Characterization of the L1-Neurocan-binding Site

IMPLICATIONS FOR L1-L1 HOMOPHILIC BINDING

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The L1 adhesion molecule is a 200–220-kDa membrane glycoprotein of the Ig superfamily implicated in important neural processes including neuronal cell migration, axon outgrowth, learning, and memory formation. L1 supports homophilic L1-L1 binding that involves several Ig domains but can also bind with high affinity to the proteoglycan neurocan. It has been reported that neurocan can block homophilic binding; however, the mechanism of inhibition and the precise binding sites in both molecules have not been determined. By using fusion proteins, site-directed mutagenesis, and peptide blocking experiments, we have characterized the neurocan-binding site in the first Ig-like domain of human L1. Results from molecular modeling suggest that the sequences involved in neurocan binding are localized on the surface of the first Ig domain and largely overlap with the G-F-C β-strands proposed to interact with the fourth Ig domain during homophilic binding. This suggests that neurocan may sterically hinder a proper alignment of L1 domains. We find that the C-terminal portion of neurocan is sufficient to mediate binding to the first Ig domain of L1, and we suggest that the sushi domain cooperates with a glycosaminoglycan side chain in forming the binding site for L1.

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Proteoglycans are assumed to have important roles for modulating cell-cell and cell-matrix interactions during nervous tissue histogenesis in the embryonic and early postnatal phase (11, 12). L1 has been found to bind to the proteoglycan neurocan (13). Neurocan has been identified as one of the most abundant chondroitin sulfate proteoglycans in the developing rat brain (14). The extracellular matrix of the developing brain is particularly rich in chondroitin sulfate proteoglycans (15). These molecules have in various instances been implicated in the formation of barriers for cell migration and in the inhibition of neuronal outgrowth (16). However, in the thalamocortical system, L1 bearing thalamocortical afferent fibers were found to extend into neurocan-immunoreactive regions while growing through the proteoglycan-enriched cortical subplate (17). Earlier in vitro studies have shown that neurocan can inhibit neural adhesion and neurite outgrowth on L1-coated surfaces (14) and is capable of blocking the homophilic L1 interaction in aggregation assays using purified L1-coated covaspheres (18). However, the biochemical basis of this binding and the mechanism of inhibition have not been established.

A recent study has proposed a model for homophilic L1-L1 binding (19). By using the crystal structure of hemolin, a related member of the I class of Ig domain molecules, it was proposed that in the nonengaged resting state the N-terminal Ig domains adopt a horseshoe-like structure due to an intramolecular binding between domains 1 and 4 or 2 and 3, respectively (19). The backfolding of domains is facilitated by an amino acid spacer leading to a sharp bend between domains 2 and 3 (19). When engaged in homophilic binding between adjacent cells, L1 could undergo a conformational change leading to a pairwise antiparallel alignment of Ig domains 1–4 and 2–3 (19). In previous work we have studied the interaction of L1 with neurocan and integrins (20). In order to understand better the mechanism of inhibition of L1-L1 homophilic binding by neurocan, we have now examined the binding in more detail. Our results show that the first domain of L1 is the only binding site and that neurocan contacts the same site in the first domain used for interaction with the domain 4. We conclude that neurocan binding inhibits homophilic binding by preventing a proper domain alignment. We also demonstrate that the binding site in neurocan is located in the C-terminal part and that the sushi domain and a GAG side chain cooperate in the formation.

MATERIALS AND METHODS

Cell Culture—The mouse T cell hybridoma IH3-1-expressing cell surface neurocan, CHO cells, and CHO-L1 cells stably transfected with

1 The abbreviations used are: CHO, Chinese hamster ovary; CAM, cell adhesion molecule; GAG, glycosaminoglycan; IgSF, immunoglobulin superfamily; mAb, monoclonal antibody; PBS, phosphate-buffered
mouse L1 were described elsewhere (20, 21). IH3-1 cells were kept at 37 °C, 5% CO₂, and 100% humidity and were cultivated in RPMI supplemented with 10% fetal calf serum, 2-mercaptoethanol, and glutamine. CHO cells were kept in Dulbecco's modified Eagle's medium with 10% fetal calf serum and glutamine.

