

## Netrin Binds Discrete Subdomains of DCC and UNC5 and Mediates Interactions between DCC and Heparin\*

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**Netrins are secreted proteins that elicit both attractive and repulsive responses in migrating cells in the central and peripheral nervous systems. Netrins interact with members of two distinct families of transmembrane receptors, represented by DCC (deleted in colorectal cancer) and UNC5. A human netrin fragment (soluble netrin; sNetrin) was purified from an engineered Chinese hamster ovary cell line and used in a pull-down assay to map the interactions between netrin and its receptors. We find that sNetrin binds exclusively to the fifth fibronectin type III repeat of DCC and to each immunoglobulin repeat of UNC5. Both DCC and UNC5 bind to sNetrin with 1:1 stoichiometry in solution, and the minimal receptor fragments behave similarly to larger fragments in cross-linking experiments with purified sNetrin. We find no evidence for formation of a ternary complex between sNetrin and soluble forms of DCC and UNC5. We also find no evidence for an interaction between DCC and heparin and instead demonstrate that a loop on the fifth fibronectin type III repeat of DCC previously implicated in mediating interactions with heparin is important for sNetrin binding. Since netrin binds heparin, our results suggest that interactions between DCC and heparin are probably mediated by netrin.**

Netrins are secreted proteins that elicit both attractive and repulsive responses in extending axonal processes and migrating cells in the central and peripheral nervous systems (1–7). Members of two distinct protein families, represented by DCC (deleted in colorectal cancer) and UNC5, bind netrin with nanomolar affinity and transmit netrin-dependent signals across the membranes of migrating neural cells (8, 9). In addition, both netrin and DCC have been reported to bind heparin (8, 10, 11). Netrin, DCC, and UNC5 are all composed of multiple domains that are homologous to those found in other extracellular proteins (12) (Fig. 1). Netrin family members share an N-terminal Type VI laminin repeat (domain VI), followed by three cysteine-rich epidermal growth factor modules (domains V1, V2, and V3, respectively), and a positively charged C-

terminal domain. DCC family members are type I glycoproteins with extracellular regions containing four Ig repeats followed by six type III fibronectin repeats (Fn).<sup>1</sup> Members of the UNC5 family are also single-pass transmembrane proteins with extracellular regions consisting of two Ig repeats followed by two thrombospondin type-I modules.

Genetic and biochemical studies indicate that in general DCC mediates attractive responses to netrin, whereas UNC5 is required for netrin-mediated repulsion (8, 13–15). DCC mediates attractive responses in the absence of UNC5, but the presence of UNC5 is sufficient to convert DCC-mediated attraction to repulsion (16). In some cases, UNC5-mediated repulsion requires the presence of DCC (16), but other studies suggest that UNC5 alone is capable of mediating repulsion (17).

Netrin signaling requires the cytoplasmic regions of DCC and UNC5, both of which appear to function by recruiting signaling/adaptor proteins (16, 18, 19). Attractive responses require netrin-induced dimerization of the cytoplasmic region of DCC, whereas repulsion arises from netrin-induced association of the DCC and UNC5 cytoplasmic regions (16, 20). In the absence of the extracellular domains of the receptors, the cytoplasmic region of DCC interacts constitutively with itself and with the UNC5 cytoplasmic region (16, 20). These interactions, however, are repressed by the presence of the receptor extracellular domains (16), and netrin binding to either the DCC or UNC5 extracellular domains appears to initiate signaling by relieving this repression.

To better understand the nature of netrin-mediated signaling, we have undertaken a biochemical characterization of the interactions between netrin and its receptors. Using a recombinant form of human soluble netrin-1 (sNetrin) (8), we have mapped the regions of DCC and UNC5 that interact with sNetrin and find that discrete domains of DCC and UNC5 are both necessary and sufficient for sNetrin binding. These domains recapitulate the behavior of the receptors' ectodomains in both pull-down and cross-linking experiments. sNetrin binds to DCC and UNC5 fragments with 1:1 stoichiometry, and interactions of the receptors with sNetrin appear to be mutually exclusive. We also find that netrin binding is mediated by the same 11-amino acid loop on DCC previously identified as a heparin binding site (11). Since heparin does not compete with netrin for DCC binding and we are unable to detect direct interactions between heparin and DCC, interactions between DCC and heparin observed in earlier studies appear to have been mediated by netrin.

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<sup>1</sup> The abbreviations used are: Fn, type III fibronectin; hGH, human growth hormone; PBS, phosphate-buffered saline; BS<sup>3</sup>, bis(sulfosuccinimidyl)suberate; CHO, Chinese hamster ovary; PNGase F, peptide-N<sup>4</sup>-(N-acetyl- $\beta$ -D-glucosaminyl)asparagine amidase F; TEV, tobacco etch virus protease.

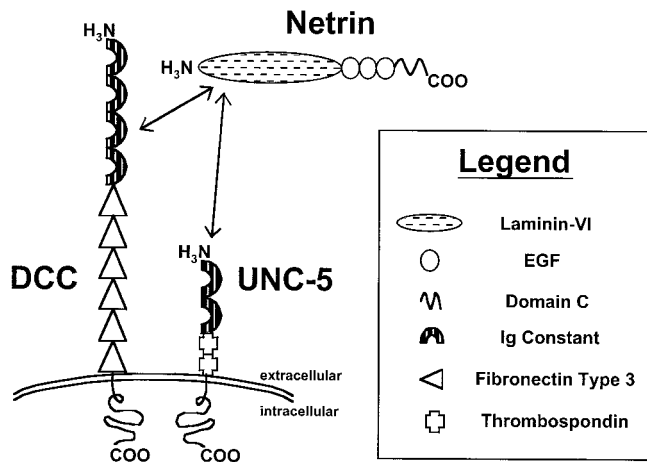


FIG. 1. Domain architecture of netrin and its receptors DCC and UNC5. Domain identities are given in the key and are drawn roughly to scale. Proteins are depicted in relation to the plasma membrane.

## MATERIALS AND METHODS

### Domain Assignments

We used the SMART algorithm (Simple Modular Architecture Research Tool; available on the World Wide Web at [smart.embl-heidelberg.de/](http://smart.embl-heidelberg.de/)) to make preliminary assignments of domain boundaries for human netrin-1, DCC, and UNC5C (Table I). These boundaries were cross-validated with secondary structure predictions using the PsiPred algorithm (available on the World Wide Web at [bioinf.cs.ucl.ac.uk/psipred/](http://bioinf.cs.ucl.ac.uk/psipred/)). Predicted sites of signal peptide cleavage were identified using the SignalP server (available on the World Wide Web at [www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)).

