0023-6837/01/8102-201$03.00/0
LABORATORY INVESTIGATION
Vol. 81, No. 2, p. 201, 2001
Printed in U.S.A.

Truncated DCC Reduces N-Cadherin/Catenin Expression and Calcium-Dependent Cell Adhesion in Neuroblastoma Cells

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SUMMARY: The deleted in colorectal cancer (DCC) protein is important in the pathway guidance of cells and cell processes during neural development, and DCC has also been implicated in the aberrant cellular migrations of neuroblastoma dissemination. We attempted to further define DCC protein function by the overexpression of full-length and truncated DCC constructs in a human neuroblastoma cell line. Overexpression of the truncated DCC protein resulted in a less epithelioid morphology. This was accompanied by decreases in expression of N-cadherin and α- and β-catenin by immunoblot and Northern blot analysis. Levels of desmoglein were relatively less affected, whereas endogenous DCC protein levels were increased in the truncated transfectants. N-cadherin immunofluorescence was consistent with the immunoblot studies and localized the protein to the cytoplasm and sites of cell-cell contact. Cell aggregation studies demonstrated diminished calcium-dependent aggregation in the truncated transfectants. In conclusion, overexpression of a truncated DCC protein in neuroblastoma cells resulted in the loss of an epithelioid morphology, diminished expression of N-cadherin and α- and β-catenin, and diminished calcium-dependent cell adhesion. These studies provide the first evidence of an apparent functional link between DCC and N-cadherin/catenin-dependent cell adhesion. (*Lab Invest 2001, 81:201–210*).

The human *DCC* gene is located on chromosome 18q21 and encodes a 1447 amino acid transmembrane protein with extracellular immunoglobulin and fibronectin type III domains typical of the neural cell adhesion molecule (NCAM) family of proteins (Fearon et al, 1990). DCC and neogenin, a protein implicated in chicken neural development, define an NCAM subfamily on the basis of their unique constellation of extracellular domain motifs and cytoplasmic domain (Vielmetter et al, 1994). Several *DCC* homologs have been isolated and cloned from invertebrates and vertebrates (Chan et al, 1996; Keino-Masu et al, 1996; Kolodziej et al, 1996; Pierceall et al, 1994b; Rieger-Christ et al, 1997), and significant conservation of the *DCC* gene in vertebrate evolution has been demonstrated (Pierceall et al, 1994b; Rieger-Christ et al, 1997).

Several recent studies have demonstrated that the DCC protein functions in the guided migration of cells and cell processes during neural development as an integral component of the netrin response pathway (Keino-Masu et al, 1996). Netrins, a family of laminin-related proteins, function as diffusible chemoattractants/repellents for developing axons. Mutational studies in *Caenorhabditis elegans* (Chan et al, 1996; Hedgecock et al, 1990) and *Drosophila* (Kolodziej et al, 1996) have been central in defining this pathway. In *C. elegans*, products of the *unc-5* gene, which encodes a transmembrane protein with some similarity to the ZO-1 tight junction protein (Leonardo et al, 1997), *netrin/unc-6,* and *DCC/unc-40* genes are required for proper dorsoventral migrations of commissural axons. Recent studies of netrin-1 (Serafini et al, 1996) and DCC-deficient (Fazeli et al, 1997) mice have demonstrated defects in commissural axon projections that are essentially identical to those of invertebrates. Moreover, these knockout studies demonstrated defective commissure development in the brain. Importantly, the developmental role of DCC also includes guidance of cell migrations. Migrations of the Q neuroblast and other cell types are abnormal in *C. elegans DCC/unc-40* mutants (Chan et al, 1996). Also, in $DCC^{-/-}$ mice, the pontine nuclei of the rostral midbrain are absent, apparently due to a mismigration (Fazeli et al, 1997). There is now biochemical evidence for a ligand:receptor relationship between netrins and *DCC* and/or *unc-5* (Keino-Masu et al, 1996; Leonardo et al, 1997). A simplistic model is that DCC mediates attractive responses to a netrin gradient, whereas unc-5

Received October 11, 2000.
This work was supported in part by National Institutes of Health grants CA63297 and CA72894 (to MAR), and GM57604 (to DLR). JAM was supported by a predoctoral fellowship from the Howard Hughes Medical Institute. DLR was supported by the William and Catherine Weldon Donaghue Foundation for Medical Research.
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mediates a repulsive response. Refinement of this model awaits characterization of the receptor complex, because one interpretation is that DCC and unc-5 are modifiers of the actual receptor (Leonardo et al, 1997).

