) BSA and 0.05% Tween 20. After overnight incubation, strips were washed, dried and exposed for autoradiography. A similar assay was performed with purified skin and bone DCN.

In the dot-blot overlay assay, purified proteins (1 µg) were dot-blotted onto nitrocellulose (Millipore) in Tris buffer. After saturation with BSA, the nitrocellulose sheet was incubated with [^{125}I]TSP and exposed for autoradiography as described above.

Enzyme-Linked Immunosorbent Assay

The binding of TSP to bone and skin DCN was studied by enzyme-linked immunosorbent assay (ELISA) according to a method described by Clezardin et al. [1988], with minor modifications. Microtiter plates (Nunc, ref. 269620) were coated with DCN (2 µg/ml, 100 µl/well) in Tris buffer (10 mM Tris–HCl, pH 7.4) overnight at 4°C. After saturation with BSA, plates were incubated with increasing concentrations of TSP (0.1–20 µg/ml, 100 µl/well) for 3 h at 37°C, and bound TSP was detected with a peroxidase-conjugated rabbit polyclonal anti-TSP antibody (Stago). The amount of DCN effectively bound to the substrate was calculated using skin and bone [^{125}I]DCN. Results were expressed as moles of TSP bound per mole of DCN. In competitive binding experiments, TSP (10 µg/ml) together with increasing concentrations (0.1–50 µg/ml) of DCN were added to DCN-coated plates; the assay was performed as described above.

Solid-Phase Radioimmunoassay (SPRIA)

Microtiter plates (Nunc, ref. 269620) were coated for 3 h at 37°C with 100 µl/well of TSP (0.022 µM) or synthetic peptides (100 µM) in Tris buffer (10 mM Tris.HCl, pH 7.4). After washing, plates were saturated with Tris buffer containing 1% (w/v) BSA. Untreated or chondroitinase-treated skin [^{125}I]DCN (16.25 nM, 100 µl/well) diluted in Tris buffer containing 150 mM NaCl and 1 mM $CaCl_2$ was added and incubated overnight at 4°C. After washing, bound [^{125}I]DCN was solubilized with 20% (w/v) SDS (200 µl/well) and counted in a gamma counter (Riastar®, Packard Instruments Company, Downers Grove, IL). In competitive binding experiments, [^{125}I]DCN together with TSP fusion proteins (1 µM, 100 µl/well) were added to TSP-coated plates, and the assay performed as described above.

Gravity Cell Attachment Assay

Cell attachment to 60-mm bacteriologic petri dishes (Falcon) coated with peptides was performed as described by Clezardin et al. [1997], with minor modifications. Briefly, dots (10 µl) of fusion proteins of TSP (25 µg/ml) or synthetic peptides (10 µM) diluted in HBS/$CaCl_2$ buffer were incubated overnight at 4°C. Unbound material was discarded, and 10 µl of HBS/$CaCl_2$ buffer containing 0.1% (w/v) BSA was added to each dot. After a 30-min incubation at room temperature, BSA was removed and 10 µl of DCN (12.5 µg/ml) was added to each dot and incubated overnight at 4°C. Remaining steps were performed as described [Clezardin et al., 1997]. Mouse NIH 3T3 fibroblastic and human MG-63 osteoblastic cells that adhered to the substrate were fixed and counted microscopically using an eyepiece reticle (Zeiss, plate No. VI). Quadriplicate determination was performed for each protein or peptide concentration. Results were expressed as the number of cells bound/mm^2. Statistical analysis was performed using a Wilcoxon test.

RESULTS

TSP Binds to Bone Proteoglycans

Bovine bone proteins were gel-filtered on a Sepharose CL-6B column (Fig. 1A), and each collected fraction was subjected to electrophoresis on a 5:3 Tris–tricine polyacrylamide gel, Western-blotted onto nitrocellulose and incubated with [^{125}I]TSP. [^{125}I]TSP only bound to fractions 10–38 containing high-molecular-weight proteins ranged between 250 and 120 kDa (Fig. 1B). As described by Fisher et al. [1987], fractions 10–28 and 16–34 contained biglycan and DCN, respectively. Biglycan and DCN eluted from the Sepharose CL-6B column were therefore purified, dot-blotted onto nitrocellulose, and then incubated with [^{125}I]TSP. As shown in Figure 1C, [^{125}I]TSP strongly bound to DCN and biglycan (Bgn) but not to other puri-

