NIH3T3 Cells Expressing the Deleted in Colorectal Cancer Tumor Suppressor Gene Product Stimulate Neurite Outgrowth in Rat PC12 Pheochromocytoma Cells

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Abstract. The Deleted in Colorectal Cancer (DCC) gene is a candidate tumor suppressor gene that is predicted to encode a transmembrane polypeptide with strong similarity to the neural cell adhesion molecule (N-CAM) family. Previous studies have suggested that several different N-CAMs, when expressed in non-neuronal cell types can stimulate neurite outgrowth from PC12 rat pheochromocytoma cells. Based on the predicted structural similarity of DCC to N-CAMs, we sought to determine whether NIH3T3 cells expressing DCC could stimulate neurite outgrowth in PC12 cells. We found that NIH3T3 cell lines expressing DCC could stimulate PC12 cells to extend neurites. Supernatants from DCC-transfected NIH3T3 cells did not induce neurite outgrowth above background levels, suggesting that cell-cell interaction was required. NIH3T3 cells expressing a truncated form of DCC, lacking the majority of the cytoplasmic domain sequences, also failed to induce neurite outgrowth above the levels seen with control NIH3T3 cells, suggesting that the cytoplasmic domain of DCC was necessary for its neurite-promoting function. In contrast to NGF-mediated neurite outgrowth, the DCC-mediated response was inhibited by treatment with pertussis toxin or the combination of N- and L-type calcium channel blockers, and was unaffected by the transcriptional inhibitor cordycepin. The data suggest that the DCC protein can function in a fashion analogous to other N-CAMs to alter PC12 cell phenotype through intracellular pathways distinct from those involved in NGF signaling.
teractions. At present, however, little is known about molecules that interact with the extracellular domain of DCC or the means by which DCC functions in normal or neoplastic cell growth.

Initial studies suggested that the DCC transcripts are present in most adult tissues at very low levels, with highest levels in brain tissues (Fearon et al., 1990). DCC expression appears to be higher in fetal brain compared with the levels in adult brain tissues (Fearon et al., 1990), although detailed studies of DCC gene expression in developing neural tissues have not yet been carried out. In addition, immunohistochemical analysis has demonstrated DCC expression in central and peripheral nervous system tissues of the adult (Hedrick, L., K. R. Cho, E. R. Fearon, T.-C. Wu, K. W. Kinzler, and B. Vogelstein, manuscript submitted for publication). Recent studies using an in vitro model of neuronal differentiation and anti-sense DCC expression constructs suggest that persistent DCC expression may be necessary in some cells for the morphological differentiation induced by nerve growth factor (NGF) (Lawlor and Narayanan, 1992). Specifically, PC12 cells, a rat adrenal pheochromocytoma cell line that exhibits features of neuronal differentiation (such as neurite outgrowth) after treatment with NGF, were transfected with an anti-sense DCC expression construct. There was inhibition of NGF-mediated morphological differentiation in cells expressing high levels of the anti-sense DCC transcripts despite the induction of several early response genes by NGF. In addition, it was observed that the addition of anti-sense DCC oligonucleotides to NGF-differentiated PC12 cells caused a morphological reversion of the neuronal phenotype. These data have been interpreted as evidence that expression of DCC in PC12 cells is necessary for induction and maintenance of morphological differentiation.

Previous studies have provided evidence for the critical role of at least three different classes of cell-surface proteins—integrins, cadherins, and immunoglobulin superfamily CAMs—in mediating cell-cell recognition and differentiation in the nervous system in vivo and in vitro model systems of neuronal differentiation and axonal outgrowth (Neugebauer et al., 1988; Reichert and Tomasselli, 1991; Hynes, 1992; Takeichi, 1991; Edelman and Crossin, 1991; Walsh and Doherty, 1991). While in most studies purified preparations of N-CAM have not been found to be active in promoting neurite outgrowth, NIH3T3 fibroblast monolayers expressing high levels of N-CAM or N-cadherin will stimulate PC12 neurite outgrowth (Doherty et al., 1991; Walsh and Doherty, 1992). Furthermore, the studies of Doherty, Walsh, and their colleagues have suggested that the morphoregulatory activities of N-CAM and N-cadherin involve activation of G-protein-dependent and calcium channel-dependent signaling pathways. Because DCC shares common structural motifs with members of the N-CAM family (Ig-like domains and fibronectin type III repeats), we sought to investigate whether the expression of DCC in NIH3T3 fibroblasts could stimulate PC12 neurite outgrowth, and if so, to explore some of the mechanisms by which this response is regulated. In this report, we demonstrate that DCC can function in a fashion similar to other cell adhesion molecules, like N-CAM and N-cadherin, to stimulate morphological differentiation of PC12 cells. These data support the proposal that the DCC tumor suppressor gene product may function in differentiation pathways and cell fate determination through cell-cell interactions.

Materials and Methods

Plasmid Constructs

The mammalian cell expression vector pCMVNeoXhoI was used to generate all DCC expression constructs (see Fig. 1). In this vector cDNA expression is driven by a potently regulated retrovirus promoter/enhancer element; the vector also confers resistance to G418 in mammalian cells under control of herpes simplex thymidine kinase promoter and poly-adenylation elements (Baker et al., 1990). The vector pCMV/DCC-S contains full-length DCC cDNA sequences (nucleotides −15 to 4496), encoding amino acids 1 to 1447. The pCMV/DCC-T vector encodes a truncated form of DCC lacking amino acids 1156-1447; the extracellular domain of DCC is predicted to begin at amino acid 1121. A vector encoding an extremely truncated (mutant) DCC polypeptide with only the signal sequence and a portion of the first immunoglobulin-like domain of DCC (pCMV/DCC-M) was generated by deleting the two central nucleotides from a unique StuI site at nucleotide 189. The ~50-amino acid polypeptide produced from cells expressing DCC-M is predicted to be secreted into the media. Superoxoled plasmid DNAs were purified on CsCl gradients.

