**Deleted in Colorectal Carcinoma (DCC) Binds Heparin via Its Fifth Fibronectin Type III Domain**

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DCC (deleted in colorectal carcinoma) is a broadly expressed cell-surface receptor. Netrin-1 was recently identified as a DCC ligand in brain, but the possibility of other DCC ligands was suggested by the finding that an anti-DCC antibody (clone AF5) neutralized netrin-1-dependent commissural axon outgrowth without blocking DCC/netrin-1 interactions. Here we have searched for alternative cell-surface DCC ligands. A DCC-Ig fusion protein bound to neural and epithelial derived cell lines, indicating that these lines express ligand(s) for DCC. The cell-surface binding activity was mediated by the loop between β-strands F and G of the fifth fibronectin type III repeat FNIII-D5. The loop included the sequence KNRR, which resembles heparin-binding motifs in other proteins. Heparinase and heparitinase treatment of cells reduced binding of DCC-Ig, suggesting that heparan sulfate proteoglycans are cell-surface DCC ligand(s). This was further supported by heparin blocking experiments and by binding of DCC-Ig to immobilized heparan sulfate. The interaction between DCC-Ig and heparan sulfate/heparin, both on the surface of cells and immobilized on plastic, was blocked by the same anti-DCC antibody that blocks netrin-1-dependent commissural axon outgrowth. Taken together, these findings suggest that the DCC-Ig/heparin interaction may contribute to the biological activity of DCC.

Allelic deletions on chromosome 18q in >70% of primary colorectal tumors prompted the search for a tumor suppressor gene at that locus. An early result of this search was the cloning of a putative cell-surface receptor, DCC (deleted in colorectal carcinoma) (1). While the cloning of DCC brought much excitement that a tumor suppressor gene responsible for many colorectal cancers had been identified, 7 years later, the evidence that DCC is the putative tumor suppressor located on chromosome 18q remains inconclusive. Nonetheless, a role for DCC in tumor progression is suggested by the large number of different tumor types that have been reported to have lost DCC expression, including carcinomas of the pancreas, breast, prostate, bladder, and stomach; leukemias; neuroblastomas; and gliomas. The evidence for DCC as a tumor suppressor was examined in a recent review by Fearon (2).

While the question of DCC as a tumor suppressor is still being debated, understanding of the normal physiological role of DCC has moved forward. Recent studies provide biochemical, functional, and genetic data suggesting that DCC is a receptor for the diffusible neural chemotractant netrin-1 (3–5). Netrin-1 bound specifically to cells expressing DCC, and DCC mediated netrin-1-dependent outgrowth of commissural axons from dorsal spinal cord explants. This outgrowth was blocked by an anti-DCC mAb that did not block the interaction between netrin-1 and DCC, suggesting that the interaction between netrin-1 and DCC may require additional factors. Furthermore, genetic analysis of UNC-40, a DCC homolog from *Caenorhabditis elegans*, suggests that there are several developmental functions attributed to UNC-40 that do not require netrin-1 (UNC-6). Taken together, these data suggest that while DCC/netrin-1 interactions are important, all the components for this guidance system have yet to be identified, and the functional role of DCC is not fully understood.

A functional role of DCC in epithelial cells has also been suggested. Chuong et al. (6) showed that a Fab fragment of an anti-DCC mAb disrupted normal dermal condensation during feather bud formation in an embryonic chicken dorsal skin explant culture, a process that involves epithelial/mesenchymal cell interaction (6). In addition, the same mAb blocked aggregation of stage 34 (embryonic day 8) skin epithelial cells. These findings suggested that DCC participated in Ca²⁺-independent cell/cell interactions.

DCC encodes a transmembrane protein with an extracellular domain composed of four Ig C2-like repeats, six fibronectin type III (FNIII)-like repeats, a single membrane-spanning region, and a 325-amino acid cytoplasmic domain (7). This complicated extracellular domain structure of DCC provides many candidate domains for mediating intracellular interactions. In this study, we set out to identify counter cell-surface DCC ligand(s) by using a DCC-Ig fusion protein, which binds to neural and epithelial derived cell lines. We have further demonstrated that the molecular basis for this interaction is mediated by binding of the fifth FNIII domain of DCC to heparin and/or heparan sulfate proteoglycans.