### Oligonucleotides, PCR, Plasmids, and Mutagenesis

cDNAs encoding human Netrin-1 and DCC were supplied by E. Fearon (University of Michigan), and the UNC5H2 cDNA was provided by S. Ackerman (Jackson Laboratory). These were used as templates for all PCRs. A complete list of all receptor/cDNA fragments generated and the corresponding oligonucleotides (Invitrogen) used in this study is presented in Table I. All PCRs were performed using the Expand High Fidelity system (Roche Applied Science) according to manufacturer's suggestions. Following separation by agarose gel electrophoresis and subsequent purification, each PCR product was digested with either *Bam*HI and *Not*I (for DCC fragments), *Xba*I and *Not*I (for both UNC5 and netrin fragments), or *Sal*I and *Not*I (DCC-Fn(5) for prokaryotic expression), subcloned into the corresponding sites of the appropriate expression vector, and then completely sequenced. For eukaryotic expression, the vector pSGHV0 was used (21); this vector allows for expression and secretion from mammalian cells of proteins fused to the C terminus of an octahistidine-tagged variant of human growth hormone (21). The vector pT7 was used for protein overexpression in *Escherichia coli* (22).

Alanine or "loop-swap" mutations in either DCC-Fn(1) or DCC-Fn(5) were identical to those described by Bennett *et al.* (11) and were generated in two rounds of PCR. In the first reaction, two partially overlapping oligonucleotides encoding the multiple mutations needed were annealed and amplified according to standard protocols. The product of this PCR was purified and used in conjunction with the necessary upstream primer to generate the desired full-length mutant cDNA for subcloning into the pSGHV0 expression vector.

### Transfections, Cell Culture, and Preparation of Cell Lysates

DNA used for transfection was purified by equilibrium CsCl density gradient ultracentrifugation and dissolved in deionized water to a concentration greater than 1.0 mg/ml. Transient transfections were performed in six-well clusters using the Superfect reagent (Qiagen) according to the manufacturer's instructions. Following transfection, both *dhfr*<sup>-/-</sup> CHO and HEK293 cells were cultured in  $\alpha$ -minimal essential medium supplemented with 1% (v/v) fetal bovine serum, hypoxanthine/thymidine, and penicillin/streptomycin (Invitrogen) for 72 h (as described in Ref. 21). The conditioned medium was then harvested and clarified by centrifugation (10 min at 13,000  $\times$  g) prior to analysis. All stable transfections and methotrexate selections of mutant CHO cells

were performed essentially as described (21). High expressing cell lines were expanded for culture in roller bottles, and the cells were maintained in serum-free medium (Excell 301, JRH Biosciences) supplemented with penicillin/streptomycin (21).

NMUT1 cells were a gift from A. Klingelutz (University of Iowa) and were cultured in keratinocyte serum-free medium supplemented with recombinant human epidermal growth factor and bovine pituitary extract according to the manufacturer's suggestions (Invitrogen). To prepare whole-cell lysates, NMUT1 cells were grown to confluence in a 75-cm<sup>2</sup> flask and were lysed by scraping in 200  $\mu$ l of lysis buffer (20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1% (w/v) Triton X-100) supplemented with protease inhibitors (Complete tablets, according to the manufacturer's instructions; Roche Applied Science). The cell extract was incubated on ice for 30 min, and a soluble extract was prepared by centrifugation at 4  $^{\circ}$ C (10 min at 13,000  $\times$  g). For Western analysis, 20  $\mu$ l each of conditioned medium and whole-cell lysate were analyzed.

### Enzyme-linked Immunosorbent Assay and Immunoblotting

Enzyme-linked immunosorbent assays to detect human growth hormone (hGH) were carried out as described (21). For immunoblotting, samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane according to the manufacturer's suggestions (Millipore Corp.). All procedures were performed at 25  $^{\circ}$ C. Following transfer, the membrane was blocked by a 1-h incubation in 1% gelatin (Sigma) dissolved in PBST (10 mM sodium-P<sub>i</sub>, 137 mM NaCl, 2 mM KCl, with 0.1% Tween 20). hGH was detected by incubating for 1 h with a rabbit polyclonal antibody that recognizes hGH (Research Diagnostics; 1:10<sup>4</sup> dilution in block solution). The membrane was washed five times with PBST, and the rabbit antibody was detected by a 1-h incubation with an horseradish peroxidase-conjugated goat antibody that recognizes rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:2  $\times$  10<sup>4</sup> dilution in block solution). Following another series of washes, membrane-bound horseradish peroxidase was detected using the SuperSignal Pico system (Pierce) according to the manufacturer's suggestions. During the course of experimentation, it was necessary to change production lots of anti-hGH; this resulted in increased nonspecific binding of the antibody during routine immunoblotting. For detection of endogenous netrin-1, an affinity-purified goat anti-human netrin-1 polyclonal antibody (Santa Cruz Biotechnology; 1:50 dilution in 5% (w/v) nonfat dry milk in PBST) was used. In this case, membrane-bound goat IgG was detected with an horseradish peroxidase-conjugated donkey antibody (Santa Cruz Biotechnology; 1:5000 dilution in 5% (w/v) nonfat dry milk in PBST).

### Eukaryotic Protein Expression and Purification

All manipulations were carried out at 4  $^{\circ}$ C unless otherwise indicated. Conditioned medium was harvested every third day, and cellular debris was removed by centrifugation (30 min at 9,000  $\times$  g). In a typical preparation, 4–8 liters of medium were concentrated  $\sim$ 20-fold in a Pellicon tangential flow filtration apparatus equipped with 30-kDa nominal molecular mass cut-off membranes (Millipore). The concentrate was exchanged into binding buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10 mM imidazole) according to the manufacturer's instructions and was sterile-filtered, and glycerol was added to 10% (v/v). The sample was applied to a 10-ml column of Ni<sup>2+</sup>-Sephacryl Fast-Flow (Amersham Biosciences), the column was washed in binding buffer, and bound proteins were eluted in binding buffer containing 500 mM imidazole. Protein-containing fractions were pooled and prepared for TEV digestion by adding EDTA and aprotinin to concentrations of 2 mM and 1  $\mu$ g/ml, respectively; recombinant TEV protease (Invitrogen) was added as described (21); and the reaction was incubated at 25  $^{\circ}$ C until complete. Following proteolysis, samples were dialyzed into binding buffer and reappplied to Ni<sup>2+</sup>-Sephacryl, and the unbound proteins were collected for further purification. Below, we describe the subsequent purifications of individual proteins, which in all cases led to preparations greater than 95% pure as judged by SDS-PAGE.

*sNetrin* ( $M_r$   $\sim$ 48,000)—The sample was applied to a 6-ml Resource S column (Amersham Biosciences) equilibrated in 20 mM sodium-P<sub>i</sub> (pH 6.0) and eluted with a gradient to 500 mM NaCl. At this point, *sNetrin* was essentially pure, but an additional gel filtration step was typically performed (Superdex 75 (26/60) gel filtration column (Amersham Biosciences)).