Cell Lines

NIH3T3 cells were obtained from American Type Culture Collection (Rockville, MD) and were routinely maintained in DME supplemented with 10% fetal bovine serum (FBS), 100 IU/ml of penicillin and 100 µg/ml of streptomycin (P/S) at 37°C/5% CO2 (GIBCO BRL [Life Technologies, Inc.] Gaithersburg, MD). PC12 cells were obtained from ATCC and were maintained in RPMI 1640 medium supplemented with 5% FBS, 10% horse serum (heat inactivated at 56°C for 30 min) and P/S at 37°C/5% CO2. For the neurite outgrowth assay both PC12 and NIH3T3 cells were cultured in SATO media (Bottenstein, 1985), containing DME (GIBCO BRL), progesterone (0.062 µg/ml), putrescine (16.6 µg/ml), thyroxine (0.4 µg/ml), selenium (0.039 µg/ml), human transferrin (100 µg/ml), bovine pancreas insulin (10 µg/ml), and triiodothyronine (0.377 µg/ml) (all reagents other than DME from Sigma Chemical Co., St. Louis, MO).

DNA Transfections

To generate NIH3T3 cell lines expressing the various DCC cDNA constructs, DNA transfections were carried out using Lipofectin Reagent (GIBCO BRL). In brief, NIH3T3 cells were transfected with a mixture of 3 µg of plasmid DNA and cationic liposomes in serum-free media. After an overnight incubation, the cells were washed and complete media was added. On the second day after transfection, selection in G418 at a concentration of 600 µg/ml was begun. Single G418-resistant clones were tried and established as clonal cell lines. Once established, the clonal cell lines were maintained in G418 at 400 µg/ml.

Ribonuclease Protection Assay

Total RNA was isolated from the various cell lines as described previously (Chomczynski and Sacchi, 1987). A radiolabeled DCC antisense riboprobe transcript was prepared from the plasmid pBSII/DCC-RR, which contains a 400-bp EcoRI-BamHI DCC cDNA fragment (containing sequences encoding the distal extracellular, transmembrane, and proximal cytoplasmic domains of DCC—amino acids 1021-1155), using T7 RNA polymerase (GIBCO BRL). The approximately 480-bp 32p-labeled transcript was purified by electrophoresis, and 50 × 10⁶ cpm of transcript was incubated overnight at 48°C with 25 µg of total RNA in hybridization solution (80% deionized formamide; 40 mM 1,4-piperazinediethane sulfonic acid [Pipes], pH 6.4; 400 mM NaCl; 1 mM EDTA). Ribonuclease digestion was then carried out for 1 h at 30°C using 7.5 U of RNase T2 (GIBCO BRL) in 250 µl of digestion buffer (50 mM sodium acetate, pH 4.4; 100 mM NaCl; 10 mM EDTA). RNA was precipitated with isopropanol, resuspended in RNA loading buffer (90% deionized formamide; 10 µM EDTA; 0.2% bromophenol blue; 0.2% xylene cyanol), heated for 3 min at 90°C, and electrophoresed on a denaturing polyacrylamide gel. After drying of the gel, autoradiography was carried out with intensifying screens and Hyperfilm (Amersham Corp., Arlington Heights, IL).

Western Blot Analysis of DCC Expression

Western blot analysis was carried out on lysates from the various NIH3T3 cell lines. Confluent monolayers of cells were washed and lysed directly in Laemmli sample buffer or in RIPA buffer (25 mM Tris[hydroxymethyl]-}

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aminomethane, 150 mM NaCl, 1% deoxycholate, 0.1% sodium dodecyl sulphate, 1% nonidet P-40) supplemented with 10 μg/ml phenylmethylsulfonyl fluoride (PMSF), 50 μg/ml antipain, 5 μg/ml aprotinin, and 2 μg/ml leupeptin (all protease inhibitors purchased from Sigma Chemical Co.). Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL). Proteins were separated by electrophoresis on SDS/polyacrylamide gels and transferred to Immobilon P membranes (Millipore Corp., Bedford, MA) by semi-dry electroblotting. DCC proteins were detected using each of three different affinity-purified, polyclonal, rabbit anti-DCC antisera as the primary antibody and horseradish peroxidase as the secondary reagent. One of the anti-DCC antisera was directed against an extracellular domain peptide epitope derived from sequences located between the sixth fibronectin type III repeat and the transmembrane region of DCC (sera 645); another anti-DCC antisera was directed against a juxtamembrane cytoplasmic domain peptide epitope (sera 641); and the third anti-DCC antisera was directed against a recombinantly produced fusion protein containing cytoplasmic domain sequences (sera 721). Detection of antibody complexes was carried out with the Enhanced Chemiluminescence (ECL) Western Blot Kit (Amersham Corp.) and subsequent exposure to Hyperfilm (Amersham Corp.).

**Immunofluorescence Studies of DCC Expression in Transfected Cells**

NIH3T3 cell lines were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature. The cells were then washed and stained with a primary mouse monoclonal antibody directed against DCC extracellular domain sequences (Oncogene Science, Manhasset, NY) at a concentration of 0.2 μg/ml in PBS and a secondary, rhodamine-conjugated, goat anti-mouse immunoglobulin antibody (Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA) at a concentration of 1.5 μg/ml. Coverslips were then mounted with Gel-Mount (Biomeda Corp., Foster City, CA), and the cells were examined and photographed in phase and under fluorescence optics on a Nikon FAX microscope.