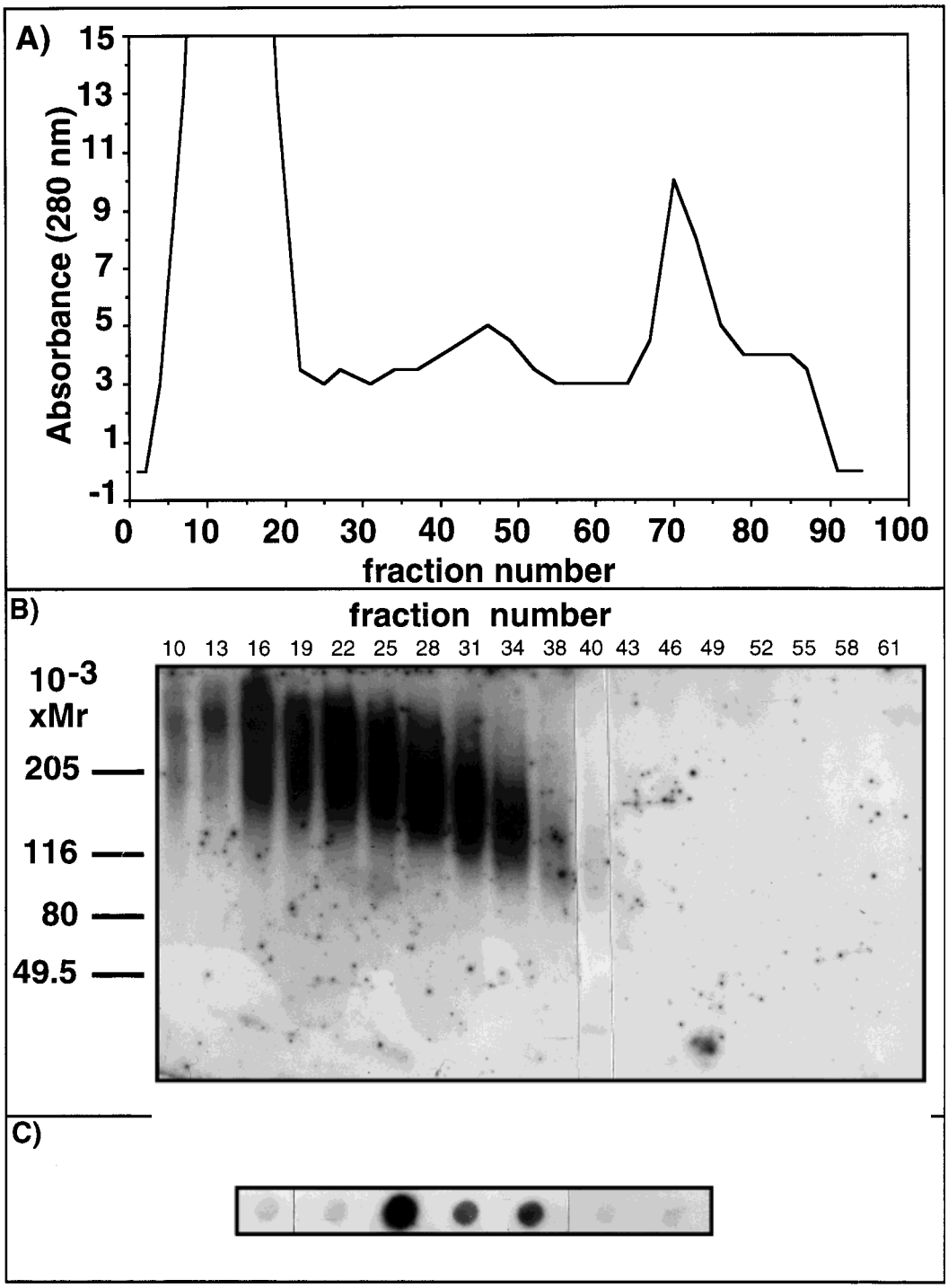

Fig. 1. Thrombospondin binding to bone proteoglycans. **A:** Sepharose CL-6B gel-filtration chromatography of noncollagenous proteins released from a bovine bone extract. Noncollagenous bone proteins were gel-filtered in 6 M guanidine–HCl and the absorbance read at 280 nm. **B:** Blot-overlay assay of [^{125}I]TSP with gel-filtered noncollagenous bone proteins eluted from the Sepharose CL-6B column. Eluted fractions were subjected to Tris–Tricine polyacrylamide gel electrophoresis, Western-blotted onto nitrocellulose, incubated with fluid-phase [^{125}I]TSP, and exposed for autoradiography. **C:** Binding of [^{125}I]TSP to purified proteins. Purified bone proteins were dot-blotted onto nitrocellulose, incubated with fluid-phase [^{125}I]TSP, and exposed for autoradiography. Ge, gelatin; Bsp, bone sialoprotein; Cn, type I collagen; Bgn, biglycan; Dcn, decorin; Vn, vitronectin; Fn, fibronectin.

