The versican C-type lectin domain recognizes the adhesion protein tenascin-R

(J1-160/180/janusin)

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ABSTRACT The core proteins of large chondroitin sulfate proteoglycans contain a C-type lectin domain. The lectin domain of one of these proteoglycans, versican, was expressed as a recombinant 15-kDa protein and shown to bind to insolubilized fucose and GlcNAc. The lectin domain showed strong binding in a gel blotting assay to a glycoprotein doublet in rat brain extracts. The binding was calcium dependent and abolished by chemical deglycosylation treatment of the ligand glycoprotein. The versican-binding glycoprotein was identified as the cell adhesion protein tenascin-R, and versican and tenascin-R were both found to be localized in the granular layer of rat cerebellum. These results show that the versican lectin domain is a binding domain with a highly targeted specificity. It may allow versican to assemble complexes containing proteoglycan, an adhesion protein, and hyaluronan.

Versican, aggrecan, neurocan, and brevican constitute a family of chondroitin sulfate proteoglycans that form aggregates with hyaluronan (1-4). The C termini of these proteoglycans contain a C-type lectin domain, as well as epidermal growth factor-like and complement regulatory-like sequence motifs. This domain arrangement is similar to that of the selectins, which are adhesion receptors involved in leukocyte homing and extravasation at inflammatory sites (5-8).

The C-type animal lectins are defined by the presence of a calcium-dependent carbohydrate-recognition domain in which a pattern of 32 amino acid residues, spaced over a stretch of 120 amino acids, is highly conserved (9). The lectin domains of human and chicken versican show 96% amino acid identity (10, 11), and the human and rat versican lectin domains differ in only one amino acid (Thr-2257 in human versican replaced by Ala; Joan Lemire and Thomas N. Wight, personal communication), suggesting that the versican family lectin domains may have an important conserved function. However, very little is known about the function of these lectin domains. The aggrecan lectin domain has been shown to bind to fucose and galactose in a calcium-dependent manner (12, 13). Recently, the recombinant C-terminal part (epidermal growth factor-like, lectin, and complement regulatory-like domains) of chicken versican was shown to bind to simple carbohydrates, as well as to heparin and heparan sulfate, in affinity chromatography (14). A C-terminal fragment of heparan has been reported to bind tenasin and the cell–cell adhesion proteins N-CAM and Ng-CAM (15-17), but it is unclear if the lectin domain is responsible for these interactions.

We have studied the potential of the lectin domain of versican to function as a binding domain. Here we report that recombinant versican lectin domain, produced in mammalian cells has carbohydrate-binding activity and that it also binds the extracellular matrix glycoprotein tenasin-R (also known as J1-160/180 and janusin).

EXPERIMENTAL PROCEDURES

Materials. Antiserum against the versican lectin domain was raised against a synthetic peptide (KMFEDFWRWTDGSTLOYEN), corresponding to amino acid residues 2244-2262 (10), and affinity purified by using the peptide. The antiversican antiserum used in immunofluorescence staining was raised against recombinant intact versican (1) and affinity purified by using a bacterially expressed fragment of versican, corresponding to amino acid residues 51-354 (10). Mouse monoclonal anti-tenasin-R antibody #596 (18) was a kind gift from Melitta Schachner (Eidgenössische Technische Hochschule, Zurich), and anti-tenasin-C antisera was from Telios Pharmaceuticals (La Jolla, CA). Agarose beads conjugated to various monosaccharides or to streptavidin were from Sigma.

Plasmid Construction and Expression of the Versican Lectin Domain. The human versican lectin domain (amino acid residues 2177-2305; ref. 10) was amplified from cDNA clone 2B (19) by PCR with upstream primer 5'-GTCAACTCGAGACAAAGATACGCCAGATGTGAC-3' and downstream primer 5'-TGTCTTAGATCAGGTGCACCTT-TCCTGACGTATAATTG-3'. The lectin-coding sequence was then fused to an immunoglobulin signal peptide (20) and inserted into pcDNA 1/I/Neo (Invitrogen). The sequence of the construct was determined by using Sequenase II (United States Biochemical). CHO/DG44 cells (21) were cotransfected with the versican lectin domain construct and pSV2-dhfr (22) by using calcium phosphate transfection (CellPhect; Pharmacia). Transfected cells were selected in nucleoside-free α-modified minimal essential medium (GIBCO/BRL) containing 9% (vol/vol) dialyzed fetal calf serum, and lectin expression in selected colonies was amplified with methotrexate according to standard procedures. Cell supernatants were screened for versican lectin secretion by immunoblotting with anti-lectin-domain antisera.