**Cell Surface Biotinylation**

NIH3T3 cell lines were grown to 80% confluence in 75-cm² tissue culture flasks. The cells were washed twice with PBS, and 3 ml of biotinylation buffer (10 mM sodium borate, 150 mM NaCl, pH 8.5) and 15 μl of 10 mg/ml D-biotinyl-epilson-amidocaproic acid N-hydroxysuccinimide ester (biotin-CNHS-ester; Boehringer Mannheim Corp., Indianapolis, IN; freshly made and dissolved in dimethyl sulfoxide) were added to the flask (Meier et al., 1992). The flask was rocked gently at room temperature for 15 min, and 30 μl of 1 M NH₄Cl was then added to terminate the reaction. The cells were washed twice with PBS and lysates were prepared in RIPA buffer with protease inhibitors. Immunoprecipitations were carried out with affinity-purified rabbit anti-DCC antisera 645 and Protein A (Pierce, Rockford, IL) as described (Harlow and Lane, 1991), and the biotinylated proteins were detected using avidin/biotin-conjugated horseradish peroxidase (Pierce) and ECL, as described above.

**Neurite Outgrowth Assay**

**Cell Culture and Reagents.** Approximately 5 × 10⁴ cells of each of the NIH3T3 cell lines were plated in DME/10%FBS and P/S on two-chamber glass slides (Nunc, Roskilde, Denmark), coated with poly-d-lysine and rat tail collagen type I (Collaborative Research, Bedford, MA), and grown to 70% confluence at 37°C/5% CO₂. To establish the NIH3T3 and PC12 co-cultures, DME/10%FCS was removed and 5 × 10⁴ PC12 cells were added to the NIH3T3 cell monolayers in 1 ml of SATO medium (Doherty et al., 1988) per chamber. For the experiments in which neurite growth factor and/or other compounds were administered to the co-cultures, these agents were added at the time of the PC12 cell addition to the NIH3T3 cells. The various reagents included: nerve growth factor subunit 2.5S subunit (GIBCO BRL); cordycepin (1 μM) (Boehringer Mannheim, Indianapolis, IN); pertussis toxin (1 μg/ml) (GIBCO BRL); cholester (1 ng/ml) (GIBCO BRL); dilitazem (10 μM) (Sigma Chemical Co.); and a-conotoxin (0.25 μM) (Sigma Chemical Co.).

**Immunofluorescence Detection of PC12 Cells and Scoring of Neurite Outgrowth**

After 48 h of co-culture at 37°C/5% CO₂, the cell cultures were fixed in 4% paraformaldehyde in PBS for 30 min. PC12 cells were stained with the addition of a mouse anti-rat Thy-1 monoclonal antibody (GIBCO BRL) at a dilution of 1:100 in PBS for 1 h. After washing with PBS, cells were incubated sequentially with a goat anti-mouse biotinylated secondary antibody and a streptavidin-Texas red conjugate (Amersham Corp.), each for 1 h at a dilution of 1:500 in PBS. Cells were then washed with PBS, coverslips were mounted, and the Thy-1 PC12 cells were visualized under rhodamine optics on a Nikon FAX microscope. The percentage of Thy-1 PC12 cells with at least one neurite >20 μm in length was determined. An eyepiece with a micrometer scale was used to assess the 20 μm length. For each experiment, >100 randomly selected Thy-1 PC12 cells were studied in each chamber. For each of the various inhibitor treatments, three to seven independent experiments were performed. For the experiments displayed in Figs. 5, 6, and 7, all slides were blinded with regard to the NIH3T3 cell line used and the particular treatment before determination of the percentage of Thy-1 cells with neurites greater than 20 μm in length. To characterize the length of the longest neurite per cell (Table I), PC12 cells were cultured on the NIH3T3 cell monolayers and stained as described above. The longest neurite on each of 100 Thy-1 PC12 cells was measured using a Zeiss Axiopt microscope and a Sony DCC-760 MD CCD camera connected to a Sony Trinitron monitor.

**Supernatant Experiments**

The transfected NIH3T3 DCC-expressing cell line 1A1 and the control cell line 3A3 were grown to 70% confluence in DME/10% FCS/PS on two-chamber slides, as described above. The DME/10% FCS was then removed, the cells were washed once with SATO media, and 1 ml of SATO media, with or without the supplementation of 50 mg/ml NPG, was added. After 48 h of culture, the conditioned SATO media was removed, filtered through 0.2 μm polycarbonate, low protein-binding filters (Acrodisc, Gelman Sciences, Ann Arbor, MI), and used as the media for PC12 neurite outgrowth in separate co-culture experiments as described above.

**Results**

**Generation of NIH3T3 Cell Lines Expressing DCC Proteins**

NIH3T3 cells were transfected with a eukaryotic expression vector encoding a full-length human DCC cDNA under control of a cytomegalovirus promoter (Hedrick, L., K. R. Cho, E. R. Fearon, T.-C. Wu, K. W. Kinzler, and B. Vogelstein, manuscript submitted for publication). This vector also confers resistance to G418. In addition, two constructs encoding altered forms of DCC were also transfected into NIH3T3 cells (Fig. 1). One cDNA, termed DCC-T, encodes a protein lacking the 292 carboxy-terminal amino acids of DCC. The other (DCC-M) encodes a severely truncated form of DCC containing only the signal sequence and a portion of the first immunoglobulin-like domain. Assuming removal of the signal sequence, only a 50-amino acid polypeptide would be produced and secreted into the media from cells expressing the DCC-M cDNA construct, despite the fact that the DCC mRNA produced from the vector is only subtly different from that produced from DCC-S (see Materials and Methods). After transfection of the constructs into NIH3T3 cells, G418-resistant colonies were selected. Upon expansion of these colonies into clonal cell lines, the cell lines were tested for DCC gene expression using anti-sense RNA probes and a ribonuclease (RNase) protection assay. A 480-bp riboprobe containing DCC sequences was synthesized and hybridized to RNA prepared from a number of cell lines, including the parental NIH3T3 cells, the G418-resistant transfected cell lines, and IMR32, a human neuroblastoma cell line that expresses endogenous DCC transcripts. As shown in Fig. 2A, a heterogeneous pattern of protected RNA fragments was observed in all transfected NIH3T3 cell lines; however, protected RNA fragments were not seen in control

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RNA preparations, such as yeast RNA or parental NIH3T3 cells. The level of endogenous DCC transcripts in the IMR32 cell line was well below that seen in the transfected cells, and protected fragments could only be detected after long exposure. The basis for the heterogeneous pattern of transcripts detected by the RNase protection assay of the transfected NIH3T3 cell lines is unknown. However, multiple different DCC transcripts may be synthesized in the transfected lines as a result of the integration of both intact and rearranged DCC cDNA sequences following DNA transfection.