The *DCC* gene has also been implicated in the malignant process as a candidate tumor suppressor. This is based on its location within a region of frequent allelic loss in a variety of malignancies, including neuroblastoma (Takita et al, 1995), limited evidence of mutations within the remaining *DCC* allele, and frequent loss of *DCC* expression in tumor tissue. However, no familial mutations in *DCC* that confer a predisposition to tumor formation have yet been described (Rieger-Christ et al, 1997). Although two xenograft studies have provided evidence in support of a suppressor function for DCC (Klingelhutz et al, 1995; Narayanan et al, 1992), the *DCC* knockout study of Fazeli et al (1997) did not demonstrate this function.

Despite the relative lack of genetic and functional data for *DCC* as a suppressor of tumor formation, there is evidence suggesting a role for *DCC* in tumor dissemination. This includes several demonstrations of reduced *DCC* expression that occur as apparently later events in the malignant process. *DCC* gene expression was reduced or absent in 59% of primary colorectal cancers, whereas 100% of liver metastases were DCC-negative (Iino et al, 1994; Itoh et al, 1993; Kikuchi-Yanoshita et al, 1992). In a study of astrocytomas that subsequently progressed to glioblastomas in individual patients, Reyes-Mugica et al (1997) demonstrated the loss of *DCC* expression with glioma progression in approximately 50% of cases, linking *DCC* loss to development of the highly invasive glioblastoma multiforme. Recent studies of *DCC* expression in neuroblastomas, a pediatric tumor of neural crest origin, showed that patients with DCC-negative primary neuroblastomas more often had disseminated disease (Kong et al, 1997; Reale et al, 1996). Moreover, neuroblastoma metastases were more frequently DCC-negative than primary tumors (Reyes-Mugica et al, 1998).

In light of DCC's role in developmental neuroblast migrations and the relationship between *DCC* expression and neuroblastoma dissemination, we examined the effect of *DCC* overexpression in neuroblastoma cells. We show that overexpression of a truncated form of the DCC protein (lacks the cytoplasmic domain) results in a less epithelioid morphology. This morphologic phenotype is accompanied by reduced expression of the calcium-dependent adherens junction proteins (N-cadherin and α- and β-catenin) and also diminished calcium-dependent cell adhesion. These studies provide the first evidence of an apparent functional link between DCC and these junctional proteins.

Results

DCC *Transfected Neuroblastoma Cells*

The parental SJNB-8 cells are a morphologically homogeneous human neuroblastoma cell line with very low-level endogenous *DCC* expression. The DCC protein expressed in SJNB-8 cells exactly comigrates with the DCC protein of the IMR-32 neuroblastoma cell line in which the DCC cDNA sequence is wild type (Reale et al, 1994) (data not shown). The DCC expression constructs used in this study included a truncated form because previous studies have implicated the cytoplasmic domain in suppression of tumor growth (Klingelhutz et al, 1995) and neurite outgrowth (Pierceall et al, 1994a) (Fig. 1). A total of 12 stably transfected clonal lines (3 negative control [*M*], 4 full-length [*S*], and 5 truncated [*T*]) were generated. The four full-length (S-2-2, 2-4, 2-5, 2-8) and five truncated clones (T-1-5, 2-3, 4-1, 4-8, 4-10) expressed their DCC construct at levels comparable to or greater than NB-20, a neuroblastoma line with high-level endogenous *DCC* expression (Reale et al, 1996) (Fig. 2A). Endogenous or transfected *DCC* expression was not evident in the negative control lines under these conditions, although very low-level endogenous *DCC* expression has been demonstrated (not shown). The transfected full-length DCC migrated as a doublet at a slightly higher apparent molecular mass than endogenous DCC. The doublet form has been observed previously in rat and mouse brain tissue and also in several other cell lines transfected with this DCC cDNA construct (Reale et al, 1994). The small difference in electrophoretic mobility is likely explained by alternative splicing of endogenous DCC (Fig. 2A). We have previously shown alternative splicing of the DCC extracellular domain in a neuroblastoma cell line that results in a transcript with a 20-codon deletion. This spliced out sequence is a potential site of O-linked glycosylation and consequently could effect a change in molecular mass evident on a protein gel (Reale et al, 1994). The endogenous DCC protein was observed in four of five truncated clones (Fig. 2A), although it was better demonstrated with antiserum 723 that does not detect the truncated form of the DCC protein (Reale et al, 1994). Interestingly, endogenous DCC levels were substantially increased in the truncated relative to the negative control and full-length transfectants (Fig. 2B).