Purification and Biotinylation of Versican Lectin Domain. Cells expressing the versican lectin were adapted to growth in serum-free medium (Nutri Base + Vita Cyte serum supplement; J. Brooks Laboratories, San Diego). Proteins from conditioned serum-free medium were precipitated in the presence of 5 mM EDTA with ammonium sulfate (50% saturation) and dissolved in Hepes-buffered saline (HBS; 50 mM Hepes, pH 7.4/0.88% NaCl) containing 5 mM CaCl2. Thereafter, the lectin was enriched through affinity chromatography on a fucose-agarose column and eluted with HBS containing 20 mM EDTA. After adding CaCl2 to 50 mM and NaOH to pH 8.5, the lectin was biotinylated with NHS-LC-biotin (Pierce) and purified by C4 reverse-phase HPLC. The samples were injected onto a 214TP54 column (Vydac Sepa-

Abbreviations: ELC, epidermal growth factor-like, lectin, and complement regulatory-like domains; TFMS, trilfuromosethanesulfonic acid; PNGase F, glycopeptide N-glycosidase; Endo H, protein fusion of endo-B-4-acetylglucosaminidase and maltose-binding protein.

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Constructions and Expression of Versican C-Terminal Domain—IgG Fusion Protein. The 3’ part of versican cDNA, corresponding to the epidermal growth factor-like, lectin, and complement regulatory-like domains (ELC; nucleotides 6530–7493; ref. 10), was amplified by PCR from clone 2B and fused in frame with the cDNA coding for the signal peptide and the first eight amino acids of α2 integrin (residues 1–49; ref. 23). This fragment was fused to human genomic DNA encoding the Fc region of IgG1 (24), inserted into plasmid pCDNA I/Neo, and sequenced. The plasmid was introduced, together with pSV2-dhfr, into CHO/DG44 cells by using Lipofectamine (GIBCO/BRL). The ELC—IgG fusion protein was purified from cell culture medium by affinity chromatography on protein A-Sepharose FF (Pharmacia).

Brain Extracts. Adult, female Sprague–Dawley rats were euthanized with carbon dioxide, and the brains were immediately removed and minced in ice-cold 0.9% NaCl. The tissue was homogenized with a Polytron in nine volumes of 4 mM Hepes, pH 7.5/0.3 M sucrose. containing 0.25 mg of N-ethylmaleimide per ml. 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride as protease inhibitors. The homogenate was centrifuged at 12,000 × g for 30 min. The supernatant was transferred to new tubes and centrifuged at 378,000 × g. The resulting supernatant was aliquotted, quick frozen in liquid nitrogen, and stored at −70°C until used.

Protein Blotting. Brain extract was boiled 5 min in sample buffer, electrophoresed through precast SDS/4–12% polyacrylamide gels (NOVEX, San Diego) with prestained molecular mass markers (GIBCO/BRL), and electroblotted onto nitrocellulose membranes (Schleicher & Schuell). The membranes were treated with 3% bovine serum albumin in TTBS (25 mM Tris-HCl, pH 7.4/0.88% NaCl/0.1% Tween 20) to inhibit nonspecific binding, incubated with biotinylated versican lectin or antibodies in 0.3% bovine serum albumin in TTBS, washed with TTBS, incubated with avidin-conjugated horseradish peroxidase (Vectastain ABC Elite; Vector Laboratories) or peroxidase-conjugated secondary antibody, washed with TTBS, and visualized by chemiluminescence (ECL; Amersham). For the lectin blots, all solutions contained either 5 mM CaCl2 or 5 mM EDTA.

Deglycosylation of Brain Extracts. Aliquots of brain extract were precipitated in acetone, dissolved in appropriate buffers for enzyme digestions, and incubated with or without recombinant glycoprotein N-glycosidase (PNGase F; EC 3.5.1.52; New England Biolabs), recombinant protein fusions of Endo-β-N-acetylgalactosaminidase (EC 3.2.1.96) and maltose-binding protein (Endo H; New England Biolabs), or Vibrio cholerae neuraminidase (EC 3.2.1.18; Boehringer Mannheim). For the incubations these were provided by the manufacturer or prepared according to the manufacturer’s instructions. After a 12-h incubation, the samples were boiled in SDS/PAGE sample buffer. Deglycosylation with trifluoromethane-sulfonic acid (TFMS) was performed as described (25).