**DCC Proteins Are Expressed on the Cell Surface**

Based on the predicted amino acid sequence of DCC, both the full-length and truncated DCC (DCC-T) proteins would be expected to be expressed on the cell surface. To demonstrate that the NIH3T3 cell lines expressing DCC proteins properly sorted and delivered the DCC protein products to the cell surface was next performed using two different, affinity-purified, polyclonal rabbit anti-DCC antibodies. Antibodies 641 is directed against a juxtamembrane peptide epitope from the cytoplasmic domain of DCC; this antibody would be expected to react with both the full-length and truncated (DCC-T) forms of DCC. In contrast, antibody 721 was generated against a bacterially-produced recombinant fusion protein containing the DCC cytoplasmic domain sequences that were deleted from the truncated DCC (DCC-T) construct. Neither of these two antibodies would be expected to react with the ~50-amino acid mutant DCC polypeptide predicted to be produced by the DCC-M cDNA. As shown in Fig. 2, B and C, the full-length DCC protein was expressed at high levels in NIH3T3 cell lines 1A1 and 1A2, as well as in IMR32 cells, but was expressed at quite reduced levels in clone 1E2. The DCC-T protein was detected in the cells transfected with the DCC-T cDNA construct (cell lines 2A3 and 2B2), although DCC-T expression in the 2B2 cells could only be detected after long exposure (Fig. 2 C and data not shown). No DCC protein was detected in the cells transfected with the DCC-M cDNA construct (cell lines 3A3 and 3A6).

**Figure 2.** DCC gene and protein expression in the transfected NIH3T3 cell lines. (A) Ribonuclease (RNase) protection assay of DCC gene expression in transfected cell lines and controls. Approximately 25 μg of total RNA from each sample was subjected to RNase protection analysis with a 480-bp 32P-labeled DCC antisense RNA probe. Approximately 1,500 cpm of the undigested probe is shown at the left. Protected fragments were seen in the transfected cell lines at this exposure, and in IMR32 upon long exposure. No protected fragments were seen in the parental NIH3T3 (3T3-wt) cell RNA or the yeast torula RNA (tRNA). The position of the predicted 400 bp protected DCC-specific fragment is indicated by the arrowhead. (B) Western blot analysis of DCC expression using a rabbit polyclonal antiserum (721) directed against DCC cytoplasmic domain sequences. Approximately 100 μg of total protein was loaded on each lane and SDS-PAGE was carried out on an 8% gel. After transfer of proteins to Immobilon membranes, DCC protein was detected by ECL. The mobility of molecular weight markers (in kilodaltons [kD]) is indicated at the left. The full-length DCC protein, migrating at approximately 170-185 kilodalton (kD), is detected in the transfected cell lines 1A1 and 1A2, and the neuroblastoma line IMR32. A cross-reactive band at ~110 kD is detected in all cell lines. Note that the ECL detection system is non-linear, and based on other exposures, the level of DCC expression in IMR32 is significantly less than those seen in the 1A1 and 1A2 transfected cell lines. (C) Western blot analysis of DCC expression using a rabbit polyclonal antiserum (641) raised against a DCC cytoplasmic, juxtamembrane peptide. Immunoreactive proteins were detected by ECL. The position of molecular weight markers (in kilodaltons [kD]) is indicated at the left. The full-length DCC protein detected in the 1A1, 1A2, and 1E2 cell lines is indicated. The DCC-T protein has a mobility of ~140 kD. DCC-T expression in 2B2 cells could be detected in longer exposures.
Figure 3. DCC is expressed on the surface of the transfected NIH3T3 cells. (A) Cells were fixed in 4% paraformaldehyde and stained with a mouse monoclonal antibody against DCC extracellular domain sequences and a rhodamine-conjugated goat anti-mouse immunoglobulin antibody. Cells were photographed under fluorescence (a and b) or phase (c and d) optics; (a and c) IA1 cells; (b and d) 3A3 cells. Specific staining was seen only in IA1 cells. (B) Biotinylation of full-length and truncated DCC proteins. Biotinylation of cell surface proteins was carried out in vitro. Cell lysates were subjected to immunoprecipitation with control purified rabbit immunoglobulin (IgG) or a polyclonal rabbit antisera (645) directed against an extracellular domain DCC peptide; and immunoprecipitates were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to an Immobilon membrane. Biotinylated cell surface proteins were detected by an avidin/horseradish peroxidase reagent and ECL. Full-length DCC protein was detected in IA1 cells; truncated DCC protein was seen in 2A3 and 2B2 cells; and no DCC protein was detected in the 3A3 cells. Several lower molecular weight protein species were also detected; some may represent partially proteolyzed DCC polypeptides. The mobility of molecular weight markers (in kD) is indicated at the left. Bar, 25 µm.