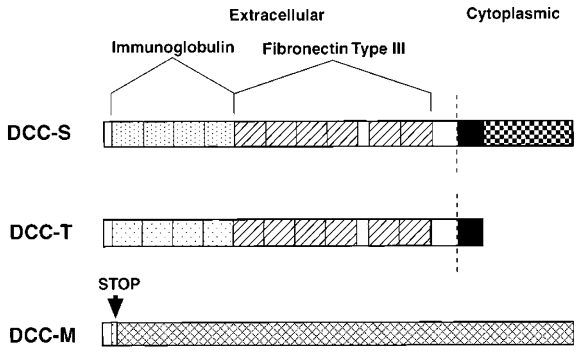

Figure 1.
The *deleted in colorectal cancer (DCC)* cDNAs cloned into the pCMV-neo eukaryotic expression vector. *DCC-M,* cDNA encoding a protein that contains signal sequence and a portion of the first immunoglobulin domain; *DCC-T,* cDNA with deletion of sequence encoding the carboxy terminal 293 amino acids; *DCC-S,* full-length cDNA.

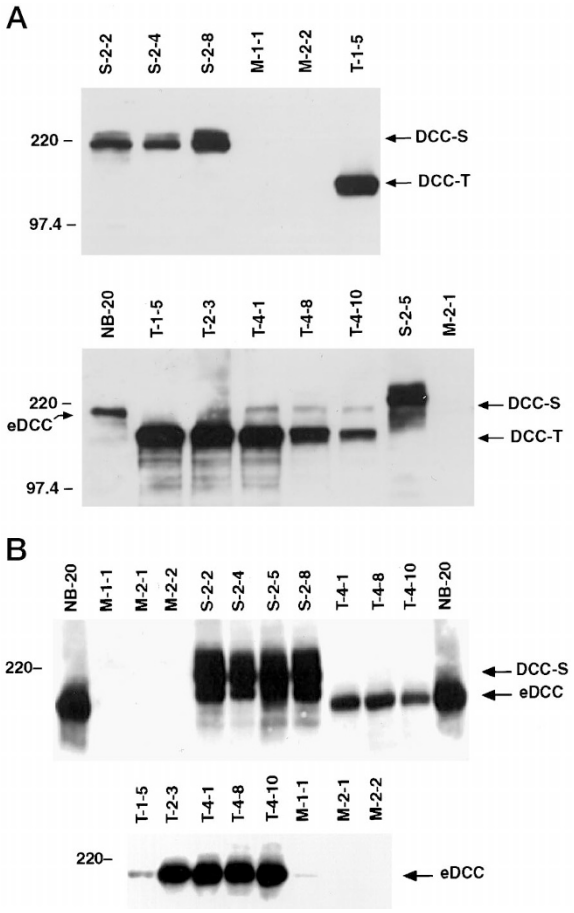

Figure 2.

Immunoblot of the DCC protein in transfected SJNB-8 neuroblastoma cells. A, Lysates (160 ug protein/lane) from all 12 DCC-transfected clonal lines (*S,* full-length DCC; *M,* negative control; *T,* truncated DCC) were examined with DCC antisera 641 (detects both full-length and truncated DCC) (8% PAGE). B, Lysates (*NB20*, 135 ug protein/lane; all others, 190 ug protein/lane) were examined with the DCC antisera 723 (does not detect the truncated DCC protein) (6% PAGE). *NB-20,* DCC-positive neuroblastoma cell line control. The full-length (*DCC-S*), truncated (*DCC-T*), and endogenous (*eDCC*) DCC protein and size markers are indicated.

Immunoprecipitation of DCC from surface biotinylated cells (Fig. 3A) and immunofluorescence studies (Fig. 3B) demonstrated that a significant proportion of the full-length and truncated constructs was expressed at the cell surface as predicted. Previous studies have also demonstrated the cell surface expression of these constructs in NIH-3T3 cells (Pierceall et al, 1994a).