*DCC-Fn(1-6)* ( $M_r$   $\sim$ 75,000)—The sample was chromatographed on a 6-ml Resource S column equilibrated in 20 mM sodium acetate (pH 5.0) buffer and eluted with a gradient from 0 to 500 mM NaCl. The DCC-Fn(1-6)-containing fractions were dialyzed against 10 mM potassium-P<sub>i</sub> (pH 6.8) and applied to a 5-ml EconoPak CHT-II hydroxylapatite car-

TABLE I  
Netrin, DCC, and UNC5 protein fragments used in this study

A list of Netrin, DCC, and UNC5 protein fragments used in this study is presented in Table I. Each protein fragment is designated as it is referred to in the paper, and the encompassing residues are listed. All fragments were generated by PCR using the indicated oligonucleotides as described under "Materials and Methods"; restriction endonuclease recognition sites are italicized for convenience.

Protein fragment (encoded residues)	5' Oligonucleotide	3' Oligonucleotide
sNetrin-(1-429) (GPGLS . . . APCIK)	<i>chgggtctagaggccgggctcagcatg</i>	<i>ggggaattcttactttatcagggggcatgg</i>
DCC-Ecto-(1-1072) (HLQVT . . . QKNSN)	<i>aaaggatccatcttcaagtaaccggtttt</i>	<i>tttgcggccgctgtgtcttctgaggagt</i>
DCC-Ig-(1-400) (HLQVT . . . AIPSS)	<i>aaaggatccatcttcaagtaaccggtttt</i>	<i>tttgcggccgaggcttgggattgcagg</i>
DCC-Mid-(252-691) (IEGKD . . . AETPE)	<i>aaaggatccatggaagaaagatgctgtc</i>	<i>tttgcggccctctgaggtctctgcagtatacca</i>
DCC-Fn(1-6)-(401-1072) (SVLPS . . . QKNSN)	<i>aaaggatccagtgtctcctctcggt</i>	<i>tttgcggccgctgtgtgttctctgaggagt</i>
DCC-Fn(1-4)-(401-791) (SVLPS . . . ATTRS)	<i>aaaggatccagtgtctcctctcggt</i>	<i>tttgcggccgagacctgtgtggcactttcata</i>
DCC-Fn(2-5)-(513-916) (PGPVE . . . ATTYE)	<i>aaaggatcccaggggccagtagaaaactg</i>	<i>tttgcggcccttcatcagtggtggcattgcagt</i>
DCC-Fn(3-6)-(594-1072) (SDVPS . . . QKNSN)	<i>aaaggatcctctgacgtgccaagtgccccg</i>	<i>tttgcggccgctgtgtgttctctgaggagt</i>
DCC-Fn(2-3)-(513-691) (PGPVE . . . AETPE)	<i>aaaggatcccaggggccagtagaaaactg</i>	<i>tttgcggccctctgaggtctctgcagtatacca</i>
DCC-Fn(3-4)-(594-791) (SDVPS . . . ATTRS)	<i>aaaggatcctctgacgtgccaagtgccccg</i>	<i>tttgcggccgctgtgtgttctctgaggagt</i>
DCC-Fn(4-5)-(698-916) (SQVPD . . . ATTYE)	<i>aaaggatccttcaagtctgatcaacca</i>	<i>tttgcggcccttcatcagtggtggcattgcagt</i>
DCC-Fn(5-6)-(816-1072) (STPML . . . QKNSN)	<i>aaaggatctccaccctatgctccacca</i>	<i>tttgcggccgctgtgtgttctctgaggagt</i>
DCC-Fn(1)-(401-500) (SVLPS . . . TQPEL)	<i>aaaggatccagtgtctcctctcggtccc</i>	<i>tttgcggccgcaactcagctgtgtggccactt</i>
DCC-Fn(4)-(698-791) (SQVPD . . . ATTRS)	<i>aaaggatccttcaagtctgatcaacca</i>	<i>tttgcggccgagacctgtgtggcactttcata</i>
DCC-Fn(5)-(816-916) (STPML . . . ATTYE)	<i>aaaggatctccaccctatgctccacca</i>	<i>tttgcggcccttcatcagtggtggcattgcagt</i>
DCC-Fn(6)-(921-1072) (AAPTSS . . . QKNSN)	<i>aaagtcagctccaccctatgctccacca</i>	<i>tttgcggccgctgtgtgttctctgaggagt</i>
DCC-Fn(5) prok.-(816-916) (STPML . . . ATTYE)	<i>aaagtcagctccaccctatgctccacca</i>	<i>tttgcggcccttcatcagtggtggcattgcagt</i>
DCC-Fn(1-Loop5)-(896-906) MVTKNRRSSTW	<i>aagccagaagccatgtacactttcgagttat</i>	<i>tttgcggccgcaactcagctgtgtggccacttgcagg</i>
	<i>ggtaacaaaaacagaaggtccagctacttgg</i>	<i>ttgagaccaagctactggacttctgtttttgtaccat</i>
DCC-Fn(5-Loop1)-(578-588) VAYNEWGPGES	<i>ctcaaccaacaacaaatgtatgaattctcggtcg</i>	<i>tttgcggccgcttcatcagtggtggcattgcagtct</i>
	<i>tggttacaatgaatggggaccggagagagt</i>	<i>actctctcccggtcccaactcattgtgaaccac</i>
DCC-Fn(5-Ala) TKNRRS→TANAAS	<i>aaaggatctccaccctatgctccacca</i>	<i>atgtgcagctatgctccaagctactggagccggcgccg</i>
		<i>gtttaccatgaccgagaattcatacat</i>
UNC5-Ecto-(1-326) (QDDDF . . . PDSDD)	<i>aaatctagacaagatgatgactttttcatgaa</i>	<i>tttgcggccgcatcatctgaatcaggagcagt</i>
UNC5-Ig-(1-222) (QDDDF . . . YVNGG)	<i>aaatctagacaagatgatgactttttcatgaa</i>	<i>tttgcggccgcccaccctgtgacatagactatgac</i>
UNC5-TSP-(220-326) (NGGWS . . . PDSDD)	<i>aaatctagaaacgggtggctggctccactg</i>	<i>tttgcggccgcatcatctgaatcaggagcagt</i>
UNC5-Ig(1)-(1-142) (QDDDF . . . VSLEQ)	<i>aaatctagacaagatgatgactttttcatgaa</i>	<i>tttgcggccgctgttccaagacacttcttcc</i>
UNC5-Ig(2)-(140-222) (LEQEV . . . YVNGG)	<i>aaatctagattggaacaggaagtctactcca</i>	<i>tttgcggccgcccaccctgtgacatagactatgac</i>

tridge (Bio-Rad). Pure DCC-Fn(1-6) was eluted with a gradient from 0 to 400 mM potassium-P<sub>i</sub> (pH 6.8).

**UNC5-Ecto** ( $M_r \sim 38,000$ )—The sample was applied to a 6-ml Resource Q column equilibrated in 20 mM Tris-HCl (pH 8.0) and eluted with a gradient from 0 to 500 mM NaCl. The UNC5-containing fractions were further chromatographed on CHT-II hydroxylapatite (as described above).