Precipitation of Tenascin-R with Versican Lectin. Brain extract was incubated for 1 h on ice with biotinylated versican lectin in the presence of 5 mM CaCl2 or 20 mM EDTA. Streptavidin-conjugated agrose beads were then added, and the incubation was continued for 1 h at 4°C with gentle rotation. The beads were washed four times in Tris-buffered saline (TBS; 25 mM Tris-HCl, pH 7.4/0.88% NaCl/5 mM CaCl2 or 20 mM EDTA) and boiled in SDS/PAGE sample buffer containing 2.5% 2-mercaptoethanol. The samples were electrophoresed and electroblotted, and tenascin-R was detected as described above.

Immunofluorescence Staining. The cerebellum from an adult, male Sprague–Dawley rat was dissected, embedded in O.C.T. compound (Miles), frozen, and cut into 5-μm cryosections. The sections were air dried for 20 min and rehydrated in TBS. Nonspecific binding was blocked by incubating sections in 5% (vol/vol) goat serum/TBS. Primary antibodies were then applied in 2% (vol/vol) goat serum/TBS. The sections were washed, incubated with fluorescein-conjugated secondary antibodies raised in goat, washed again, and mounted in Vectashield mounting medium (Vector Laboratories). The slides were inspected in a Zeiss Axiosvert 51 microscope equipped for fluorescence microscopy, and pictures were taken with Kodak T-Max 400 film.

RESULTS

Binding of the Versican Lectin Domain to Monosaccharides. The versican lectin domain was expressed in CHO cells, and a band corresponding to the expected molecular mass of the lectin (14.7 kDa) in culture medium was detected by immunoblotting with anti-versican lectin antibodies. To determine if the lectin-domain protein could bind carbohydrates, the culture medium from the CHO cells was incubated with monosaccharides coupled to agarose beads. The lectin domain bound to beads coupled with fucose or GlcNac but not to beads coupled with GalNac, mannose, or glucose (Fig. 1). Chelation of calcium with EDTA or EGTA eliminated binding (data not shown). Thus, the versican lectin domain appears to be a functional carbohydrate-binding domain.

Versican Lectin Domain Binds to a Brain Protein. The biotinylated versican lectin domain bound to two proteins of approximate molecular masses 170 and 190 kDa in rat-brain extracts (Fig. 2 A). Rat brain was chosen as a primary target since versican is prominently expressed in the brain (26, 27), as are the other members of this proteoglycan family (2, 4, 28). The 75- and 130-kDa bands on the blots appear to be nonspecific because they are also detected by the avidin–horseradish peroxidase conjugate alone (Fig. 2 A, control). The binding of the lectin to the 170- and 190-kDa bands was inhibited in the presence of EDTA (Fig. 2 A).

The 170- and 190-kDa bands were also detected when blots were probed with ELC—IgG recombinant fusion protein, and this binding was also calcium dependent (Fig. 2 B). The 170- and 190-kDa Glycoproteins React with Anti-Tenascin-R Antibodies. The sizes of the 170- and 190-kDa glycoproteins we detected with the biotinylated lectin suggested to us that they might be tenascin-R (29). Furthermore, its presence in the brain and the reduction in apparent molecular mass of ~20 kDa by PNGase F treatment (see Fig.

![Fig. 1. Versican lectin domain binds specifically to fucose and GlcNac. Culture supernatants from versican-lectin-domain-expressing CHO cells were incubated with monosaccharide-conjugated beads, the beads were pelleted by centrifugation, and beads (B) and supernatants (S) were analyzed for versican lectin by SDS/PAGE and immunoblotting with anti-versican-lectin-domain-specific antibodies. The positions of molecular mass markers (in kDa) are on the left. The control lane contains untreated culture supernatant.](image-url)
were electrophoresed, transferred to nitrocellulose, and probed with biotinylated versican lectin domain (Vcl) in the presence of 5 mM CaCl$_2$ or EDTA. Bound lectin was detected with avidin–horseradish peroxidase, chemiluminescent substrate, and autoradiography. In the control lane, the lectin was omitted to show nonspecific binding by avidin–horseradish peroxidase. (B) As in A, but probed with versican ELC–IgG fusion protein and horseradish peroxidase-conjugated anti-human IgG antibodies. The control lane (IgG) was probed with human IgG instead of ELC–IgG.

5) are in agreement with the reported expression pattern of tenascin-R and the reduction of its molecular mass after removal of N-linked carbohydrates (18).