Overexpression of Truncated DCC Alters Cell Morphology and N-Cadherin–Dependent Cell Adhesion

The truncated clones demonstrated a distinct cellular morphology and growth pattern as compared with the negative control and full-length transfected clones (Fig. 4), and also with the parental SJNB-8 cells (not shown). Whereas the parental cells, negative control transfectants, and full-length transfectants grew in tightly packed, epithelioid colonies, the truncated clones had a more scattered growth pattern. The truncated clones infrequently demonstrated colony-like growth, and they did not display the tight cell-cell contact of the negative control and full-length lines. Moreover, the truncated cells were smaller with frequent and sometimes long, neurite-like cell processes. (Although a possible differentiation effect has not yet been specifically addressed, we have not demonstrated an accompanying decrement in cell proliferation among the transfectants as assessed by doubling time and colony formation ability [not shown].) This phenotype has been evident with routine cell passage and is not due to clonal variation, because it was seen with all five truncated clones as compared with the three negative control and four full-length DCC transfectants.

The distinctive morphologic change led us to focus on the molecular basis of the apparent differences in cell-cell adhesion among the transfectants. It seemed unlikely that DCC-mediated cell-cell adhesion could explain this phenotype because there is limited experimental evidence that DCC itself mediates intercellular adhesion in the calcium-independent manner of NCAM family molecules (Chuong et al, 1994; ER Fearon, University of Michigan personal communication, 1998). Because alterations in N-cadherin expression have been associated with the epithelial-mesenchymal transition of neural crest cells (Akitaya and Bronner-Fraser, 1992; Duband et al, 1988) and cadherins in general are integral to the epithelial phenotype (Gumbiner, 1996), we addressed the role of N-cadherin and the associated catenins.

Immunoblot analysis with two distinct antibody reagents in each case demonstrated significant reductions in N-cadherin and α- and β-catenin levels in the truncated clones. Levels of the desmosomal cadherin, desmoglein (Fig. 5A), and tight junction–associated protein, ZO-1 (not shown), were relatively well maintained in the truncated clones. There were increases in N-cadherin and α-catenin and a decrease in β-catenin levels in the full-length relative to the negative control clones (Fig. 5A). Essentially identical immunoblot results were obtained with a pan-cadherin reagent, suggesting that N-cadherin is the predominant cadherin in these cells (not shown).

Northern blot analysis of N-cadherin and α- and β-catenin transcripts (Furukawa et al, 1994; Hatta et al, 1988; Tsutsui et al, 1996) demonstrated reductions in the truncated clones. N-cadherin and α-catenin transcript levels were increased in the full-length transfectants, which agrees with the immunoblot data. β-catenin transcript levels were slightly increased in the full-length clones and therefore discordant with the immunoblot results (Fig. 5B). The degree of alteration in N-cadherin protein and transcript levels was nearly identical, whereas the degree of alteration in α-catenin (DCC-S and DCC-T transfectants) and β-catenin transcripts (DCC-T) was less commensurate with the changes in protein levels (Fig. 5C).

Immunofluorescence studies of N-cadherin were undertaken to assess cellular localization with regard to its presumed junctional function. Consistent with the immunoblot and Northern blot analyses, N-cadherin immunofluorescence was relatively dimin-