Construction of Recombinant Proteins—The construction and characterization of recombinant fusion proteins containing theFc portion of human IgG1 and the entire ectodomain (L1-Fc) or the Ig domains 1, 1–5, 1–6, and 6 of mouse L1 have been described (20). Additional proteins containing Ig domains 2–5, 2–6, or 5, the fibronectin type III domain (FNIII) repeats were constructed by PCR. Ig domains 2–5 were amplified using sense primer 5'-TGCAACCCTCCACCCAGTGCAGCCCCA-3' and reverse primer 5'-ATGATTTCCATCCACCCAGGAGCCCATGCTGGTTGCG-3' reverse primer. Ig domains 2–6 were amplified using the sense primer 5'-TGCAACCCCTCCAACATGTTGCGCCTTGGAG-3' and the reverse primer 5'-ATGATTTCCATCCACCCAGGAGCCCATGCTGGTTGCG-3'. For amplification of the FNIII repeats the sense primer was 5'-CTGCTGCGTCGTCGTCCTGCTT-3' and the reverse primer was 5'-ATGAATTCACTTACCGGCCACACAACTGTAGTTGCCCT-3'.

The amplified fragment was ligated to human Ig domain in pIg previously cut with HindIII and cloned into the PCR blunt cloning vector (Invitrogen). The amplified product was digested with HindIII and cloned into the PCR blunt cloning vector (Invitrogen). Conditions for mutagenesis PCR were 95 °C (30 s) for one cycle, 95 (30 s), 64 (30 s), and 70 °C (5 min) for 16 cycles, and one cycle at 72 °C for 10 min. Mutated cDNAs were digested with EcoRI and HindIII and reinserted into the plg vector. For construction of a human 1.L1-Fc domain expressing amino acids encoded by exon 2, cDNA from a neuronal cell line Kelly, which expresses exon 2, was used as template for PCR using primer 5'-CCCAAGCTTGCAAGAGCCAGGTGCACTTG-3' and reverse primer 5'-TCGTCACCCGCTCGTCCTGCTT-3'. The amplified product was digested with HindIII and BglII.

This fragment was ligated to human Ig domain in pIg previously cut with HindIII and BglII. All sequences and point mutations were confirmed by DNA sequencing. Plasmid DNA was transfected into COS-7 cells, and supernatants containing the Fc fusion protein were collected. The recombinant proteins were quantitated by ELISA and confirmed by DNA sequencing. Plasmid DNA was transfected into COS-7 cells, and supernatants containing the Fc fusion protein were collected. The recombinant proteins were quantitated by ELISA and confirmed by DNA sequencing.

For the analysis of L1-neurocan interaction, 0.5 μg of neurocan or its fragments were adsorbed to Immobilon membranes (Millipore) using a dot blot apparatus. Membranes were incubated with Fc fusion proteins at the indicated dilutions and the respective secondary antibodies followed by ECL detection as described above for Western blots. For the binding of CHO-L1 cells to neurocan fragments was performed in the absence or presence of 2 μg/ml laminarin sulfate. Glyceranase treatment of neurocan fragment D925AT was done in the presence of 1 unit/ml chondroitinase ABC lyase (EC 4.2.2.4) or heparinase III (EC 4.2.2.8) for 1 h at 37 °C as described before (20). After enzyme treatment an aliquot of the reaction was analyzed by Western blot analysis using a polyclonal antibody to neurocan. Remaining fragments with enzymes was done in the presence of 10 mM Hepes, 1 M NaCl, and used for the assay. For the adhesion assay, cells (5 × 10⁶) were suspended in Hanks' balanced salt solution containing 10 mM Hepes, and 0.2 ml aliquots were added to the coated slides for 30 min at room temperature without shaking. Slides were then fixed in 4% glutaraldehyde in PBS after briefly dipping into PBS. For peptide-blocking studies, IH3-1 cells were preincubated with peptides at the indicated concentration for 10 min at room temperature and then transferred to the chamber slides. Antibody-mediated blocking of adhesion was performed as described before (20). Binding of CHO-L1 cells to neurocan fragments was performed in the absence or presence of 2 μg/ml laminarin sulfate (kind gift from Dr. I. Vlodavsky, Hadassah Hospital, Jerusalem, Israel) where indicated. Cell binding was measured by counting six independent 10 × fields by video microscopy using IMAGE 1.47 software.