**MATERIALS AND METHODS**

**Cell Culture—**NMUTI cells were kindly provided by Al Klingelhutz (8) and grown in keratinocyte-SFM medium (Life Technologies, Inc.). The SW-1088 (human astrocytoma), U138-MG (human glioblastoma), and I-407 (human intestinal epithelial) cell lines were purchased from American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Construction and Expression of DCC-Ig Fusion Proteins—**Expression vectors containing DCC extracellular domains were generated by the polymerase chain reaction (PCR) method using a DCC cDNA generously provided by Kathy Cho (7) as a template. The PCR primers

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1 The abbreviations used are: mAb, monoclonal antibody; FNIII, fibronectin type III; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; HS, heparan sulfate; FGF, fibroblast growth factor.
DCC Binds Heparin

Identification of the DCC Domain Responsible for Binding to the Cell-surface Ligand—We next sought to localize the domain of DCC responsible for the binding. Initially, two fusion proteins were expressed, one that included all four immunoglobulin domains (IgD-Ig) and one with six fibronectin type III domains (FNIII-D-Ig) (see Fig. 1). These fusion proteins were then used for binding analysis of SW-1088, U138-MG, and NMYUTI cells. Shown in Fig. 4A are flow cytometry profiles of stained NMYUTI cells. Only binding of FNIII-D-Ig was detectable, indicating that the binding site for cell-surface ligand(s) is contained within the FNIII domains. Similar results were obtained with U138-MG and SW-1088 cells (data not shown).

To identify the FNIII domain that mediates the cell binding, each of the six fibronectin type III repeats were independently expressed as fusion proteins (Fig. 1). All six fusion proteins were tested for binding to U138-MG and NMYUTI cells. Identical results were obtained with both cell lines. As shown in Fig. 4B, NMYUTI cells bound only to FNIII-D-Ig. Thus, the DCC binding site mapped to the fifth FNIII domain.

An 11-Amino Acid Loop Is Responsible for Binding of DCC to the Cell-surface Ligand—Inspection of the sequence alignment of the DCC FNIII domains revealed two stretches of 11 and 13 amino acids that had diverged in the fifth FNIII repeat (Fig. 5A) (7). One of the areas of divergence corresponded to the RGD loop in the tenth FNIII domain in fibronectin, which provides for the interaction between fibronectin and some integrins (11).
The crystal structure of the fibronectin domain containing the RGD motif shows the RGD loop protruding between β-strands F and G (12). This suggested that the corresponding region in DCC was a candidate for a binding region responsible for interacting with the DCC ligands. To test this hypothesis, two new DCC fusion proteins were expressed (Fig. 5B). One contained the 11 divergent amino acids from FNIII-D5 swapped into the FNIII-D1-Ig fusion protein, replacing the corresponding 11 amino acids (FNIII-D1-D5-Ig). The second consisted of FNIII-D5-Ig with the 11 amino acids from FNIII-D1 (FNIII-D5-D1-Ig).

These two fusion proteins were analyzed for binding to both SW-1088 and NMUTI cells (Fig. 6). While FNIII-D5-D1-Ig bound weakly (Fig. 6C), FNIII-D1-D5-Ig showed strong binding to the DCC ligand(s) in NMUTI cells (Fig. 6D). Identical results were found when staining SW-1088 cells. These data indicate that the 11 amino acids between β-strands F and G contain the sequences responsible for mediating DCC/ligand interactions.

Identification of DCC/HS Interactions—Since the DCC ligands were broadly distributed in many cell lines, we reasoned that it might be a known molecule. One candidate was heparin, which binds FNIII domains in both fibronectin and tenascin C. Consistent with this possibility was that embedded in the 11-amino acid loop was the sequence KNRR, a sequence reminiscent of other heparin-binding motifs.

To test if heparin and/or HS was a DCC ligand, NMUTI cells were treated with chondroitin ABC lyase or with a mixture of heparinase and heparitinase and then stained with the DCC-Ig fusion protein (Fig. 7, A and B). Chondroitin ABC lyase had a minimal effect on binding, whereas the binding activity was lost after heparitinase and heparinase treatment. In addition, binding of DCC-Ig to NMUTI cells was blocked by increasing concentrations of heparin (Fig. 7C). Chondroitin sulfate A had much less effect on binding. The partial blocking by chondroitin sulfate A is not surprising due to the similarities of the composition of the two glycosaminoglycans. These experiments suggest that DCC binds to cell-surface HS proteoglycans.