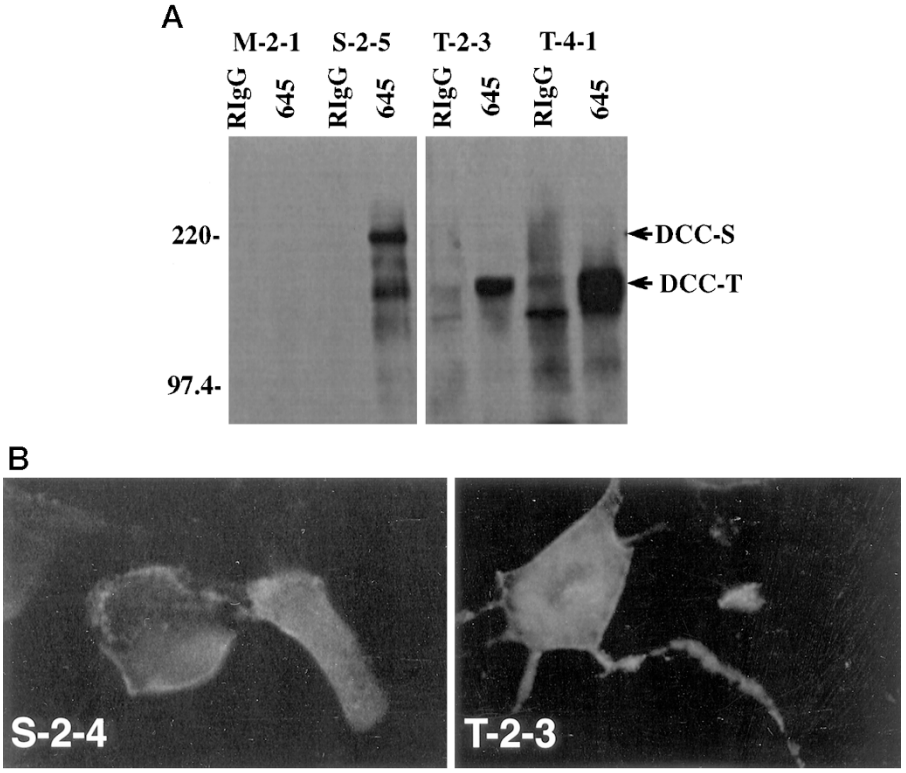

Figure 3.

Cell surface expression of DCC cDNAs in transfected SJNB-8 neuroblastoma cells. A, Lysates from surface biotinylated cells were immunoprecipitated with normal rabbit immunoglobulin (*RIgG*) and DCC-specific antisera *645* that is directed to the extracellular domain. Cell surface expression of DCC proteins was demonstrated by immunoblot analysis with an avidin-biotin-horseradish peroxidase conjugate. NB-20, DCC-positive neuroblastoma cell line control. The full-length (*DCC-S*) and truncated (*DCC-T*) DCC proteins are indicated. B, Representative DCC immunofluorescence in full-length (*S*) and truncated (*T*) transfectants. No significant staining was observed in the negative control transfectants (*M*) or with a negative control antibody reagent (not shown). Original magnification, ×60.

ished in the truncated clones. Both cytoplasmic staining and staining at sites of cell-cell contact were evident (Fig. 6).

The loss of the epithelioid characteristics of the negative control and full-length clones and the accompanying reduction in expression of the major components of an adherens-type junction strongly suggested a reduced ability of the truncated clones to mediate calcium-dependent cell-cell aggregation. Consequently, cell aggregation was assessed in all of the transfectants in the presence and absence of calcium. As predicted, calcium-dependent cell aggregation was significantly greater in the negative control ($p = 0.0146$) and full-length ($p = 0.0022$) transfectants compared with the truncated transfectants (Fig. 7). To confirm that the cell clustering is mediated by N-cadherin, the JSNB parental cell line cell aggregation was assessed in the presence of A-CAM, a monoclonal antibody against N-cadherin; predictably, cell aggregation was inhibited (Fig. 8).

Discussion

We compared the effects of overexpression of a full-length DCC, truncated DCC, and a negative control DCC construct in the SJNB-8 human neuroblastoma cell line. Although overexpression of the full-length DCC protein did affect N-cadherin and α- and β-catenin expression, the predominant phenotypic effect was seen with overexpression of truncated DCC. This resulted in a less epithelioid morphology that was accompanied by decreased N-cadherin and α- and β-catenin transcript and protein levels. Levels of the desmosomal cadherin, desmoglein, and tight-junction–associated protein, ZO-1, were relatively less affected, whereas endogenous DCC levels were increased in the truncated transfectants. N-cadherin immunofluorescence confirmed the immunoblot and Northern blot studies, and also demonstrated the protein at sites of cell-cell contact, consistent with its adherens junction role. Cell aggregation studies confirmed the functional implications of the morphology, expression, and localization studies by showing diminished calcium-dependent aggregation in the truncated transfectants.

Although analysis of protein function through constitutive overexpression may be limited by nonspecific effects, two aspects of the present study strongly suggest this is not true of the truncated DCC phenotype. Nonspecific effects are more likely in cell lines derived from tissues in which endogenous expression is not comparable to that achievable by gene transfer. This is not the case with neuroblastoma cells because we have previously demonstrated high-level *DCC* expression in subsets of neuroblastoma cell lines and