In the cell surface, we performed immunofluorescence studies with anti-DCC antibodies on the cell lines, after fixation, but without permeabilization of the cells. As shown in Fig. 3 A, a mouse monoclonal antibody directed against an extracellular domain epitope of DCC stained the DCC-expressing IA1 cells (a), but did not demonstrate specific staining of the 3A3 cells (b). The 2A3 and 2B2 cell lines did not demonstrate staining above background levels with the monoclonal anti-DCC antibody (data not shown). However, as demonstrated previously by Western blotting, the level of DCC-T protein expression in the 2A3 and 2B2 cell lines was reduced compared to the level of full-length DCC protein expression seen in the IA1 cells. Therefore, to demonstrate that the truncated DCC protein was also expressed on the cell surface, biotinylation of cell surface proteins in several of the NIH3T3 cell lines was carried out in vitro. Cell lysates were then prepared and immunoprecipitated with an antibody directed against an extracellular domain epitope of DCC was performed. Both the full-length and truncated DCC proteins were found to be biotinylated (as evidenced by their reactivity with an avidin reagent when transferred to a membrane), confirming that both full-length and truncated DCC proteins are expressed at the cell surface (Fig. 3 B). Moreover, the relative abundance of total DCC protein to cell-surface DCC protein appeared to be similar in the IA1 and 2A3 cell lines (compare Fig. 3 B with Fig. 2 C).
DCC Expression in NIH3T3 Cells Stimulates Neurite Outgrowth in PC12 Cells

We next sought to determine whether the cell lines expressing the full-length DCC protein could stimulate neurite outgrowth in PC12 cells. In brief, PC12 cells were plated in defined media on monolayers of each of the various NIH3T3 cell lines, including the parental NIH3T3 cells; the full-length DCC-expressing cell lines IA1, IA2, and IE2; and the control G418-resistant cell lines 3A3 and 3A6. After 48 h of co-culture, in the presence or absence of NGF supplementation, the degree of neuronal differentiation in PC12 cells staining positively for the Thy 1 antigen (Thy1+) was assessed by immunofluorescent microscopy. As the antibody to Thy1 only binds to the PC12 cells, PC12 cells growing on the NIH3T3 cell monolayers could be rapidly detected and neuronal differentiation assessed. In Fig. 4, representative examples of Thy1+ PC12 cells obtained after several different culture conditions are shown. The majority of Thy1+ PC12 cells failed to exhibit a significant degree of neurite outgrowth when cultured on monolayers of control NIH3T3 cell lines in the absence of NGF (Fig. 4 A). Supplementation of the defined growth media with NGF, however, stimulated significant neurite outgrowth in ~30% of the Thy1+ PC12 cells (Fig. 4 B). Moreover, co-culture of PC12 cells on DCC-expressing NIH3T3 cell lines, even in the absence of NGF addition, led to detectable neurite outgrowth in ~35–40% of the PC12 cells (Fig. 4 C).

To quantitate the effect, the percentage of PC12 cells undergoing morphological neuronal differentiation was determined after 48 h of co-culture on a monolayer of each of the various cell lines, in the presence or absence of NGF. Thy1+ PC12 cells were scored positively for neuronal differentiation if they had at least one neurite greater than 20 μm in length. This criteria for PC12 neuronal differentiation has been used previously in experiments demonstrating that N-CAM and N-cadherin can stimulate PC12 neurite outgrowth (Doherty et al., 1991; Walsh and Doherty, 1992). In the experiments shown in Fig. 5, 21.7% ± 1.4% (mean ± SEM) of the Thy1+ PC12 cells underwent neuronal differentiation when cultured, in the absence of NGF, on the control 3A3 cells. This background level of PC12 neurite outgrowth may result from the synthesis of NIH3T3 cells of a variety of soluble factors, such as fibroblast growth factors, or extracellular matrix components, such as fibronectin and laminin, which are capable of inducing neurite outgrowth in PC12 cells (Togari et al., 1985; Lumsden and Davies, 1986; Heffner et al., 1990; Reichardt et al., 1990; Sanes et al., 1990; Walsh and Doherty, 1992). After NGF addition to control 3A3 cell monolayers, the percentage of PC12 cells with at least one neurite >20 μm in length increased to 31.7% ± 1.1%. In the absence of NGF addition, the various DCC-expressing NIH3T3 cell lines induced 31.5% ± 0.3% (IA2 cells) to 39% ± 1.7% (IA1 cells) of PC12 cells to undergo neuronal differentiation. After addition of NGF to the co-cultures containing DCC-expressing NIH3T3 cells, the percentage of PC12 cells undergoing neuronal differentiation increased to 37.5% ± 1.1% to 42% ± 0.5%.

Thus, NIH3T3 cell lines expressing DCC stimulated neurite outgrowth in PC12 cells, and NGF appeared to have an additive effect on this process. Other control cell lines, such as the parental NIH3T3 cells and NIH3T3 cell lines transfected solely with a G418-resistance element, showed essentially the same results as the 3A3 control cell line (data not shown). When compared with the control 3A3 cell monolayer, the effect of DCC expression on stimulation of neurite outgrowth was highly significant (P < 0.001). In addition, the relative level of DCC expression in the transfected NIH3T3 cells did not appear to be particularly critical, as NIH3T3 cell lines that varied greatly in their level of expression of DCC protein (IA1 vs. IE2) induced a similar percentage of Thy1+ PC12 cells to undergo neuronal differentiation. The variation in percentage of Thy1+ PC12 cells with at least one neurite >20 μm in length between the various DCC-expressing NIH3T3 cell monolayers was not statistically significant. The variation observed between the DCC-expressing cell lines might simply reflect clonal variations in other factors which may influence neurite outgrowth, such
NIH3T3 cell lines expressing full-length DCC stimulate PC12 neurite outgrowth. The results of PC12 co-culture experiments, in the presence (50 ng/ml) or absence of NGF, on control NIH3T3 (3A3), three DCC-S-expressing (1A1, 1A2, 1E2), and two DCC-T-expressing (2A3, 2B2) cell monolayers are shown. The percentage (±SEM) of Thy1+PC12 cells with at least one neurite >20 μm in length after 48 h of culture is indicated. Each value was derived from the analysis of more than 100 Thy1+PC12 cells in two to four independent experiments. All experiments were blinded with regard to the identity of the NIH3T3 cell monolayer prior to the assessment of PC12 neuronal differentiation.