**DCC-Ig Binds Immobilized Heparan Sulfate**—To prove that DCC binds heparin and/or HS, we next tested whether DCC fusion proteins bound to immobilized HS. Binding of the fusion proteins to immobilized HS was detected with horseradish peroxidase-conjugated anti-human Ig antibodies. The fusion proteins that bound to the immobilized HS mirrored what was seen with cell-surface binding: FNIII-D1-Ig did not bind, whereas DCC-Ig, FNIII-D5-Ig, and FNIII-D1-D5-Ig all bound HS in a concentration-dependent fashion.
We next tested if this interaction between immobilized HS and DCC-Ig was blocked by the anti-DCC mAb. As shown in Fig. 8B, increasing concentrations of a control IgG1 antibody did not specifically block the DCC/HS interaction, but increasing concentrations of the anti-DCC antibody blocked the interaction. Taken together, these results demonstrate that DCC is a heparin-binding protein and that the anti-DCC mAb specifically blocked the interaction.

**DISCUSSION**

We have shown that DCC binds to HS/heparin and that an anti-DCC mAb (clone AF5) blocks the interaction. We mapped the DCC HS-binding sequence to an 11-amino acid loop in the fifth FNIII domain that separates β-strands F and IgG. Swapping the 11 amino acids from FNIII-D5 into FNIII-D1 conferred HS/heparin binding activity. Within this 11 amino acids is the sequence KNRR, a sequence reminiscent of Cardin and Wein...
traub (13) sequences (BBXB and BBXXB) that were proposed to be predictive of heparin-binding proteins.

Heparin-binding motifs within FNIII domains are found in two matrix proteins, tenascin C and fibronectin (14, 15). The fibronectin sequence responsible for binding to HS proteoglycans was identified by peptide-cell binding studies. The hepa-
A binding peptide sequence (SEPLIGRKKT) encompassing \( \beta \)-strand G was shown to contain the required sequence, RKK. The tenascin C sequence responsible for the interaction has not been demonstrated, but a FNIII domain responsible for HS binding was identified, and a consensus heparin-binding motif \((B^X^B^X^B^X^X^X^B)\) was located in the sequence. A model of this FNIII domain placed the heparin-binding motif in the loop between \( \beta \)-strands F and G, with the final basic amino acid on the G strand face. We show here that the corresponding sequence in DCC contains a heparin-binding motif.

Removing the 11 amino acids from DCC FNIII-D5-Ig and replacing them with the 11 amino acids from FNIII-D1 resulted in a loss of most (but not all) of the binding activity. This suggests that other sequences in the fifth FNIII domain also contribute to the DCC/HS interaction. An examination of the crystal structure of a four-domain segment of fibronectin shows that the loop between \( \beta \)-strands C and B is in close spatial proximity to the HS-binding loop and contains a potential heparin-binding motif, KNQK (12).

HS/heparin binding by many proteins has been demonstrated to provide a critical role in their biological function. For example, it has been established that HS/heparin is required to fully activate the basic FGF receptor, but the mode of interaction between basic FGF, the FGF receptor, and HS/heparin is poorly understood. HS/heparin binds directly to the FGF receptor (16). This binding requires a 2-O-sulfate group on the glycosaminoglycan backbone, whereas induction of a mitogenic response requires the presence of both 2-O- and 6-O-sulfation (17). In addition, heparin alone can activate FGF receptor 4; ligand binding is not required (18). Binding of FGF family members to heparin promotes receptor dimerization, a requirement for receptor activation (19).

The finding that a cell-surface DCC ligand was HS/heparin suggested two possible functions for DCC: (i) DCC is a receptor that mediates cell/cell interactions and/or cell/matrix interaction by binding HS/heparin, or (ii) DCC is a receptor for a...
soluble HS/heparin-containing or -associated molecule. In support of the first possibility is the fact that there are many other cell-surface receptors that bind HS (12, 20). These receptors collectively contribute to cell-surface HS interactions, and DCC may add to these interactions.

Support for the role of DCC as a receptor for a soluble heparin-containing or -associated molecule comes from the previous demonstration that netrin-1 can bind to cell lines expressing DCC (4). Netrin-1 is a heparin-binding chemoattractant, capable of providing long-range guidance cues to commissural axons in the spinal cord along a circumferential pathway from the dorsal spinal cord to floor plate cells at the ventral midline. Tessier-Lavigne and co-workers (4) demonstrated that while netrin-1-dependent axon outgrowth of spinal cord explants was blocked by an anti-DCC mAb, the mAb did not block DCC/netrin-1 interactions. We have shown here that the same mAb does block DCC/heparin binding. The heparin binding may stabilize DCC/netrin-1 interactions and locally concentrate netrin, especially since netrin-1 also binds HS/heparin (4). The blocking of the biological function of DCC and heparin binding by mAb AF5 suggest an important biological role for interactions between DCC and HS/heparin. HS/heparin binding may be required to activate the netrin-1 receptor (DCC).

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