Analysis of Neurocan Binding by Dot Blot Immunoassay—For the analysis of L1-neurocan interaction, 0.5 μg of neurocan or its fragments were adsorbed to Immobilon membranes (Millipore) using a dot blot apparatus. Membranes were incubated with Fc fusion proteins at the indicated dilutions and the respective secondary antibodies followed by ECL detection as described above for Western blots. For the binding of IH3-1 cells to neurocan fragments was performed in the absence or presence of 2 μg/ml laminarin sulfate. Glyceranase treatment of neurocan fragment D925AT was done in the presence of 1 unit/ml chondroitinase ABC lyase (EC 4.2.2.4) or heparinase III (EC 4.2.2.8) for 1 h at 37 °C as described before (20). After enzyme treatment an aliquot of the reaction was analyzed by Western blot analysis using a polyclonal antibody to neurocan. Remaining fragments with enzymes was done in the presence of 10 mM Hepes, and 0.2 ml aliquots were added to the coated slides for 30 min at room temperature without shaking. Slides were then fixed in 4% glutaraldehyde in PBS after briefly dipping into PBS. For peptide-blocking studies, IH3-1 cells were preincubated with peptides at the indicated concentration for 10 min at room temperature and then transferred to the chamber slides. Antibody-mediated blocking of adhesion was performed as described before (20). Binding of CHO-L1 cells to neurocan fragments was performed in the absence or presence of 2 μg/ml laminarin sulfate (kind gift from Dr. I. Vlodavsky, Hadassah Hospital, Jerusalem, Israel) where indicated. Cell binding was measured by counting six independent 10 × fields by video microscopy using IMAGE 1.47 software.

RESULTS

The First Ig Domain Is the Only Neurocan-binding Site in L1—In a previous study we have shown that a binding site for neurocan is located in the first Ig domain of L1 (20). This conclusion was based on the observation that only L1-Fc fusion proteins containing the first domain could support neurocan binding. To investigate whether the first domain was the only binding site, we generated additional fusion proteins containing Ig domains 2–5, 2–6, or the five fibronectin type III repeats. The fusion proteins were purified and characterized as described before and revealed the expected sizes of 80 kDa (2–5.L1-Fc), 91 kDa (2–6.L1-Fc), and 96 kDa (FNIII-Fc) as described above. Dot intensities were quantitated by densitometric scanning using Lumi Imager (Roche Molecular Biochemicals).
As shown in Fig. 1B, strong binding of L1-Fc, 1–5.L1-Fc, and 1–6.L1-Fc to neurocan was observed in a concentration-dependent fashion. In contrast, no binding was detected with 2–5.L1-Fc, 2–6.L1-Fc, and FNIII-Fc proteins. Collectively, these data suggested that the first Ig domain was the only neurocan-binding site in mouse L1.

Two pathological mutations in the first Ig domain of human L1 have been studied (24, 25). To examine the effects of these mutations on neurocan binding, we constructed mutated forms of human 1.L1-Fc proteins. IH3-1 cells were allowed to adhere to human L1 Fc proteins captured with preimmobilized anti-human IgG-Fc antibody. P-Selectin Fc-protein was used as negative control. The inset shows an ECL blot of the purified Fc proteins.
TABLE I  
Identification of potential L1 binding domains in neurocan

| Construct | Binding of neurocan |
|-----------|---------------------|
| L639      | 94 ± 7.1            |
| 773M      | 5 ± 0.8             |
| D925      | 96 ± 12.6           |
| D925S     | 1 ± 0.1             |
| D925T     | 88 ± 6.3            |
| NCAL      | 56 ± 8.2            |

YEGHH motive was present. As shown in Fig. 2B, the presence or absence of this motive did not influence the neurocan-mediated binding of IH3-1 cells. In contrast, cell adhesion was reduced by approximately 71% in the L120V mutant and 43% in the G121S mutant, respectively (Fig. 2B). Equal coating of the wells with recombinant proteins was controlled in parallel by performing an anti-human Fc specific ELISA (data not shown).