Immunoblotting of brain extracts fractionated by SDS/PAGE with either an anti-tenascin-R monoclonal antibody or the biotinylated lectin domain resulted in virtually identical staining patterns with reduced samples (Fig. 3A). When unreduced samples were probed, the lectin and the anti-tenascin-R antibody stained the same bands, but an additional band was detected with the antibody (Fig. 3B). This band may represent an alternative glycoform or splicing product (29) that is not reactive with the versican lectin domain and that is not seen in the reducing gel because of comigration with one of the other bands. Immunoblotting with anti-tenascin-C antiserum gave only weakly staining bands in the adult rat-brain extracts (data not shown); a strongly staining 230-kDa band and a weakly staining triplet between 210 and 220 kDa were seen in extract prepared from brains of 11-day-old rats (Fig. 34). The versican lectin did not bind tenascin-C in brain extracts prepared from either adult or 11-day-old rats (data not shown).

To confirm the identity of the gel bands detected with the versican lectin domain as tenasin-R, the ligand was precipitated from brain extracts by employing biotinylated versican lectin domain and streptavidin-conjugated agarose beads, and analyzed by immunoblotting. As shown in Fig. 4, the lectin domain-bound material reacted with anti-tenascin-R. No bands were detected in the lectin domain-bound fraction when EDTA had been added to the brain extract prior to the incubation with the lectin (Fig. 4, lane 3) or when the extract was incubated with beads without the lectin domain (Fig. 4, lane 4).

Chemical Deglycosylation of Tenascin-R Eliminates Versican Lectin Domain Binding. When all carbohydrates were removed from brain-extract proteins through chemical deglycosylation with TFMS, versican lectin domain binding to tenascin-R was abolished (Fig. 5). Incubation with PNGase F, which removes N-linked carbohydrates, reduced the apparent molecular mass of tenascin-R but did not decrease lectin domain binding (Fig. 5). Incubation with Endo H$_r$ had no effect on versican lectin binding, although concanavalin A staining of a parallel blot was decreased after the Endo H$_r$ treatment (data not shown). V. cholerae neuraminidase caused a slight reduction in the apparent molecular mass without affecting lectin domain binding (Fig. 5). The effect of TFMS did not seem to have been caused by degradation of the protein backbones in the extracts, as judged from Ponceau S staining of the blots (data not shown) and the continued presence of the 75- and 130-kDa avidin-binding bands in the TFMS-treated lane (Fig. 5). The size of the tenasin-R core protein in the TFMS treatment was evaluated by stopping the reaction after different incubation times. As shown in Fig. 6B, the versican

![Image](image_url)

**Fig. 2.** Calcium-dependent binding of versican lectin domain to 170- and 190-kDa proteins in rat brain extracts. (A) Rat-brain extracts were electrophoresed, transferred to nitrocellulose, and probed with biotinylated versican lectin domain (Vcl) in the presence of 5 mM CaCl$_2$ or EDTA. Bound lectin was detected with avidin–horseradish peroxidase, chemiluminescent substrate, and autoradiography. In the control lane, the lectin was omitted to show nonspecific binding by avidin–horseradish peroxidase. (B) As in A, but probed with versican ELC–IgG fusion protein and horseradish peroxidase-conjugated anti-human IgG antibodies. The control lane (IgG) was probed with human IgG instead of ELC–IgG.

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**Fig. 3.** Versican lectin domain ligand comigrates with tenasin-R. Rat-brain extract was boiled in SDS/PAGE sample buffer with (A; Reducing) or without (B; Nonreducing) 2-mercaptoethanol, electrophoresed, transferred, and probed with biotinylated versican lectin domain (Vcl), as in Fig. 2, with mouse monoclonal anti-tenasin-R antibody #596 (anti-TN-R), or with polyclonal anti-tenasin C (anti-TN-C). Tenasin-C staining in adult rat-brain extract was very weak; thus, the anti-tenasin-C lane in A shows staining in postnatal day 11 rat-brain extract, while the sample in all other lanes is adult rat-brain extract. The control lane shows the nonspecific staining by avidin–horseradish peroxidase in the absence of versican lectin domain.