**UNC5-Ig** ( $M_r \sim 25,500$ )—The sample was chromatographed on a 6-ml Resource Q column as described above followed by chromatography on a Superdex 75 (26/60) gel filtration column.

#### Prokaryotic Protein Expression and Purification

The plasmid pT7-DCC-Fn(5) was transformed into *E. coli* strain B834(DE3). Fresh transformants were cultured, induced, and harvested as previously described (22). Induced cells from 1 liter of culture were resuspended in 25 ml of 20 mM Tris-HCl (pH 7.5) and lysed by two passes through a French pressure cell (30,000 p.s.i.) at 4 °C. DNase I (Sigma) was added to a concentration of 20 µg/ml, and the cell homogenate was centrifuged at 4 °C (30 min at 25,000 × *g*). The supernatant was sterile-filtered, diluted to 100 ml, and applied to a 25-ml column of Q Sepharose Fast-Flow at pH 7.5. The unbound fraction was dialyzed against a buffer of 20 mM Hepes (pH 7.0) and applied to a 6-ml Resource S column. Bound proteins were eluted with a gradient from 0 to 500 mM NaCl, and the DCC-Fn(5)-containing fractions were pooled and concentrated. This protein mixture was chromatographed on a Superdex 75 (26/60) column, after which the DCC-Fn(5) was greater than 95% pure as judged by SDS-PAGE ( $M_r \sim 11,200$ ).

#### Preparation of Netrin Affinity Resin

All *in vitro* binding assays made use of netrin coupled to CNBr-activated Sepharose Fast-Flow (Amersham Biosciences). To prepare this resin, purified netrin was dissolved in a coupling buffer (100 mM sodium-P<sub>i</sub>, 500 mM NaCl, 2 mM EDTA) and mixed with resuspended CNBr-activated Sepharose Fast-Flow so that the protein coupling density was 1 mg of netrin/ml of resin. The coupling reaction was carried out at 25 °C for 16 h and quenched with 200 mM Tris-HCl (pH 8.0) according to the manufacturer's suggestions. The netrin-Sepharose was stored as a 50% slurry in PBS supplemented with 2 mM EDTA.

#### Pull-down Binding Assays

All binding assays were performed in 1 ml of total volume of PBS supplemented with 1% Tween 20 (PBST). Each reaction contained a

10-µl bed volume of netrin-Sepharose (effective netrin concentration of 200 nM) and a 1 nM total concentration of hGH-tagged ligand. Binding reactions were carried out at 25 °C for 1 h, and the affinity resin was captured by centrifugation (2 min at 5,000 × *g*). After the resin was washed three times in PBST, it was resuspended in 25 µl of 2× Laemmli sample buffer and heated to 100 °C for 5 min. Netrin complexes were detected by processing 10 µl of each sample for SDS-PAGE and immunoblotting with antibodies against hGH.

#### Protein Cross-linking

Netrin receptor complexes were covalently coupled through the water-soluble, amine-reactive cross-linking agent bis(sulfosuccinimidyl)suberate) (BS<sup>3</sup>; Pierce). Proteins were dialyzed into PBS prior to cross-linking to avoid inappropriate aminolysis of BS<sup>3</sup>. All reactions were carried out in 20 µl with 5 µM final concentration of each reactant protein. In conditions where both DCC and UNC5 fragments were present, a stock solution containing both proteins was prepared so that the receptors could be added simultaneously. For each reaction, the protein mixture was allowed to equilibrate for 1 h at 25 °C before cross-linking was initiated by the addition of BS<sup>3</sup> to a final concentration of 300 µM. The reactions were allowed to proceed for an additional 20 min at 25 °C. Excess BS<sup>3</sup> was quenched by adding Tris-HCl (pH 6.8) to 100 mM final concentration and incubating for an additional 15 min. The samples were prepared for analysis by adding 6× Laemmli sample buffer and heating to 100 °C for 5 min. Complexes were separated by SDS-PAGE and visualized by staining with Coomassie Blue.

#### Analytical Gel Filtration Chromatography

All separations were performed at 4 °C using a flow rate of 0.5 ml/min on a Superdex 200 (10/30) column equilibrated in PBS. 1-ml fractions were collected, and elution of protein from the column was monitored by UV spectrophotometry at 280 nm. 250 µg of purified protein (concentration 1 mg/ml) were injected for control separations, and complexes were prepared by combining 250 µg of each protein in a final volume of 250 µl. The approximate molecular masses of eluted species were determined by comparison with a calibration curve generated from a mixture of globular protein standards (Amersham Biosciences). Fractions of interest were analyzed by SDS-PAGE and staining with Coomassie Blue.

#### Heparin Binding Assays

Purified proteins (5 µg) were incubated with 10 µl of heparin-Sepharose in a total volume of 1 ml of PBST. Binding reactions were carried

out at 25 °C for 1 h, and the heparin resin was captured by centrifugation (2 min at 5,000 × *g*). The resin was washed three times in PBST and then resuspended in 10 μl of 2× Laemmli sample buffer and heated to 100 °C for 5 min. Proteins were separated by SDS-PAGE and visualized by staining with Coomassie Blue.

#### Immunofluorescence and Cell Surface Binding Assays

sNetrin binding to DCC-expressing HEK293 cells was measured essentially as described by Keino-Masu *et al.* (8). HEK293 cells were transiently transfected with either pCMV-DCC (a plasmid for expression of full-length human DCC; E. Fearon, University of Michigan) or pCMV alone as described above. After 24 h, the cells were trypsinized and plated on sterile, gelatin-coated coverslips. After another 24 h, the cells were incubated with conditioned culture medium (see above) containing either hGH-sNetrin, hGH-sNetrin with 500 μg/ml heparin, or hGH (each protein at a final concentration of 40 nM). The cells were incubated at 37 °C for 1 h further, at which time the unbound proteins were removed by washing three times in sterile PBS. The samples were fixed 10 min in 3.7% (w/v) paraformaldehyde/PBS and washed twice with sterile PBS. Each sample was incubated in 5% (w/v) normal goat serum/PBST (block solution) followed by a 1-h incubation with a mixture of rabbit antibodies specific for hGH (Research Diagnostics; 1:1000 in block solution), mouse antibodies specific for DCC (Oncogene Sciences; 1 μg/ml monoclonal AF5 in block solution), and 4',6-diamidino-2-phenylindole (Molecular Probes, Inc., Eugene, OR). The cells were washed three times with PBST, and the following secondary antibodies were incubated for 1 h: Alexa-Texas Red-anti-rabbit (1:400 in block) and Alexa-fluorescein isothiocyanate-anti-mouse (1:400 in block) (Molecular Probes). Excess fluorescent antibodies were removed by washing in PBST, and the coverslips were mounted on microscope slides using AquaPoly/Mount (Polysciences, Inc.).