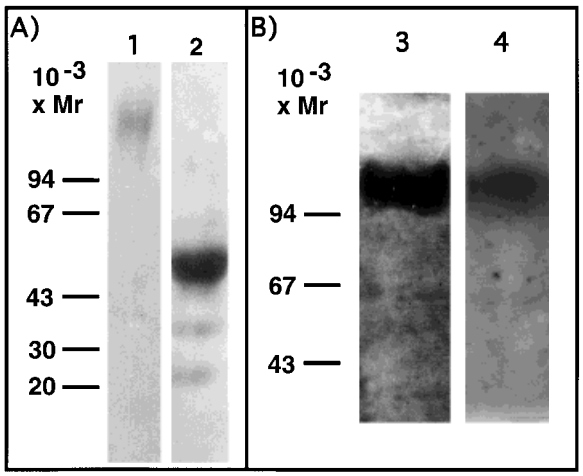

Fig. 2. Thrombospondin binding to bone and skin DCN, using blot-overlay assay: **A:** Untreated (**lane 1**) and chondroitinase-treated (**lane 2**) bone DCN were subjected to electrophoresis on a 4–15% SDS-polyacrylamide gel, Western-blotted onto nitrocellulose, and incubated with a monoclonal antibody against bovine DCN, followed by incubation with a radiolabeled anti-mouse secondary antibody and exposed for autoradiography. **B:** Skin (**lane 3**) and bone (**lane 4**) DCN were subjected to electrophoresis on a 10% SDS-polyacrylamide gel, Western-blotted onto nitrocellulose, incubated with fluid-phase [^{125}I]TSP and exposed for autoradiography.

fied bone proteins [bone sialoprotein (Bsp), vitronectin (Vn), and fibronectin (Fn)], as compared to gelatin (Ge). As previously published by Mumby et al. [1984], [^{125}I]TSP also strongly bound to type I collagen (Cn).

TSP Binds to a Similar Extent to Bone and Skin DCN

In order to confirm the identity of purified bone DCN, aliquots of native and chondroitinase-treated bone DCN were subjected to electrophoresis on a 4–15% SDS-polyacrylamide gel, Western-blotted onto nitrocellulose and immunodetected with a monoclonal antibody against bovine DCN. Figure 2 showed immunoreactive bands at 155 and 48 kDa for native (lane 1) and chondroitinase-treated (lane 2) DCN, respectively. The 22 and 33-kDa bands corresponded to immunoreactive peptidic fragments. As shown in figure 2B, [^{125}I]TSP bound to skin and bone DCN immobilized onto a nitrocellulose sheet in a blot-overlay assay. This result was confirmed by ELISA; TSP bound skin and bone DCN in a dose-dependent manner, reaching a plateau at 5–10 µg/ml (Fig. 3A). TSP binding experiments to solid-phase adsorbed skin DCN (2 µg/ml) were also performed in the presence of increasing concentrations of fluid-phase skin