The Cytoplasmic Domain of DCC Is Required for Mediating Neurite Outgrowth

Previous studies of several CAMs, including E-cadherin and N-cadherin, have established that the cytoplasmic domain sequences are critical for mediating appropriate cell–cell interaction (Takeichi, 1991). Thus, we sought to determine whether the cytoplasmic domain of DCC was necessary for inducing neurite outgrowth in PC12 cells. The 2A3 and 2B2 cell lines expressing the DCC-T protein were assayed for their ability to stimulate neurite outgrowth in PC12 cells. As shown in Fig. 5, in the absence of NGF, the 2A3 and 2B2 cell lines failed to stimulate neurite outgrowth above the levels seen with the 3A3 control cell line. We also found that the 2A3 and 2B2 cell lines failed to increase the mean neurite length of the longest neurite per cell (Table I). As demonstrated above (Fig. 3B), both the 2A3 and 2B2 cell lines express DCC-T protein on the cell surface. Thus, a possible explanation for the results is that the absolute levels of cell surface DCC expression in the 2A3 and 2B2 lines may not be sufficient for inducing PC12 neurite outgrowth. However, the 1E2 cell line, which expressed less total DCC protein than 2A3 (see Fig. 2C), was able to induce PC12 neurite outgrowth despite low level expression of DCC. The data, therefore, suggest that DCC cytoplasmic domain sequences are necessary for inducing PC12 neurite outgrowth.

Supernatants from DCC-expressing Cells Fail to Stimulate Neurite Outgrowth

To demonstrate that the stimulation of PC12 neurite outgrowth by DCC resulted from cell–cell interactions and not from a soluble factor(s) secreted by the DCC-expressing cells, we determined if supernatants from the 1A1 cell line were sufficient for induction of PC12 neurite outgrowth. Monolayers of DCC-expressing 1A1 cells and the control cell line 3A3 were prepared in the same manner as for the co-culture experiments, except that no PC12 cells were added. After culturing the monolayers for 48 h in the SATO media, at least one neurite greater than 20 μm was used to analyze all further studies. As expected NGF treatment increased the mean neurite length of the longest neurite per cell in all cultures (Table I).

Table I. Mean Neurite Length of the Longest Neurite per PC12 Cell When Plated on Control or DCC-expressing NIH3T3 Cell Monolayers

| Monolayer cell line | Neurite length (μm)/cell* (-NGF) | Neurite length (μm)/cell* (+NGF) |
|---------------------|---------------------------------|---------------------------------|
| DCC-M-transfected (control) (3A3) | 11.7 ± 2.2 | 28.7 ± 4.2 |
| DCC-S-transfecteds (1A1) | 15.7 ± 2.1 | 35.6 ± 3.9 |
| (1E2) | 17.0 ± 2.5 | 24.8 ± 3.6 |
| (1A2) | 16.9 ± 2.7 | 24.7 ± 3.7 |
| DCC-T-transfecteds (2A3) | 8.1 ± 1.6 | 13.1 ± 2.1 |
| (2B2) | 10.6 ± 2.2 | 19.1 ± 2.6 |

* Mean ± standard error of the mean; 100 Thy1+PC12 cells analyzed for each monolayer and each NGF condition.
† NGF supplemented at 50 ng/ml in the culture media.
Supernatants from DCC-expressing cells fail to stimulate PC12 neurite outgrowth. Conditioned media (supematant) was obtained from the indicated cell lines after 48 h of culture, in the presence (50 ng/ml) or absence of NGF. The conditioned media was then used for culture of PC12 cells on monolayers of control 3A3 cells or DCC-expressing 1A1 cells. The mean percentage (±SEM) of Thy1+ PC12 cells with at least one neurite >20 μm in length was determined from three independent experiments and is indicated. The identity of all experiments was blinded with respect to the transfected NIH3T3 monolayer and treatment, before assessment of PC12 neuronal differentiation.

The conditioned media was collected, filtered through a 0.2-μm filter, and then used for co-culture experiments with monolayers of either 1A1 or 3A3 cells. As shown in Fig. 6, conditioned media collected from the 1A1 cells appeared to have a minimal effect on the induction of neurite outgrowth when PC12 cells were co-cultured on 3A3 monolayers. To demonstrate that soluble neurite-inducing factors like NGF would still retain potency after the prolonged incubation in SATO media, NGF was added at 50 ng/ml to the 1A1 and 3A3 monolayer cultures before the 48-h incubation period used for conditioned media collection. Even after 48 h, conditioned media obtained from cultures supplemented with NGF retained most of the potency expected of NGF for induction of neurite outgrowth (Fig. 6). Thus, the data suggest that supernatants from DCC-expressing NIH3T3 cells are not sufficient for induction of neurite outgrowth, and that DCC-mediated PC12 neurite outgrowth requires interaction with cells expressing DCC.