#### Biochemical Analysis

PNGase F was obtained from New England Biolabs and was used to deglycosylate sNetrin according to the manufacturer's suggestions. N-terminal protein sequences were determined by the Edman method at the Department of Biological Chemistry core facility (Johns Hopkins University School of Medicine). Purified human antithrombin-III, heparin (178 units/mg), and heparin-Sepharose (960 μg/ml bed) were obtained from Sigma.

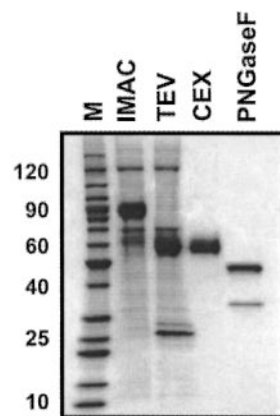
#### RESULTS

**Expression and Purification of a Human Netrin-1 Fragment—**To study the molecular properties of netrin-receptor complexes, we first generated a stable CHO cell line secreting a recombinant form of human netrin-1 (8) (sNetrin) comprising the laminin-VI domain through the epidermal growth factor repeats fused to the C terminus of an octahistidine-tagged variant of hGH (Table I). The hGH-sNetrin fusion protein expressed at ~4 mg of hGH/liter of conditioned medium and purified, cleaved sNetrin could be obtained with an approximate yield of 0.25 mg/liter of starting material (Fig. 2).

N-terminal sequencing of sNetrin revealed the expected residues, but purified sNetrin migrated as a diffuse band near 60 kDa despite a calculated molecular mass of 48 kDa. The primary sequence of sNetrin contains four consensus sites for asparagine-linked glycosylation, and each complex asparagine-linked glycosylation site typically adds 3–4 kDa to the protein's molecular mass. To test whether the increased mass of our preparation resulted from glycosylation, the purified sNetrin was treated with PNGase F. This resulted in sNetrin migrating as a 48-kDa species in SDS-PAGE (Fig. 2).

**Identification of the sNetrin-binding Regions in DCC and UNC5—**To identify specific subdomains of DCC and UNC5 involved in sNetrin binding (Fig. 1, Table I), we developed an *in vitro* pull-down assay. In this assay, conditioned medium from CHO cells secreting hGH-tagged receptor fragments was incubated with an sNetrin-coupled resin and binding was assessed by immunoblotting for bound hGH (see "Materials and Methods").

Three distinct fragments of DCC were initially generated and expressed transiently in CHO cells in addition to the entire DCC ectodomain: the Ig repeats (DCC-Ig), the Fn repeats



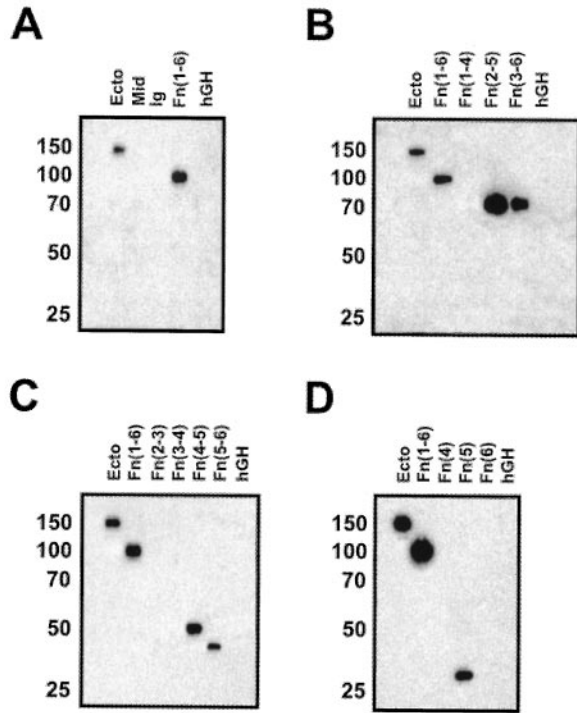
**FIG. 2. Expression and purification of a human netrin fragment (sNetrin) from CHO cells.** An active fragment of human netrin-1, denoted sNetrin (Table I) was expressed and secreted from CHO cells fused to the C terminus of human growth hormone. The fusion protein was purified by immobilized metal affinity chromatography (IMAC) and digested with recombinant TEV protease (TEV). sNetrin was purified further following cation exchange chromatography (CEX). Purified sNetrin migrated on SDS-PAGE as a 60-kDa species but migrated at its predicted mass (48 kDa) following deglycosylation with PNGase F (PNGaseF). The 35-kDa band present in lane 4 corresponds to PNGase F. *M*, molecular mass standards.

(DCC-Fn(1–6)), and a middle fragment (DCC-Mid) composed of the last two Ig repeats and the first three Fn repeats. As shown in Fig. 3A, sNetrin bound to DCC ectodomain and DCC-Fn(1–6) but not DCC-Ig or DCC-Mid (20). We further mapped this interaction by testing two series of hGH-tagged DCC fragments, those composed of four (Fig. 3B) and those with two contiguous Fn repeats (Fig. 3C), respectively. Only DCC fragments containing the fifth Fn repeat bound sNetrin. We then tested the single Fn repeat proteins DCC-Fn(4), DCC-Fn(5), and DCC-Fn(6) and observed sNetrin binding only for DCC-Fn(5) (Fig. 3D), showing this repeat to be necessary and sufficient to mediate the sNetrin/DCC interaction.

sNetrin binding to UNC5 was also examined using this approach. Five distinct UNC5 fragments were tested, including the entire UNC5 ectodomain (UNC5-Ecto), the entire Ig-containing region (UNC5-Ig), the thrombospondin repeats (UNC5-Tsp), and the individual Ig repeats (UNC5-Ig(1) and UNC5-Ig(2)). We observed that UNC5-Ecto and UNC5-Ig bound sNetrin, whereas UNC5-Tsp did not (Fig. 4A). Surprisingly, both individual Ig repeats bound sNetrin in a manner indistinguishable from the larger UNC5 fragments (Fig. 4B).

**Reconstitution of sNetrin Complexes Using Purified Proteins—**Ligand-induced cross-linking of receptors is a well established signaling mechanism, and the stoichiometry of sNetrin interactions with DCC and UNC5 may reflect important features of netrin signaling. To address this issue, we developed overexpressing CHO cell lines and used these to purify DCC-Fn(1–6), DCC-Fn(5), UNC5-Ecto, and UNC5-Ig (see "Materials and Methods") (Fig. 5). This allowed us to examine the behavior of mixtures of sNetrin with these proteins following cross-linking with the bifunctional amine-reactive reagent bis(sulfosuccinimidyl)suberate.

We first tested the larger fragments of DCC (DCC-Fn(1–6)) and UNC5 (UNC5-Ecto) (Fig. 6A). Cross-linking a mixture of sNetrin and DCC-Fn(1–6) resulted in the appearance of a discrete species with an apparent molecular mass of ~150 kDa. Similarly, a cross-linked sample of sNetrin and UNC5-Ecto resulted in the appearance of a 105-kDa species. In both cases, the results were consistent with these proteins forming 1:1 complexes with sNetrin. Specific higher order oligomers were not observed.