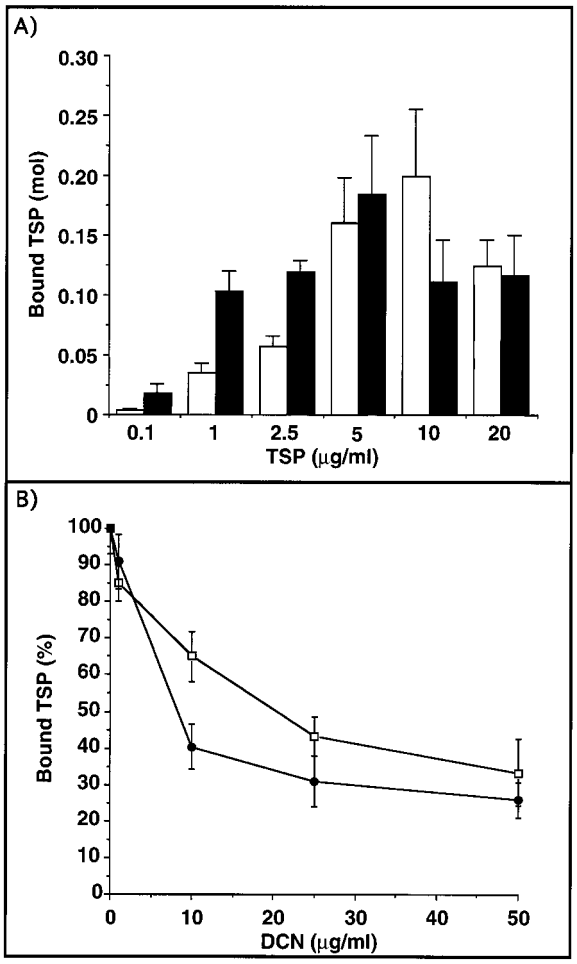

Fig. 3. Thrombospondin binding to bone and skin DCN, using ELISA. **A:** Wells of a microtiter plate were coated with bone or skin DCN (2 µg/ml). Increasing concentrations of TSP were added, and the amount of bound TSP was detected using a peroxidase-conjugated anti-TSP polyclonal antibody. Results were expressed as the number of TSP moles bound per mole of skin DCN (□) or bone DCN (■) immobilized on plastic wells. Results are the mean ± SE of six separate experiments. **B:** Wells of a microtiter plate were coated with skin DCN (2 µg/ml). TSP (10 µg/ml) was incubated in the presence of increasing concentrations of skin (□) or bone DCN (●), and bound TSP was detected as indicated in A. Results were expressed as the percentage of the amount of TSP bound in the absence of competitors. Results are the mean ± SD of three to five separate experiments.

and bone DCN (0.1–50 µg/ml) (Fig. 3B). Both skin and bone DCN dose-dependently inhibited TSP binding to solid-phase adsorbed DCN, reaching 65–72% inhibition when a 25-fold excess of proteoglycans was used. Although TSP bound to bone DCN with a higher affinity than that observed for skin DCN, the binding capacity of bone and skin DCN for TSP was similar (Fig. 3). Collectively, these data suggested that

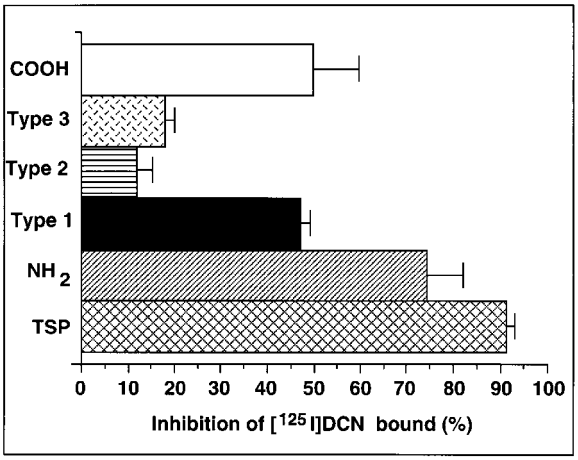

Fig. 4. Inhibitory effect of fusion proteins on [^{125}I]DCN binding to TSP. [^{125}I]DCN (2 μg/ml, 16.2 nM) was incubated in TSP-coated wells (10 μg/ml, 0.022 μM), together with fusion proteins encoding for the N-terminal (▨) and C-terminal (□) domains, type 1 (■), type 2 (▤), and type 3 (□) repeats of TSP (1 μM), or with an excess of fluid-phase TSP (▦) (0.55 μM). [^{125}I]DCN bound to TSP was measured in a gamma counter. Results are expressed as the percentage of inhibition of [^{125}I]DCN binding to TSP in the presence of excess fluid-phase fusion proteins or TSP. Data are the mean ± SD of three separate experiments.

skin and bone DCN were quantitatively identical, with respect to their ability to interact with TSP. All subsequent experiments were performed with skin DCN.

DCN Binds to the N-Terminal Domain of TSP Through a Heparin-Binding Sequence

TSP is a 450-kDa trimeric glycoprotein. Each subunit of the trimer is made up of several domains including N-terminal, C-terminal, procollagen homology domains, and type 1, 2, and 3 repeats [Adams et al., 1994]. With the aim of identifying DCN binding sites present within TSP, [^{125}I]DCN binding to solid-phase adsorbed TSP was performed in the presence of excess fluid-phase fusion proteins corresponding to the different TSP structural domains. When compared to excess fluid-phase TSP (91% inhibition), binding of [^{125}I]DCN to solid-phase adsorbed TSP was drastically inhibited in the presence of excess fluid-phase N-terminal domain (74% inhibition) (Fig. 4). A moderate decrease of [^{125}I]DCN binding to TSP was also observed with type 1 repeats (47% inhibition) and the C-terminal domain (50% inhibition). By contrast, type 2 and 3 repeats had no significant inhibitory effect (12% and 18% inhibition, respectively).