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**Fig. 4.** Precipitation of tenasin-R by biotinylated versican lectin domain and streptavidin beads. Brain extract was incubated with biotinylated versican lectin in the presence of 5 mM CaCl$_2$ or 20 mM EDTA or without versican lectin (beads only). The lectin was then collected by addition of streptavidin-conjugated agarose beads, which were then washed and boiled in SDS/PAGE sample buffer containing reducing agent, and the solubilized sample was analyzed by immunoblotting with the anti-tenasin-R monoclonal antibody. Lane 1, brain extract only; lane 2, brain extract, lectin, CaCl$_2$ and beads; lane 3, brain extract, lectin, EDTA, and beads; and lane 4, brain extract, CaCl$_2$, and beads.
lectin domain binding to tenascin-R was lost after a 10-sec incubation in the acid, whereas anti-tenascin-R antibodies detected a band with the size of the intact core polypeptide even after 120 min (Fig. 6A).

**Versican and Tenascin-R Immunolocalization in Rat Cerebellum.** Immunofluorescence staining of rat cerebellum showed versican immunoreactivity predominantly in the granular layer and to some extent also in the white matter tracts (Fig. 7A), in agreement with previous reports (27, 30). Tenascin-R staining was mainly found in the granular layer, but staining could also be found in the molecular layer and to a lesser extent in the white matter (Fig. 7B), also in agreement with previous findings (18).

**DISCUSSION**

We show that the lectin domain of versican interacts with fucose and GlcNAc and that it binds specifically and in a calcium-dependent manner to tenascin-R. The carbohydrate binding of versican lectin domain was demonstrated in two ways. First, the lectin domain (as well as the ELC-IgG chimera; data not shown) bound to some, but not all, simple carbohydrates. This binding was dependent on divalent cations. Similar binding to simple sugars has been observed for the lectin domain of aggrecan, another member of the same proteoglycan family (12, 13), and recently also for the C-terminal part of the chicken homolog of versican (14). In the latter study, only a fragment containing the ELC was found to be active in carbohydrate binding, whereas a smaller fragment composed of the epidermal growth factor-like and lectin domains was inactive. The reason for the inactivity of the smaller fragment may be that, as a bacterially produced protein, it may not have folded correctly. We have attempted bacterial expression of the versican lectin domain in two different bacterial expression systems, but in both cases the product has been inactive (unpublished results). In contrast, the eukaryotic expression system used here yielded versican lectin domain that has carbohydrate-binding activity.

The second line of evidence supporting the function of the versican lectin domain as a lectin is the loss of binding to tenascin-R in chemically deglycosylated brain extract. Thus, the versican lectin domain would appear to be a lectin, the binding of which to tenascin-R is calcium dependent and carbohydrate directed, as is characteristic of C-type lectins (9). However, these data regarding the possible carbohydrate nature of the versican lectin binding site in tenasin R have to be interpreted cautiously. At least one C-type lectin, the IgE
receptor FcεR2, binds its ligand in a carbohydrate-independent manner (31). Moreover, the chemically deglycosylated tenascin-R could have lost its ability to bind versican lectin domain as a result of a modification in the protein backbone. Thus, whether the tenascin-R binding is purely carbohydrate mediated or might also depend on protein recognition remains to be determined.

The highly selective binding of the versican lectin to tenascin-R in brain extracts suggests that this interaction is physiologically significant. The similarity of the tissue localizations of versican and tenascin-R in cerebellum provides an additional indication of such significance. We found strong staining for both proteins in the granular layer of cerebellum and also detected both of them in the white matter. In addition, tenascin-R, but not versican, staining was observed in the molecular layer with the anti-tenascin-R used in this study. This antibody reacts with all of the isoforms of tenascin-R (18). As one of the isoforms of tenascin-R detected in nonreducing SDS/PAGE did not react with versican lectin domain, it is possible that one or more of the tenascin-R isoforms are not present in the molecular layer and that versican-binding tenascin-R codistributes with versican more completely than is apparent from our study.

The various proteoglycan lectin domains may have different ligand specificities and functions; the versican lectin domain bound to both fucose and GlcNAc, whereas aggregan lectin domain binds to fucose and galactose but only weakly to GlcNAc (12, 13). Thus, the saccharide-binding specificities of the proteoglycan lectins may be related but distinct. As neuronal may bind tenascin-C (15, 32, 33), versican and neurocan may each bind to a different member of the tenascin protein family. Both tenascins and proteoglycans have been classified as antiadhesive and have been found to be expressed in barriers of neuronal outgrowth in vivo (for review see ref. 34). Such specialized matrixes consisting of a proteoglycan, tenascin-family glycoprotein, and hyaluronan may be formed as a result of the lectin interactions described in this paper.

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