Mapping of the Neurocan-binding Site Using b-Strand Peptides—To map closer the neurocan-binding site in the first Ig domain, peptide inhibition studies were employed. Peptides of variable length were selected on the basis of the predicted folding of the L1 first Ig domain. We concentrated on peptide sequences expressed on b-strands as originally proposed by Su et al. (19) based on the crystal structure of hemolin. The sequence and localization of the selected peptides is depicted in Fig. 3A. As shown in Fig. 3B, at 2 mM peptide concentration none of the peptides affected the integrin-mediated binding of IH3-1 cells to immobilized vitronectin used for control purposes. In contrast, the neurocan-mediated adhesion to the human L1-L1-Fc was clearly inhibited by peptides C and G (see Fig. 3C). Additional experiments shown in Fig. 3D demonstrated that the two peptides inhibited the binding of IH3-1 cells to L1-L1-Fc in a dose-dependent fashion, whereas peptide F showed only a weak inhibitory effect. The control peptide did not reveal any inhibition.

To visualize the topography of b-strands and point mutations affecting L1-neurocan binding, the first Ig domain of L1 was subjected to computer-based modeling using molecular coordinates from hemolin (19). As shown in Fig. 4, the results from this study indicated a close proximity of the mutations and strands G and C. The b-strands C, F, and G in the first Ig domain have been proposed to be involved in the intramolecular and intermolecular interaction of domains 1 and 4 (19). Thus, neurocan binding to the first Ig domain can be expected to block partially these contact sites and thereby affect homophilic L1-L1 binding.

Identification of the L1 Binding Region in Neurocan—To localize the potential L1-binding site in the neurocan molecule, we used recombinant neurocan forms differing in domain composition (see “Materials and Methods”). A schematic representation of the fragments is given in Fig. 5. The fragment 773M contains the N-terminal half of neurocan (amino acid 1–773) and was expressed in insect cells. The fragment L639 starts with amino acid leucine in position 639 and represents the C-terminal half of the molecule. The D925 series of constructs start with aspartic acid in position 925. D925 is composed of the last 25 amino acids of the central region, including the last potential GAG attachment site at serine 944, and the complete C-terminal domain. The D925AS construct included a stop codon after threonine 1153 and after arginine 1216, respectively, resulting either in a deletion of the sushi domain together with the appending non-homologous C-terminal tail of 40 amino acids or in a deletion of just the appending tail (Fig. 5). Both truncated molecules were modified with chondroitin sulfate chains and secreted in similar amounts by transfected HEK 293 EBNA cells (23). The NCAL construct was deficient for the second EGF module and the C-type lectin module but contained the sushi domain and the appending tail (Fig. 5). This molecule was modified with chondroitin sulfate chains and secreted in similar amounts as recombinant wild type neurocan (23).

We studied first the binding of the fragments to human L1-L1-Fc protein using a dot blot immunoassay. The neurocan fragments (0.5 mM) were adsorbed to an Immobilon membrane which was then incubated with the human L1-L1-Fc fusion protein followed by an anti-human Fc-specific antibody for detection. As shown in Table I, high binding to the protein fragments D925 and L639 nearly reaching the level of full-length neurocan (100% control) was observed. The 773M fragment interacted only weakly with L1-L1-Fc (~5% of control) suggesting that the potential binding site for L1 was located C-terminally between residues 774 and 1257. This C-terminal part of neurocan consists of a non-homologous domain followed by two EGF modules, a C-type lectin module ending with a sushi module and the appending non-homologous tail.

The C-terminal deleted fragment D925AS was deficient for the sushi module, did not bind to the L1-L1-Fc protein (1%) whereas the construct D925AT did interact (88% binding of control). The NCAL fragment showed decreased binding of 56% relative to the control (Table I). Both constructs (D925AT and NCAL) contain the sushi module (Fig. 5). From these results it was concluded that the molecular interaction between L1 and neurocan is dependent on the presence of the sushi module located in the C terminus of neurocan.

Cooperation of Sushi Motive and a GAG Side Chain—To corroborate the results obtained from the molecular interaction of L1 and neurocan fragments, we analyzed the binding of CHO-L1 cells, stably transfected with mouse L1 (21) to full-length neurocan and its C-terminal fragments D925, D925AT, and D925AS. Whereas the full-length neurocan, the D925, and the D925AT fragment mediated adhesion of CHO-L1 cells nearly to the same level, the D925AS fragment deficient for the sushi module did not support the binding of CHO-L1 cells (Fig. 6). Non-transfected CHO cells failed to adhere to any of the substrates (data not shown). The adhesion of CHO-L1 cells to full-length neurocan or the D925 and D925AT fragments, respectively, was reduced by ~90% in the presence of 2 mM laminarin sulfate (Fig. 6) which is a linear polymer consisting of 1,3-β-glucan derived from the wall of seaweed and modified by chemical O-sulfation (27). These results corresponded to our previous data showing that sulfated polysaccharides like heparin and laminarin sulfate can inhibit neurocan binding to L1 (20).