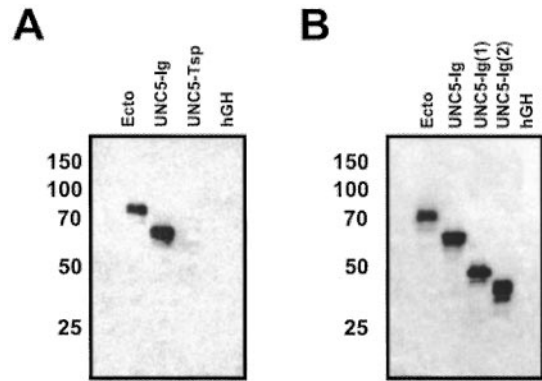
**FIG. 3. DCC binds sNetrin through its fifth Fn repeat.** Extracellular fragments of DCC (Table I) were fused to hGH and expressed transiently in CHO cells. These fusion proteins were subjected to a pull-down binding assay using sNetrin-Sepharose, and bound proteins were analyzed by anti-hGH Western blots. *A*, sNetrin binding to DCC ectodomain, DCC-Mid, DCC-Fn(1–6), and hGH alone. *B*, sNetrin binding to overlapping four-repeat segments of the Fn region. *C*, sNetrin binding to overlapping two-repeat segments of the Fn region. *D*, sNetrin binding was isolated to DCC-Fn(5) by testing individual Fn repeats.

Our pull-down experiments indicated that sNetrin binding is mediated by discrete regions within each receptor's ectodomain. As an independent test of these results, we examined the behavior of minimal functional regions of both DCC (DCC-Fn(5)) and UNC5 (UNC5-Ig) in cross-linking experiments (Fig. 6B). Consistent with our previous results, we observed the formation of 1:1 complexes between sNetrin and DCC-Fn(5) ( $M_r \sim 70,000$ ) and sNetrin and UNC5-Ig ( $M_r \sim 95,000$ ).

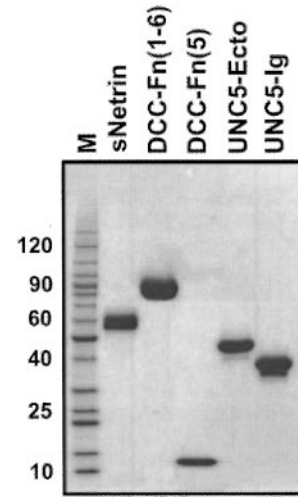
To test whether sNetrin, DCC, and UNC5 were capable of forming a ternary complex, we examined the effects of cross-linking on samples containing either sNetrin, DCC-Fn(1–6), and UNC5-Ecto (Fig. 6A) or sNetrin, DCC-Fn(5), and UNC5-Ig (Fig. 6B). In neither case did we observe formation of a discrete, ternary complex. Instead, we observed formation of binary complexes similar to those seen when the individual receptor fragments were cross-linked to sNetrin. Likewise, we failed to detect any complexes between the DCC and UNC5 receptor fragments (Fig. 6, *A* and *B*).

To examine the sNetrin complexes through a complementary approach, we incubated purified sNetrin, DCC-Fn(1–6), and UNC5-Ecto with one another and analyzed the samples by analytical gel filtration chromatography (data not shown). We observed the formation of binary complexes of sNetrin-DCC-Fn(1–6) and sNetrin-UNC5-Ecto similar to those seen in cross-linking experiments. We again found no evidence for the formation of a ternary complex, nor did we detect any interaction between DCC-Fn(1–6) and UNC5-Ecto.

**DCC Binds Netrin via a Specific Loop in DCC-Fn(5) but Does Not Bind Heparin**—It has been reported that DCC contains a heparin-binding site within its fifth Fn repeat (11). Consequently, our observation that sNetrin binding mapped to this



**FIG. 4. UNC5 binds sNetrin through its immunoglobulin repeats.** Extracellular fragments of UNC5 (Table I) were fused to hGH and expressed transiently in CHO cells. These fusion proteins were subjected to a pull-down binding assay using sNetrin-Sepharose, and bound proteins were analyzed by anti-hGH Western blots. *A*, sNetrin binding to UNC5-Ecto, UNC5-Ig, UNC5-Tsp, and hGH alone. *B*, sNetrin binding to individual Ig repeats.

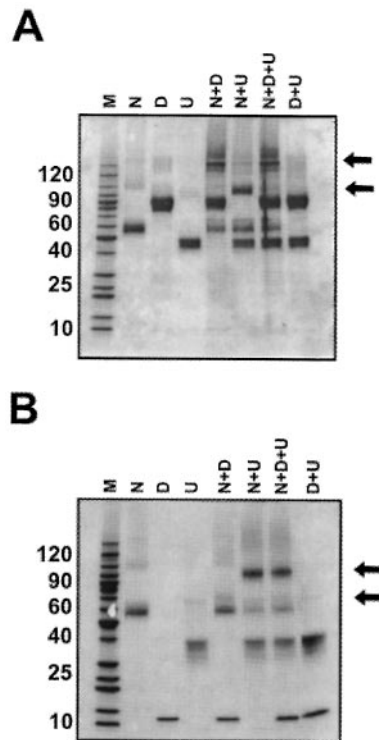


**FIG. 5. Expression and purification of netrin receptor fragments.** sNetrin-binding fragments of the DCC and UNC5 ectodomains were overexpressed and purified to homogeneity as visualized by Coomassie Blue staining of SDS-PAGE gels (see "Materials and Methods"). *M*, molecular mass standards.

same Fn repeat of DCC (Figs. 3 and 6) prompted us to investigate the relationship between heparin and sNetrin binding to DCC.

We utilized a pull-down assay to test whether heparin had any effect on the ability of sNetrin to bind DCC. sNetrin-Sepharose was incubated with hGH-DCC-Fn(1–6) in the presence of increasing concentrations of soluble heparin, and the bound proteins were analyzed by Western blotting. As shown in Fig. 7A, no change in the ability of sNetrin to bind DCC-Fn(1–6) was observed even in the presence of high concentrations of heparin. Since we failed to observe any effects of heparin on the sNetrin/DCC-Fn(1–6) interaction *in vitro*, we tested whether heparin might have any effect on the ability of sNetrin to bind specifically to the surface of DCC-expressing cells (Fig. 7, *B–E*) (8). Mirroring the *in vitro* results, sNetrin bound specifically to HEK293 cells expressing full-length DCC despite the presence of high concentrations of heparin.