Because a 15-fold excess fluid-phase heparan sulfate drastically inhibited DCN binding to solid-phase adsorbed TSP (80% inhibition) (results not shown) and the N-terminal domain of TSP contains high-affinity heparin-binding sites [Murphy-Ullrich et al., 1988; Lawler et al., 1992], our data suggested that DCN could bind to the N-terminal domain of TSP through interaction with heparin-binding sequences. To address this question, [^{125}I]DCN binding to a series of synthetic peptides overlapping known heparin-binding sequences was tested (Table I). [^{125}I]DCN strongly interacted with peptides VDAVRTEKGFLLLASLRQMKKTRGT and, to a lesser extent, KKTRGTLLALERKDHS. By contrast, [^{125}I]DCN poorly bound to high-affinity heparin-binding sequences MKKTRG, ELTGAARKGSGRRLVKGPDP and RLRIAGGVNDN and did not bind at all to peptides VDAVRTEKGFLLLASLRQ and TLLALERKDHS (Table I). Lack of [^{125}I]DCN binding to these peptides was not due to their inability to bind to the well surface as the capacity of adsorption of all the peptides tested was very similar [Clezardin et al., 1997]. Five additional synthetic peptides encompassing residues 99–136 and 190–216 of the N-terminal domain of TSP were synthesized. [^{125}I]DCN did not bind to any of these peptides (data not shown). Chondroitinase ABC treatment of DCN reduced [^{125}I]DCN binding to peptides VDAVRTEKGFLLLASLRQMKKTRGT and KKTRGTLLALERKDHS by 65% and 46%, respectively. Chondroitinase-treated [^{125}I]DCN did not bind to the other synthetic peptides tested (Table I).

DCN Inhibits Cell Adhesion by Binding to a KKTR-Containing, Heparin-Binding Sequence Present Within the N-Terminal Domain of TSP

Cell attachment to TSP involves multiple domains working together to support adhesion in a cell type-dependent fashion [Roberts et al., 1987; Kosfeld et al., 1993; Adams and Lawler, 1993, 1994; Clezardin et al., 1997]. In addition, a wide variety of cells, including NIH 3T3 fibroblastic and MG-63 osteoblastic cells, attach to the N-terminal domain of TSP through a cell adhesive site located between residues 61 and 95 (VDAVRTEKGFLLLASLRQMKKTRGTLLALERKDHS) [Clezardin et al., 1997]. The effect of DCN on NIH 3T3 and MG-63 cell attachment to peptides VDAVRTEKGFLLLASLRQMKKTRGT and KKTRGTLLALERKDHS was therefore investigated. NIH 3T3 and

TABLE I. [^{125}I]DCN Binding to Heparin-Binding Peptides From the N-Terminal Domain of TSP

	Peptide sequence[a]	[^{125}I]DCN bound to synthetic peptides (fmol)[b]	[^{125}I]DCN core protein bound to synthetic peptides (fmol)[b]
[17–36]	ELTGA**ARKGSGRRL**VKGPDP	33 ± 19	16 ± 12
[61–85]	VDAVRTEKGFLLLASLRQM**KKTR**GT	461 ± 17	163 ± 6
[61–78]	VDAVRTEKGFLLLASLRQ	0	1.4 ± 0.6
[80–95]	**KKTR**GTLLALERKDHS	161 ± 22	87 ± 13
[79–84]	M**KKTR**G	17 ± 5	0
[85–95]	TLLALERKDHS	0	0
[178–189]	**RLRIAKGGVNDN**	11 ± 14	0

[a]Heparin binding sequences present within synthetic peptides are indicated in boldface. Numbers in brackets correspond to the positions of the residues in TSP.
[b][^{125}I]DCN or [^{125}I]DCN core protein (16.2 nM) was incubated with coated N-terminal synthetic peptides (100 μM). The amount bound was counted in a gamma counter.