The DCC-mediated Response Is Not Dependent on mRNA Synthesis

Morphological differentiation of PC12 cells in response to NGF treatment requires de novo synthesis of mRNA (Greene, 1984). To determine whether DCC-mediated PC12 neurite outgrowth was a transcription-dependent process, we compared the effect of cordycepin, an inhibitor of polyadenylation, on neurite outgrowth mediated by DCC-expressing 1A1 and control 3A3 monolayers. As was previously observed (Doherty et al., 1991), cordycepin substantially inhibited the NGF-mediated response when PC12 cells were plated on control cells (Fig. 7). Specifically, 30.2% ± 0.6% of the Thy1+ PC12 cells were stimulated to differentiate when 50 ng/ml NGF was added to co-cultures with control 3A3 cells, while only about 24.2% ± 1.4% of Thy1+ PC12 cells had undergone morphological differentiation when cordycepin was added. Of note, the background level of neurite outgrowth seen on control 3A3 cells in the absence of NGF addition was not changed appreciably by cordycepin treatment (19.5% ± 0.6% vs. 18.1% ± 0.5%). Moreover, as shown in Fig. 7, the DCC-mediated response was not inhibited by cordycepin treatment (38.4% ± 0.7% vs. 37% ± 0.7%); however, the additive effect of NGF on neurite outgrowth induced by the DCC-expressing 1A1 cells was lost upon treatment with cordycepin (42.4% ± 1.3% vs. 37.3% ± 1.0%). The effect of cordycepin on NGF-mediated outgrowth on control 3A3 monolayers was statistically significant (P < 0.02), but no statistically significant effect on DCC-mediated outgrowth was noted. These results suggest that DCC-stimulated neurite outgrowth, unlike NGF-mediated outgrowth, does not require de novo transcription.

Inhibition of DCC-mediated Neurite Outgrowth by Pertussis Toxin and Calcium Channel Antagonists

Previous studies of N-CAM- and N-cadherin-mediated PC12 neurite outgrowth have shown that CAM-dependent neurite outgrowth, but not NGF-mediated outgrowth, could be inhibited by pertussis toxin and calcium channel antagonists. Thus, CAM-mediated neurite outgrowth may be effected through the activation of L- and N-type calcium channels, as well as through a pertussis toxin-sensitive G protein (Doherty et al., 1991; Walsh and Doherty, 1992). To identify intracellular signaling pathways involved in DCC-mediated neurite outgrowth, studies were undertaken to determine if treatment with pertussis toxin or calcium channel blockers...
inhibited DCC-mediated neuritogenesis. Pertussis toxin has been shown to ribosylate and inhibit the function of a variety of trimeric G proteins of the G_{i/o} family. We observed that pertussis toxin treatment had little effect on the background level of neurite outgrowth on 3A3 control monolayers, and also failed to inhibit significantly NGF-stimulated neurite outgrowth on 3A3 monolayers (Fig. 7). In contrast, pertussis toxin strongly inhibited the DCC-mediated response (38.4% ± 0.7% vs. 22.4% ± 1.6%; P < 0.001). In addition, in the presence of pertussis toxin, NGF addition to DCC-expressing 1A1 monolayers failed to fully restore the response to control levels (Fig. 7). Thus, the ability of DCC to stimulate neurite outgrowth appears to be dependent upon a pertussis-toxin-sensitive signaling pathway(s).

To determine whether DCC-mediated neurite outgrowth was a calcium channel-dependent process, we studied the effect of the combination of the L-type channel blocker diltiazem and the N-type channel blocker ω-conotoxin. The agents were studied only in combination, as previous studies had suggested that the effect of the two classes of calcium channel antagonist were additive, and that CAM-mediated neurite outgrowth was strongly inhibited by the combination (Doherty et al., 1991). The combination of calcium channel antagonists had little effect on the background level of PC12 neurite outgrowth induced by control 3A3 cells, nor was there any inhibition of NGF-mediated outgrowth on 3A3 monolayers (Fig. 7). The DCC-mediated response was inhibited by the Ca^{2+} antagonist combination (38.4% ± 0.7% vs. 29.3% ± 2.2%; P < 0.005), but less markedly than was seen with pertussis toxin treatment. These results suggest that Ca^{2+} channel blockers, while having no effect on NGF-mediated neurite outgrowth, inhibit DCC-mediated neurite outgrowth in PC12 cells.

**Discussion**

While previous studies have suggested that the loss of DCC gene function may be critical to the later stages of colorectal cancer development, and perhaps to the development of other cancers, little is known about the means by which DCC may function to mediate tumor suppression. Similarly, the function of the DCC gene in development and cell differentiation is unknown. In the studies described here, we have used a transfection-based approach to examine the role of DCC in mediating neuronal cell differentiation in an in vitro model system. Our studies suggest that DCC functions to stimulate PC12 neurite outgrowth in a fashion similar to well-characterized CAMs, such as N-CAM and N-cadherin. The stimulation of PC12 morphological differentiation by DCC appears to require cell–cell interaction. In addition, our results suggest that the cytoplasmic domain of DCC is critical for inducing differentiation. Furthermore, like N-CAM- and N-cadherin–mediated PC12 neuritogenesis, the induction of neurite outgrowth by DCC appears to be transcription-independent, and utilizes pertussis toxin-sensitive G-protein(s) and calcium channel-dependent signaling pathways. Thus, in addition to the predicted structural similarity of the extracellular domain sequences of DCC to those of a number of N-CAMs, DCC may share some similarities in its ability to alter cell phenotype through cell-cell interactions.

A sizeable number of different CAMs can induce contact-dependent neuritogenesis in PC12 cells and other neuronal cell types. In addition to N-CAM, N-cadherin, and DCC, neurite outgrowth has been shown to be supported by non-neuronal cells expressing L1, F3/F1/contactin, and the myelin-associated glycoprotein MAG or P (Williams et al., 1992; Gennarini et al., 1991; Johnson et al., 1989; Yazaki et al., 1991). Neurite outgrowth can also be triggered by the binding of anti-Thy-1 antibodies to PC12 cells. Similar intracellular signaling pathways may be activated by a number of these CAM interactions, because like N-CAM–, L1–, N-cadherin–, and DCC-mediated neurite outgrowth, the induction of neurite outgrowth by anti-Thy-1 antibodies could be inhibited by the addition of N- and L-type calcium channel blockers or by the addition of pertussis toxin (Doherty et al., 1993). Nevertheless, not all neurite-inducing agents use the signaling pathways employed by N-CAMs and N-cadherin, as neither integrin-mediated nor NGF-mediated PC12 neurite outgrowth is inhibitable by pertussis toxin or calcium channel blockers (Bixby and Jhabvala, 1990; Walsh and Doherty, 1992).