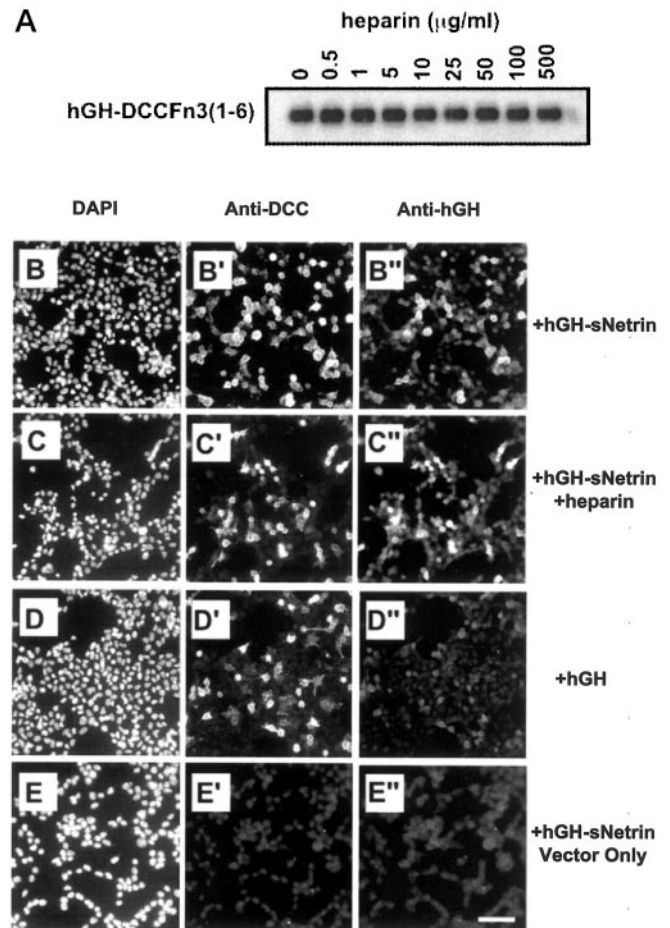
Bennett *et al.* (11) mapped the heparin binding site on DCC to a specific loop connecting the F and G  $\beta$ -strands of DCC-Fn(5), and we next tested whether this loop was also important for mediating interactions with netrin. In experiments mimicking those of Bennett *et al.* (11), we swapped the FG-loops of DCC-Fn(5) and DCC-Fn(1) to create chimeric DCC Fn domains



**FIG. 6. Detection of sNetrin complexes using purified proteins.** Netrin complexes were reconstituted *in vitro*, cross-linked, and analyzed by Coomassie Blue-stained SDS-PAGE. *A*, analysis of complexes formed between sNetrin and large fragments of the DCC and UNC5 ectodomains. Control reactions using only purified sNetrin (*N*), DCC-Fn(1-6) (*D*), and UNC5-Ecto (*U*) are shown alongside sNetrin plus DCC-Fn(1-6) (*N + D*), sNetrin plus UNC5-Ecto (*N + U*), sNetrin plus DCC-Fn(1-6) and UNC5-Ecto (*N + D + U*), and DCC-Fn(1-6) plus UNC5-Ecto (*D + U*). *B*, complexes formed between sNetrin and minimal binding fragments of each receptor as identified through pull-down assays. Control reactions using only purified sNetrin (*N*), DCC-Fn(5) (*D*), and UNC5-Ig (*U*) are shown alongside sNetrin plus DCC-Fn(5) (*N + D*), sNetrin plus UNC5-Ig (*N + U*), sNetrin plus DCC-Fn(5) and UNC5-Ig (*N + D + U*), and DCC-Fn(5) plus UNC5-Ig (*D + U*). *M*, molecular mass standards. Bands corresponding to discrete cross-linked complexes are denoted by arrows.

and compared the ability of these mutants to bind sNetrin in our pull-down assay (Fig. 8). Despite the fact that wild type DCC-Fn(1) lacked sNetrin-binding activity, we observed that DCC-Fn(1-Loop5) (DCC-Fn(1) with the transplanted FG loop from DCC-Fn(5)) bound sNetrin efficiently, although not as well as DCC-Fn(5). We also observed that DCC-Fn(5-Loop1) exhibited reduced sNetrin binding. These results suggested that the FG loop contributes a significant component of the sNetrin-binding site within the fifth Fn repeat of DCC. Mutation of the residues KNRR in the FG loop of DCC-Fn(5) to all alanines, however, resulted in only a marginal decrease in sNetrin binding, suggesting that the FG loop contains determinants outside of these residues that are important for netrin binding.

Mapping sNetrin binding to the same loop on DCC-Fn(5) that was reported to mediate heparin binding led us to question whether the observed DCC/heparin interaction was indirectly mediated by netrin, which binds heparin at a site distinct from its DCC-binding site (8, 10). We first compared the abilities of purified DCC-Fn(1-6), DCC-Fn(5), and human antithrombin-III (a positive control) to bind immobilized heparin under physiological buffer conditions (Fig. 9A). Although antithrombin-III bound heparin efficiently, we could not detect heparin binding for either DCC-Fn(1-6) or DCC-Fn(5). Since the previous study of interactions between DCC and heparin primarily examined



**FIG. 7. sNetrin binds DCC independently of heparin.** *A*, hGH-DCC-Fn(1-6) was subjected to a pull-down sNetrin-binding assay in the presence of increasing concentrations of heparin, and bound proteins were analyzed by anti-hGH Western blot. The heparin concentration (in  $\mu\text{g/ml}$ ) for each reaction is shown above the blot image. *B-E*, HEK293 cells expressing full-length DCC were incubated with medium containing either hGH-sNetrin (*B*), hGH-sNetrin plus 500  $\mu\text{g/ml}$  heparin (*C*), or hGH alone (*D*); cells not expressing DCC are shown as a control (*E*). Cells were then examined for fluorescence following staining with 4',6-diamidino-2-phenylindole (DAPI) (left column), antibodies specific for DCC (center column/prime), and antibodies specific for hGH (right column/double prime). Scale bar, 100  $\mu\text{m}$ .

DCC binding to heparin on the surface of NMUT1 cells, we then examined whether NMUT1 cells express netrin-1 (11). A polyclonal antibody specific for human netrin-1 (Fig. 9B) detected endogenous netrin-1 in Western blots of both conditioned medium and whole-cell extract prepared from NMUT1 cells (Fig. 9C). The slower electrophoretic mobility of endogenous netrin-1 is expected because of the presence of the C-terminal domain (omitted in recombinant sNetrin).