TABLE II. Effect of DCN on NIH 3T3 Cell Attachment to TSP Structural Domains*

	Adherent cells (cells/mm^2)							
	N-terminal domain				Type 3 repeats		C-terminal domain	
	Peptide [61–85]		Peptide [80–95]					
Cell type	−DCN	+DCN	−DCN	+DCN	−DCN	+DCN	−DCN	+DCN
NIH 3T3	334 ± 119 (n = 6)	136 ± 37 (n = 6)	81 ± 40 (n = 6)	16 ± 23 (n = 6)	70 ± 29 (n = 3)	77 ± 14 (n = 3)	132 ± 51 (n = 6)	68 ± 41 (n = 6)

*Fusion proteins were used at a concentration of 25 μg/ml and peptides at 10 μM. DCN was added at a concentration of 12.5 μg/ml. Data are expressed as the mean ± SD of n separate experiments.

MG-63 cells strongly attached to peptide VDAVRTEKGFLLLASLRQMKKTRGT (residues 61–85) and, to a lesser extent, to peptide KKTRGTLLALERKDHS (residues 80–95) [Clezardin et al., 1997] (Table II). DCN itself did not support cell attachment (not shown). DCN binding to peptides (61–85) and (80–95) strongly inhibited NIH 3T3 cell attachment (55% and 80% inhibition, respectively, $P < 0.05$) (Table II). Similarly, DCN inhibited MG 63 cell attachment to peptides (61–85) and (80–95) by 48% and 77%, respectively (not shown). In addition to the N-terminal domain of TSP, NIH 3T3 cells attached to type 3 repeats and the C-terminal domain, but not to type 1 and 2 repeats [Clezardin et al., 1997]. DCN inhibited NIH 3T3 cell attachment to the C-terminal domain (50% inhibition, $P < 0.05$), while attachment to type 3 repeats remained unaffected (Table II).

DISCUSSION

Skin DCN interacts with TSP through its dermatan sulfate chain and protein-core and inhibits fibroblast cell adhesion to TSP [Winnemöller et al., 1992]. However, molecular mechanisms by which skin DCN interacts with TSP and inhibits cell adhesion to TSP are unknown. In addition, the function of DCN may vary considerably depending of the proteoglycan source [Yamaguchi et al., 1990; Takeuchi et al., 1994]. In this study, the interaction of bone DCN (chondroitin sulfate-rich proteoglycan) and skin DCN (dermatan sulfate-rich proteoglycan) with TSP was first examined. Although the GAG chain of DCN was involved in binding to TSP (see below), we showed that skin and bone DCN interacted to a similar extent with TSP. With the aim of identifying the molecular mechanisms by which DCN interacted with TSP, a series of TSP fusion proteins was used to identify the different structural domains required for DCN/TSP interaction. We found that binding of DCN to TSP was mainly dependent of the N-terminal domain and, to a lesser extent, of the type 1 repeats and the C-terminal domain of TSP. These findings are consistent with the observations that DCN binding to TSP

involves two classes of binding sites [Winnemöller et al., 1992] and that these TSP structural domains (i.e., the N- and C-terminal domains and type 1 repeats) have GAG-binding consensus sequences as opposed to type 2 and type 3 repeats (Frazier et al., 1993). In addition, the involvement of the N-terminal domain in binding to DCN extends previous findings showing that DCN binds to a 27-kDa N-terminal heparin-binding fragment of TSP [Winnemöller et al., 1992]. Because the N-terminal domain and heparan sulfate drastically inhibited DCN binding to TSP (80% inhibition) (Fig. 4; and results not shown) and heparin-binding sequences have been identified in the N-terminal domain of TSP [Murphy-Ullrich et al., 1988; Lawler et al., 1992], our data suggested that DCN could bind to the N-terminal domain of TSP through interaction with heparin-binding sequences. To examine this hypothesis, a series of synthetic peptides, overlapping heparin-binding sequences A**RK**GSG**RR** (residues 22–29), **KKTR** (residues 80–83) and **R**L**R**IA**K**GGVNDN (residues 178–189), were synthesized and tested for their ability to interact with DCN. Although all these synthetic peptides bound to heparin agarose with high affinity [Clezardin et al., 1997], DCN only interacted with peptides VDAVRTEKGFLLLASLRQMKKTRGT and KKTRGTLLALERKDHS containing the heparin-binding consensus sequence **KKTR.** These peptides contained GAG-dependent and -independent binding sites because DCN binding to VDAVRTEKGFLLLASLRQMKKTRGT and KKTRGTLLALERKDHS was partially reduced upon removal of the GAG chain (65% and 46% inhibition, respectively). However, DCN poorly bound to subpeptide MKKTRG and did not bind at all to subpeptides VDAVRTEKGFLLLASLRQ and TLLALERKDHS. Guo et al. [1992a] have observed that inhibition of TSP binding to heparin by peptide SHWSPWSS from TSP type 1 repeats is markedly enhanced when flanked with the heparin-binding consensus sequence **KR**F**K** (KRFKQDGGWSHWSPWSS), while the motif KRFK was not sufficient to inhibit TSP binding to heparin. It has been proposed that addition of flanking residues to the minimal functional sequence contributes to the maintenance of an active conformation of these peptides [Guo et al., 1992b]. It is therefore most conceivable that the heparin-binding sequence M**KK**T**R**G defines a recognition binding domain for DCN when flanked with peptides VDAVRTEKGFLLLASLRQ and TLLALERKDHS.