The role of kinases in N-CAM– and N-cadherin–mediated neurite outgrowth has also been studied using a variety of kinase inhibitors (Doherty et al., 1991). Staurosporine and H7, both of which are potent inhibitors of protein kinase C and several other protein kinases (Hidaka et al., 1984), showed no inhibitory effect toward N-CAM and N-cadherin responses (Doherty et al., 1991). In contrast, K-252b, which is an inhibitor of cAMP- and cGMP-dependent kinases (Kozumi et al., 1988), strongly inhibited neurite outgrowth by N-CAM and N-cadherin (Doherty et al., 1991). In addition, these authors noted that K-252b also lowered the background level of neurite outgrowth promoted by control fibroblast monolayers. We found that K252b at 200 ng/ml demonstrated no specificity in its inhibition of neurite outgrowth mediated by DCC, NGF, or control fibroblasts. Similarly, K-252a, which is a more potent inhibitor of cAMP- and cGMP-dependent kinases than K-252b, was not useful for demonstrating a role for specific protein kinases in the DCC-mediated response (Pierceall, W. E., and E. R. Fearon, unpublished observations). Thus, at the present, little is known about the role of various protein kinases in DCC-induced neurite outgrowth.

Given that control NIH3T3 fibroblast monolayers exhibit a relatively consistent background effect on inducing PC12 neurite outgrowth, it has been proposed that the exogenous expression of CAMs in the cells stimulates increased outgrowth from predetermined neurites on the PC12 cells (Walsh and Doherty, 1992). The NIH3T3 cell monolayer might then serve as a permissive substrate for CAM-mediated neurite outgrowth. However, transfected NIH3T3 cell monolayers expressing CAMs do not appear to be a unique non-neuronal cell type for stimulating neurite outgrowth (Gennarini et al., 1991). In this regard, we have also examined the ability of Chinese hamster ovary (CHO) cells expressing DCC to induce neurite outgrowth in PC12 cells. These cells were capable of inducing neurite outgrowth above the background level seen with control CHO cell lines; however, the absolute magnitude of both the DCC- and NGF-mediated responses was less than that observed with NIH3T3 cell monolayers (Pierceall, W. E., and E. R. Fearon, unpublished observations). In addition, in the studies reported here we observed no clear-cut relationship between the level of

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DCC expression in the transfected NIH3T3 cell lines and the ability of those lines to stimulate neurite outgrowth. This may reflect the fact that lines expressing the full-length DCC construct expressed more than the threshold level of DCC needed to stimulate PC12 neurite outgrowth.

The failure of supernatants from DCC-transfected cell lines to induce neurite outgrowth in PC12 cells cultured on control fibroblast monolayers suggests that DCC-induced neurite outgrowth is mediated predominantly by cell-cell contact. To date, no ligand has been identified for DCC. Preliminary studies suggest that DCC expression does not increase cell-cell aggregation when expressed in NIH3T3 cells (Fearon, E. R., unpublished observations). However, as reviewed above, anti-sense experiments have suggested that DCC expression in PC12 cells is necessary for NGF-mediated neurite outgrowth (Lawlor and Narayan, 1992). Thus, a role for homotypic interactions between DCC molecules in PC12 neurite outgrowth perhaps should not be excluded at this time. Based on the studies described here, we infer that PC12 cells synthesize a ligand(s) for DCC. In addition, our studies suggest that the cytoplasmic domain of DCC is necessary for stimulation of PC12 neurite outgrowth. Presumably, this reflects the fact that the DCC cytoplasmic domain is necessary for the appropriate functional interaction between DCC on the NIH3T3 cell surface and its ligand(s) on the PC12 cells, perhaps mediated by proper attachment of DCC to cytoskeletal elements in the NIH3T3 cells.

Further studies will be necessary to define the DCC sequences that are responsible for inducing neuronal differentiation in PC12 cells. Presently, little is known about which domains in other N-CAM family members are necessary for stimulation of neuritogenesis. However, recent data indicate that the fourth immunoglobulin domain of the N-CAM molecule is involved in neurite outgrowth (Horstkorte et al., 1993). Specifically, neurite outgrowth was blocked when oligomannosidic glycans specific for binding to the fourth Ig domain of N-CAM were added to the cultures; no effect was observed when unrelated oligosaccharides or oligosaccharide derivatives were added. The blockage of neurite outgrowth may be due to a destabilization of a N-CAM/L1 complex that interacts with L1 on opposing cells (Horstkorte, 1993). Note, the sequences in the fourth immunoglobulin domain of N-CAM implicated in this process match the consensus for the eight carbohydrate recognition domains of the human mannose receptor (consensus-Cxxxxxxx[V/I/L][S/T][V/I]xxxx[E/S] [Taylor et al., 1990]). The fourth immunoglobulin domain of DCC has a sequence which shares similarity with this consensus (CTVSGKPVPVTYVM-KN). It will be of interest to determine if DCC uses this sequence to participate in cis-associations with other surface molecules like L1 or N-CAM to mediate neurite outgrowth.

In summary, our studies have provided evidence that DCC functions in a fashion analogous to other N-CAMs by inducing PC12 neurite outgrowth through specific intracellular signaling pathways. Although this suggests a possible physiological role for DCC in effecting changes in cell phenotype through cell-cell interactions, much remains to be learned about the function of DCC in vivo. Further studies of the mechanisms by which DCC stimulates PC12 neurite outgrowth may provide insights into its function in other tissues, including the means by which DCC may mediate differentiation and tumor suppression in epithelial cells.

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