#### DISCUSSION

Netrin recognition by DCC and UNC5 plays a fundamental role in nervous system development, and we present here the first analysis of the specific subdomains in DCC and UNC5 that mediate netrin binding. We find that sNetrin binding is an intrinsic property of the fifth Fn repeat in DCC and of the Ig repeats in UNC5. We also show that the soluble, extracellular domains of DCC and UNC5 each bind sNetrin in 1:1 complexes but find no evidence for a ternary complex of sNetrin and these receptor fragments, suggesting that the DCC- and UNC5-binding sites on netrin may overlap. Contrary to an earlier report (11), we find no evidence for a direct interaction between DCC and heparin; nor does heparin appear to interfere with sNetrin/

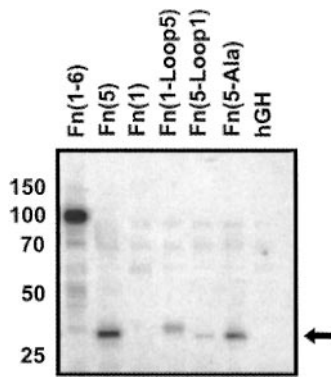


FIG. 8. The 11-residue FG loop of DCC-Fn(5) contains an sNetrin-binding site. Chimeric fragments of DCC were constructed as in Bennett *et al.* (11) such that the loop connecting the F and G  $\beta$ -strands of both Fn(1) and Fn(5) were exchanged and used to map the sNetrin-binding site on DCC. DCC-Fn(1) and DCC-Fn(5) chimeras were fused to hGH, expressed transiently in CHO cells, and subjected to a pull-down binding assay using sNetrin-Sepharose, and bound proteins were analyzed by anti-hGH Western blots. The location of the single, hGH-tagged Fn repeats is denoted with an arrow.

DCC interactions either *in vitro* or at the cell surface. Instead, we demonstrate that the loop connecting strands F and G of DCC-Fn(5), which had previously been reported to mediate an interaction between DCC and heparin (11), is an important determinant of netrin binding. Our observation of netrin-1 expression by the heparin-bearing NMUT1 cells used in these earlier studies strongly suggests that the reported DCC/heparin interaction was in fact mediated by netrin-1, which binds heparin through its C-terminal domain (8, 10).

Our failure to observe a ternary complex of sNetrin, DCC-Fn3(1–6), and UNC5-Ecto was unexpected, because full-length DCC or DCC lacking its cytoplasmic region will co-immunoprecipitate with itself or UNC5 when expressed on the surface of cells exposed to netrin (8, 20). Two possibilities may explain these results. First, associations between DCC and UNC5 may require the transmembrane regions to mediate the interaction directly or to increase the effective concentration of these receptors through confinement to the plasma membrane. The restriction of interaction partners to a particular orientation within a two-dimensional membrane may increase their effective concentration by several orders of magnitude and favor otherwise weak interactions (23). Alternatively, the netrin-mediated associations between DCC and itself or UNC5 may result from the cross-linking of occupied netrin receptors by multivalent heparin sulfate proteoglycans or another factor such as netrin synergizing activity (10, 16, 23).

The interaction between netrin and heparin results in the accumulation of netrin at the cell surface (8), which may be necessary to concentrate or spatially limit the netrin signal but does not seem essential for netrin signaling. For example, recombinant netrin lacking its heparin-binding domain (sNetrin) retains the ability to stimulate axon outgrowth in spinal cord explants (8). Thus, it remains to be seen whether the netrin/heparin interaction provides a function that may be unnecessary or overridden in certain circumstances or is an essential component of *in vivo* signaling. In this respect, it would be of interest to examine the phenotypes of transgenic animals expressing sNetrin instead of netrin-1.

The results reported here refine and focus models of the molecular mechanisms of netrin signaling and provide a basis for future studies of this system. First, mapping the sNetrin-binding regions of DCC and UNC5 to specific subdomains demonstrates that these proteins, like many other multirepeat proteins, exhibit functional as well as structural modularity.

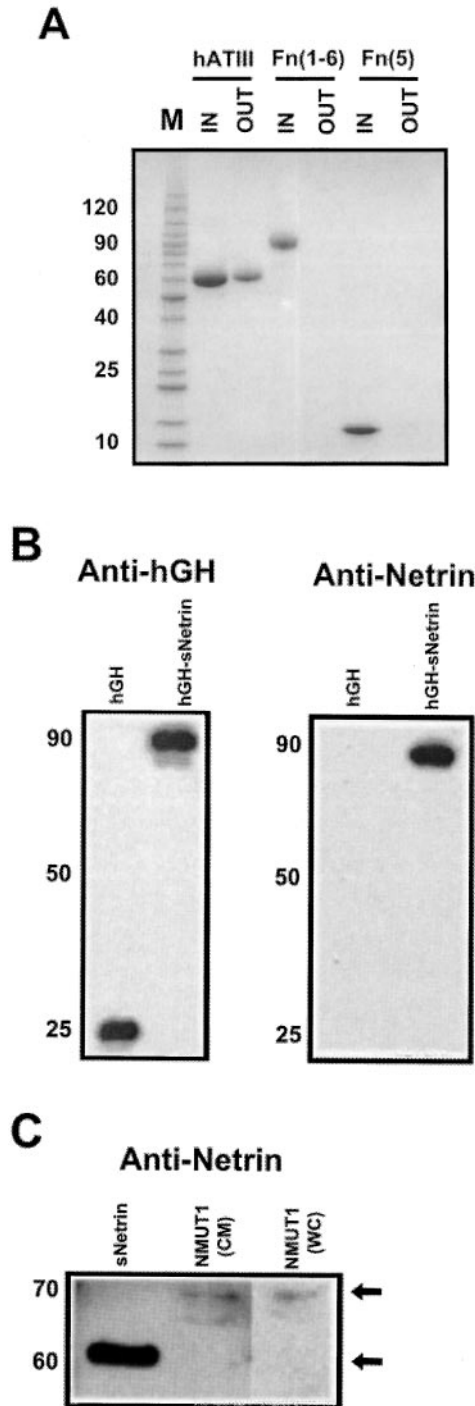


FIG. 9. Netrin-1 is expressed and secreted by NMUT1 cells. A, the heparin-binding activities of purified DCC-Fn(1–6), DCC-Fn(5), and human antithrombin-III (positive control) were assayed by SDS-PAGE and Coomassie Blue staining. An input loading control (IN) (5  $\mu$ g) is shown along with the heparin-bound fraction of each protein (OUT); M, molecular mass standards. B, the specificity of an anti-netrin-1 antibody was evaluated by blotting samples of conditioned medium containing either hGH or hGH-sNetrin with either anti-hGH or anti-netrin-1. C, conditioned medium (CM) and whole-cell extracts (WC) from NMUT1 cells were assayed for the presence of netrin by anti-netrin Western blots; purified sNetrin (1 ng) is shown for comparison.

Second, the functional role of regions of DCC and UNC5 not involved directly in netrin binding may also now be more specifically addressed through studies of receptors in which segments outside of the netrin-binding region have been deleted. Likewise, it may also be significant, particularly in the case of

DCC, that netrin binding occurs in a membrane-proximal region; studies of receptors with permuted domains will allow a critical assessment of the importance of netrin-binding domain's context for successful signaling. Finally, knowledge of the netrin-binding regions of DCC and UNC5 will facilitate structural studies of netrin/receptor interactions, the success of which is likely to depend on the use of functional but more compact subdomains.

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