Interestingly, we recently provide evidence that cell attachment to MKKTRG is markedly enhanced when flanked with peptides VDAVRTEKGFLLLASLRQ and TLLALERKDHS, indicating that residues 61–95 (sequence VDAVRTEKGFLLLASLRQMKKTRGTLLALERKDHS) constitute a cell adhesive active site in the N-terminal domain of TSP [Clezardin et al., 1997]. In this study, we found that DCN inhibited attachment of fibroblastic and osteoblastic cells to peptides VDAVRTEKGFLLLASLRQMKKTRGT and KKTRGTLLALERKDHS by about 50% and 80%, respectively. Besides the N-terminal domain of TSP, NIH 3T3 cells attach to type 3 repeats and the C-terminal domain, but not to type 1 and 2 repeats [Clezardin et al., 1997]. DCN inhibited NIH 3T3 cell attachment to the C-terminal domain, while cell attachment to type 3 repeats remained unaffected (Table II). These results were in agreement with the observation that DCN interacted with the C-terminal domain but not with type 3 repeats (Fig. 4). Two cell adhesive active sites (IRVVM and RFYVVMWK) have been identified in the C-terminal domain of TSP [Kosfeld et al., 1993]. These two VVM-containing cell adhesive sites are flanked with GAG-binding sequences [Kosfeld et al., 1993]. It is possible that DCN binds to these GAG-binding sites to inhibit NIH 3T3 cell attachment to adhesive active sites of the C-terminal domain of TSP.

Overall, our study provides, for the first time, evidence that DCN inhibits cell adhesion to TSP by interacting with a KKTR-dependent cell adhesive site located within the N-terminal domain of TSP. Moreover, DCN also binds to type 1 repeats and the C-terminal domain of TSP, and it is most conceivable that DCN inhibits cell adhesion to the C-terminal domain through a mechanism similar to that observed with the N-terminal domain. A modulation by steric exclusion of cell adhesion to adhesive sites present in the N- and C-terminal domains of TSP could therefore explain the antiadhesive properties of DCN.

ACKNOWLEDGMENTS

We are particularly grateful to Dr. HU Choi and to Dr. LC Rosenberg for providing the skin DCN used in this study. Many thanks are also due to Miss Laurence Durussel for excellent

technical assistance. This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM) (P.C.) and by the National Institutes of Health (grant HL-28749) (J.L.).

REFERENCES

Adams JC, Lawler J (1993): Diverse mechanisms for cell attachment to platelet thrombospondin. J Cell Sci 104: 1061–1071.

Adams JC, Lawler J (1994): Cell-type specific adhesive interactions of skeletal myoblasts with thrombospondin-1. Mol Biol Cell 5:423–437.

Bidanset DJ, Lebaron R, Rosenberg L, Murphy-ullrich JE, Höök M (1992): Regulation of cell substrate adhesion: Effects of small galactosaminoglycan-containing proteoglycans. J Cell Biol 118:1523–1531.

Chiquet-Ehrismann R (1995): Inhibition of cell adhesion by anti-adhesive molecules. Current Opin Cell Biol 7:715–719.

Choi HU, Johnson TL, Pal S, Tang L, Rosenberg L, Neame PJ (1989): Characterization of the dermatan sulfate proteoglycans, DS-PGI and DS-PGII, from articular cartilage and skin isolated by octyl-sepharose chromatography. J Biol Chem 264:2876–2884.

Clezardin P, McGregor JL, Manach M, Robert F, Dechavanne M, Clemetson KJ (1984): Isolation of thrombospondin released from thrombin-stimulated human platelets by fast protein liquid chromatography on an anion-exchange Mono-Q column. J Chromatogr 296:249–256.

Clezardin P, Malaval L, Ehrensperger AS, Delmas PD, Dechavanne M, McGregor J (1988): Complex formation of human thrombospondin with osteonectin. Eur J Biochem 175:275–284.