To analyze whether the binding of 1.L1-Fc protein was dependent on the presence of an intact GAG, the D925AT construct was pretreated with chondroitinase ABC or for control with heparanase III before use as substrate in the dot blot immunoassay. As shown in Fig. 7A, a core protein of ~44 kDa was detected by Western blot analysis following chondroitinase ABC but not heparanase III treatment in agreement with previous observations (23). The ability to bind to the membrane and the amount of the D925AT construct was not affected by enzymatic treatment as shown by the reactivity with the C-terminal fragment-specific antibody 1D1 (see Fig. 7B, inset). As shown in Fig. 7B, only treatment with chondroitinase ABC
reduced the binding of the 1.1L1-Fc protein, whereas heparinase III treatment had no effect. Consistent with the results presented in Fig. 6, the presence of laminarin sulfate at 1 μg/ml during the assay decreased binding also. Collectively, the data suggested that both the sushi module and the GAG side chain in position 944 were necessary for the binding to L1.

DISCUSSION

In the present report we have carried out a detailed analysis of the interaction between L1 and neurocan in order to map the contact sites in both molecules. We previously localized a neurocan-binding site in the first Ig domain of L1 (20). We now present evidence that this domain is the only neurocan-binding site in L1 as none of the L1-Fc fusion proteins devoid of the first domain were capable of supporting neurocan interaction. This was evident both when neurocan was offered in solution or in a cell surface-bound form when using IH3-1 cells in adhesion assays. Inhibition studies in the latter assay system using β-strand peptides revealed amino acid motives in the first Ig domain of L1 likely to be relevant for the neurocan interaction. Peptides derived from the C and G β-strands were the only ones giving a clear-cut dose-dependent inhibition. A number of mutations in the human L1 gene product are associated with hereditary brain malformations and neurological disorders (5, 24, 25, 28, 29). Two of these mutations are localized in the first Ig domain of L1. We generated recombinant first Ig domain Fc fusion proteins containing the L120V and G121S mutations, respectively. These mutations significantly reduced the binding of IH3-1 cells when compared with the wild type form. To localize the amino acid sequences involved in neurocan binding, the first Ig domain was subjected to molecular modeling based on the crystal structure of hemolin (19). Hemolin is a member of the IgSF, containing four Ig-like domains (30). Analysis
ysis of the crystal structure has revealed that the four domains adopt a horseshoe-like structure due to antiparallel alignment of the domains (19). In the crystal the Ig domains 1 and 4 or 2 and 3, respectively, were engaged in intramolecular binding (19). The first four Ig domains of the L1 family members may also adopt a horseshoe structure similar to that observed for hemolin as many of the key structural residues are conserved between these proteins (19). In fact, a recent study in which the crystal structure of the first four domains of the L1 family member axonin-1/TAG-1 was revealed confirmed the presence of a horseshoe structure (31). Our model of the first Ig domain of human L1 based on hemolin revealed that the G and C β-strand peptides as well as the two point mutations affecting neurocan binding were located at one side of the surface suggesting the formation of a contact area. Su et al. (19) have observed for hemolin that the G-F-C β-strands of the first domain form the contact site with the fourth Ig domain in the horseshoe structure configuration (19). Assuming similar type of domain interactions for human L1, our results predict that the very same β-strands in the first domain are involved in both neurocan and fourth Ig domain interaction.

Although the horseshoe configuration may be a common feature of certain neural CAMs of the IgSF, the way homophilic interaction is mediated is not well understood. For hemolin it was proposed that transient occurrence of an open form would lead to the formation of domain-swapped dimers or multimers through homophilic interactions with open proteins on an adjacent cell (19). However, in the axonin-1/TAG-1 crystal two of the U-shaped molecules arrange with antiparallel long axes so that the edge of one molecule contacts the face of the other (31). This interaction is brought about by a loop from domain 3 contacting a central hole formed by residues provided by domains 2 and 3 in the adjacent axonin-1 molecule. Thus, it was proposed that axonin-1-mediated cell-cell adhesion involves a zipper-like string of domains in a horseshoe conformation (31). Whatever model will turn out to be correct, it seems clear that neurocan can only bind to the first domain when the binding site is not hidden by domain 4, i.e. most likely in the open form. Our results are therefore in agreement with the notion that an open conformation indeed exists. As outlined in Fig. 8, we propose that the inhibition by neurocan is most likely mediated by preventing a crucial interaction with the fourth Ig domain due to competitive binding. The remaining interactions between domains 2 and 3 and the still possible contributions of other sites in the molecule may be either too weak to support homophilic adhesion or are sterically hindered by the bulky neurocan molecule. In addition, due to neurocan binding the open form is removed from the equilibrium as refolding into the horseshoe conformation is blocked. Another possible model in which neurocan prevents a conformational change by locking the horseshoe-like structure seems less likely as this would possibly require the interaction of neurocan with more than one L1 domain.

It has become clear from several studies that homophilic L1 binding is mediated by more than one Ig domain (25, 32, 33). Recent studies on the molecular analysis of point mutations found in the L1 gene product of MASA (mental retardation, adducted thumbs, shuffling gait, aphasia) patients have shown

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**FIG. 4. Molecular modeling of the first Ig domain of human L1.** The first Ig-like domain of human L1 was modeled according to the domain folding of hemolin (19). β-Sheets as predicted from the amino acid sequence are indicated by letters. The positions of point mutations are given in black. The locations of blocking peptides are given in red and blue.

**FIG. 5. Schematic presentation of recombinant neurocan and its fragments.** Different neurocan domains are marked by hatched or shaded boxes. Positions of potential N-glycosylation and GAG side chains are marked by black and white arrows. Amino acid positions are given according to the scale of full-length neurocan.

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**Neurocan-binding Sites in L1**

Recent studies on the molecular analysis of point mutations found in the L1 gene product of MASA (mental retardation, adducted thumbs, shuffling gait, aphasia) patients have shown...
that a surprisingly high number of mutations scattered along the molecule can affect homotypic and heterotypic binding of L1 indicating that several extracellular domains are required (25). In this study also the two mutations G121S and L120V, respectively, were analyzed (25). It was found that the G121S mutation strongly reduced homophilic and heterophilic binding of L1 to L1, F11 and axonin-1, whereas the L120V mutation did not have a significant effect compared with wild type L1 (25). The G121S mutation was also predicted to alter the structure of the first Ig domain as it affects a key residue required for correct folding (24). In contrast, the L120V mutation was proposed to exert its effect on L1 function by affecting mRNA splicing rather than disruption of ligand interactions (25). In our hands, both mutations reduced the neurocan-dependent adhesion of IH3-1 cells to the 1.L1-Fc substrate suggesting a disturbance of the integrity of the first Ig domain by the mutations. This suggests that if expressed as protein, the L120V mutant might only affect neurocan binding but not homophilic or heterophilic binding. However, it has to be kept in mind that we studied the neurocan interaction with the isolated first Ig domain, whereas the homophilic and heterophilic analysis by De Angelis et al. (25) was carried out with recombinant proteins representing the entire L1 ectodomain.

L1 is expressed in two alternatively spliced forms as follows: a neuronal one, containing exon 2 coding for amino acid sequence YEGHH, and a non-neuronal form deficient for this exon (26). Exon 2 precedes the first Ig domain of L1. To answer the question whether the presence of exon 2 had an effect on the interaction of the predominantly neuronally expressed proteoglycan neurocan with L1, we generated human first Ig domain proteins representing the entire L1 ectodomain.