Clezardin P, Lawler J, Amiral J, Quentin G, Delmas PD (1997): Identification of cell adhesive active sites in the N-terminal domain of thrombospondin-1. Biochem J 321: 819–827.

Dalton SL, Scharf E, Briesewitz R, Marcantonio EE, Assoian RK (1995): Cell adhesion to extracellular matrix regulates the life cycle of integrins. Mol Biol Cell 6:1781–1791.

Fisher LW, Hawkins T, Termine JD (1987): Purification and partial characterization of small proteoglycans I and II, bone sialoproteins I and II, and osteonectin from the mineral compartment of developing human bone. J Biol Chem 262:9702–9708.

Frazier WA, Prater CA, Jaye D, Kosfeld MD (1993): "Thrombospondin." Boca Raton, FL: CRC Press, pp 91–109.

Franzén A, Heinegard D (1984): Characterization of proteoglycans from the calcified matrix of bovine bone. Biochem J 224:59–66.

Guo NH, Krutzsch HC, Nègre E, Vogel T, Blake DA, Roberts DD (1992a): Heparin- and sulfatide-binding peptides from the type I repeats of human thrombospondin promote melanoma cell adhesion. Proc Natl Acad Sci USA 89:3040–3044.

Guo NH, Krutzsch HC, Nègre E, Zabrenetzky VS, Roberts DD (1992b): Heparin-binding peptides from the type I repeats of thrombospondin. J Biol Chem 267:19349–19355.

Hunter WM, Greenwood FC (1983): Preparation of iodine-131-labelled human growth hormone of high specific activity. Nature 194:485–497.

Huttenlocher A, Sandborg RR, Horwitz AF (1995): Adhesion in cell migration. Current Opin Cell Biol 7:697–706.

Juliano RL, Haskill S (1993): Signal transduction from the extracellular matrix. J Cell Biol 3:577–585.

Kosfeld MD, Frazier W (1993): Identification of a new cell adhesion motif in two homologous peptides from the COOH-terminal cell binding domain of human thrombospondin. J Biol Chem 268:8808–8814.

Kresse H, Hausser H, Schönherr E (1993): Small proteoglycans. Experientia 49:403–416.

Lawler J, Ferro P, Duquette M (1992): Expression and mutagenesis of thrombospondin. Biochemistry 31:1173–1179.

Mumby SM, Raugi GJ, Bornstein P (1984): Interaction of thrombospondin with extracellular matrix proteins: selective binding to type V collagen. J Cell Biol 98:646–652.

Murphy-Ullrich JE, Westrick LG, Esko JD, Mosher DF (1988): Altered metabolism of thrombospondin by Chinese hamster ovary cells defective in glycosaminoglycan synthesis. J Biol Chem 263:6400–6406.

Murphy-Ullrich JE, Lightner VA, Aukhil I, Yan YZ, Erickson HP, Höök M (1991): Focal adhesion integrity is downregulated by the alternatively spliced domain of human tenascin. J Cell Biol 115:1127–1136.

Roberts DD, Sherwood JA, Ginsburg V (1987): Platelet thrombospondin mediates attachment and spreading of human melanoma cells. J Cell Biol 104:131–139.

Sage EH, Bornstein P (1991): Extracellular proteins that modulate cell-matrix interactions. J Biol Chem 266: 14831–14834.

Schägger H, Von Jagow G (1987): Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in a range of 1 to 100 kDa. Anal Biochem 166:368–372.

Takeuchi Y, Kodama Y, Matsumoto T (1994): Bone matrix decorin binds transforming growth factor-β and enhances its bioactivity. J Biol Chem 269:32634–32638.

Termine JD, Belcourt A, Cohn KM, Kleinman HK (1981): Mineral and collagen binding of foetal calf bone. J Biol Chem 256:10403–10408.

Winnemöller M, Schmidt G, Kresse H (1991): Influence of decorin on fibroblast adhesion to fibronectin. Eur J Cell Biol 54:10–17.

Winnemöller M, Schön P, Visher P, Kresse H (1992): Interactions between thrombospondin and the small proteoglycan decorin. Eur J Cell Biol 59:47–55.

Yamaguchi Y, Mann DM, Ruoslahti E (1990): Negative regulation of transforming growth factor-β by the proteoglycan decorin. Nature 346:281–284.

Yost JC, Sage EH (1993): Specific interaction of SPARC with endothelial cells is mediated through a carboxyl-terminal sequence containing a calcium-binding EF hand. J Biol Chem 268:25790–25796.