Neurocan belongs to the group of structurally related proteoglycans of the aggrecan family that are characterized by an N-terminal hyaluronan binding domain, a mucin-like middle part of variable length and numbers of chondroitin sulfate and glycosaminoglycan attachment sites, and a C-terminal domain with epidermal growth factor, C-type lectin, and complementarity (or sushi) modules (34). Interaction of neurocan with cell surface glycosyltransferase (Gal-Nac phosphotransferase) is restricted to the N-terminal domain of neurocan containing one Ig and two link modules (35), whereas Tenascin-C interacts with the C-terminal domain (36). Whereas axonin-1/TAG-1 interacts as well with the core protein of neurocan (37), the interaction of L1/Ng-CAM was shown to be strongly reduced after the chondroitin sulfate chains had been removed by chondroitinase, and the interaction with N-CAM was almost eliminated by this treatment (14). In these studies the C-terminal half of neurocan was shown to have binding properties essentially identical to those of the full-length proteoglycan (14). Nevertheless, a detailed localization of the L1-binding site in neurocan is still missing. In the present study we provide evidence that the C-terminally located sushi module is necessary for the L1 neurocan interaction. L1-mediated cell adhesion of CHO-L1 cells to eukaryotically expressed recombinant neurocan fragments was only observed in the presence of the sushi module. The missing of the sushi module in the C-terminal D925S fragment of neurocan reduced the adhesion by 99%. The presence of the sushi module in the D925AT fragment restores adhesion to 88% of the full-length neurocan. Inhibition of D925AT-L1 interaction by the sulfated polysaccharide laminarin sulfate indicated that L1 binding was also dependent on GAG side chains. Indeed, removal of the GAG side chain of D925AT by chondroitinase treatment reduced the binding of 1.L1-Fc by 92% compared with the non-treated construct. Since it has recently been reported that the chondroitin sulfate modification of versican fragments consisting of parts of the central and C-terminal region that were recombinantly produced in

![Figure 6](image6.png) Binding of CHO-L1 cells to immobilized neurocan subfragments. Adhesion of CHO cells stably transfected with mouse L1 (CHO-L1) in the presence or absence of 2 μg/ml laminarin sulfate to recombinant neurocan and its C-terminal fragments coated at 10 μg/ml.

![Figure 7](image7.png) Function of the neurocan GAG side chain in position 944 for L1 binding. A, Western blot analysis of neurocan fragment D925AT after chondroitinase ABC or heparinase III treatment. Note that the appearance of the ~44-kDa core protein following chondroitinase ABC treatment. B, dot blot analysis of human 1.L1-Fc-protein binding to neurocan fragment D925AT following chondroitinase ABC, heparinase III treatment, or in the presence of 2 μg/ml laminarin sulfate. Binding in medium without treatment was set as 100% control. Dot intensities are represented as percent binding compared with medium control. Equal amounts of D925AT fragment on the blot were demonstrated in parallel with mAb 1D1 to the C terminus of neurocan (see inset).
COS-7 cells were dependent on the sushi (complement-binding-protein-like) module (38), it should be mentioned again that both neurocan fragments, 925T and 925AS, secreted by HEK 293 cells were modified with chondroitin sulfate chains to a similar extent (23) and have actually been recovered from conditioned medium by DEA/E-ion exchange chromatography. Therefore, we conclude that both the sushi module and the GAG side chain are important for the interaction with L1. We propose that these structures cooperate to form a binding site for the first L1 Ig domain.

Sushi modules, also called complement regulatory modules, since they were observed in various regulatory components of the complement system, are found in a number of proteins from vertebrates and invertebrates as well as viruses, sharing the common feature of binding to other proteins. It appears likely that sushi structures function as protein-binding modules (for review see Ref. 39). Nevertheless, the function of the domains is still not known. It seems possible that basic amino acid residues in the sushi domain play a role in binding to GAG chains since it was observed that in the C-terminal domain of versican the sushi domain is important for heparin binding (40). The sushi module might stabilize and orient the negative charged GAG side chain in position 944 and thereby contribute to the generation of a binding site for L1.

Chondroitin sulfate proteoglycans are assumed to have fundamental functions during brain development shown by the expression pattern during brain development and the regulation of cell migration (41–44). The interaction of neurocan but also of phosphacan with CAMs like N-CAM and L1 are important events for modulation of cell adhesion as well as axonal outgrowth and pathfinding (17, 45–47). The coexpression of LN/GnCAM and neurocan in concert with N-CAM during cerebellar histogenesis together with the fact that neurocan inhibits its neural adhesion and neurite outgrowth on L1 (14), but does not inhibit cell adhesion on laminin or tenasin-C-coated surfaces (48), suggests that binding of neurocan generates transmembrane signals to the cell influencing cellular properties like adhesion and migration. The identification of binding sites in both L1 and neurocan is an important step to advance our understanding how this interaction can regulate neurological processes.

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