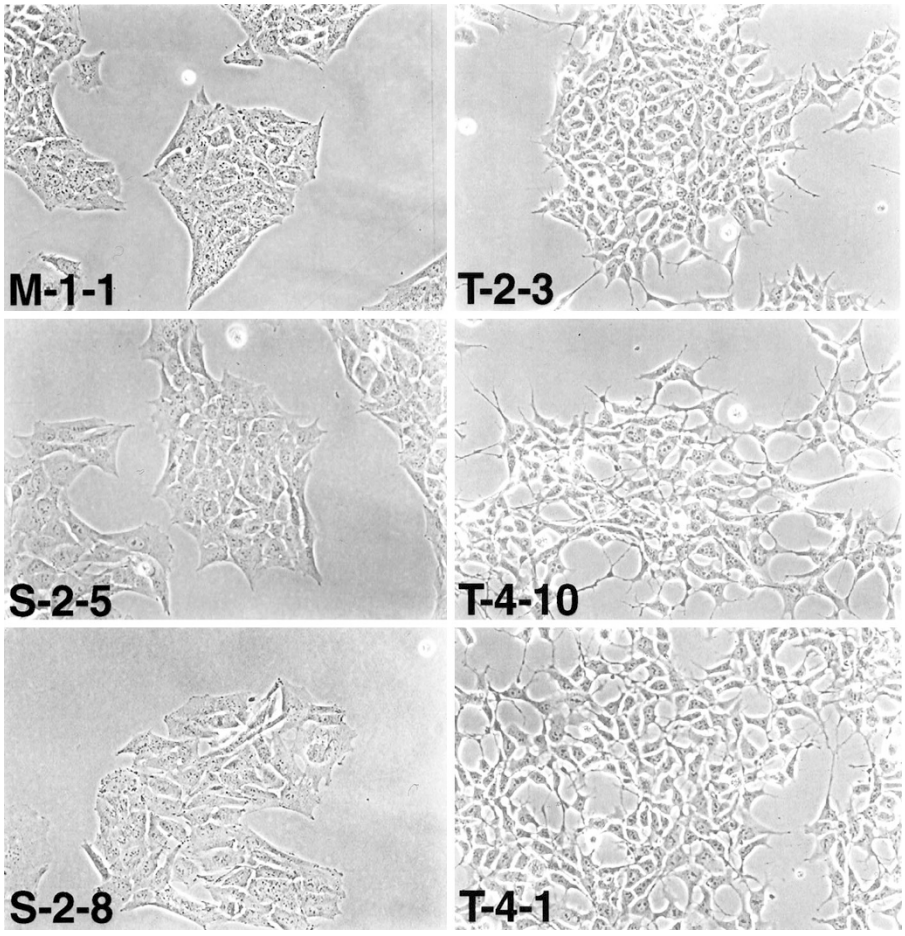

Figure 4.

Representative phase contrast photomicroscopy of SJNB-8 DCC transfected cell lines in culture. Cell lines at approximately equal density were photographed 3 days after plating. Original magnification, ×20.

tumors (Reale et al, 1994, 1996). Most importantly, the increased levels of the endogenous DCC protein in the truncated transfectants provide an internal specificity control. Although we do not yet fully understand the functional implications of this phenomenon, it strongly argues that the truncated DCC phenotype reflects alterations in endogenous DCC function.

A fundamental question is the nature (gain versus loss of function) of the truncated DCC phenotype. Although the present study does not directly address this question of mechanism, based on several lines of evidence we propose the hypothesis that this phenotype reflects a dominant negative effect: (1) The phenotype is transdominant because it occurs in the presence of increased levels of the endogenous DCC protein. (2) The N-cadherin and α-catenin expression pattern is consistent with DCC loss of function because the diminished expression in the truncated transfectants can be contrasted to increases in the full-length DCC transfectants. (3) There is evidence from *C. elegans* studies that DCC/unc-40, a putative netrin receptor, may be heteromeric. Truncated forms of the DCC/unc-40 protein have been shown to produce more severe phenotypes than DCC/unc-40 null mutations, presumably by inhibiting the interaction with a normal binding partner (Chan et al, 1996). (4) The increase in endogenous *DCC* expression in the truncated transfectants is consistent with DCC loss of function. One may speculate that it represents a compensatory increase.

We suggest that the truncated DCC phenotype results from alterations in a yet-undefined signaling pathway or pathways that ultimately modulate N-cadherin and α- and β-catenin expression and consequently adhesion function. There is little basis for a direct effect of truncated DCC through targeted protein degradation or alterations in adherens junction nucleation/assembly in light of the lack of evidence for colocalization of cadherins and immunoglobulin family adhesion molecules (Ayalon et al, 1994). The diminished levels of N-cadherin and α- and β-catenin transcripts in the truncated DCC transfectants also argue against this model. Moreover, there is a great deal of evidence that DCC functions as a receptor for soluble ligands. Specifically, DCC seems to be a component of the netrin receptor complex (Chan et al, 1996; Keino-Masu et al, 1996; Kolodziej et al, 1996). Consequently, the primary candidate for an effector ligand in the present study would be the netrin family of proteins. However, it is important to note that netrin-independent phenotypes of *DCC/unc-40* mutants in *C. elegans* have been described, implying the

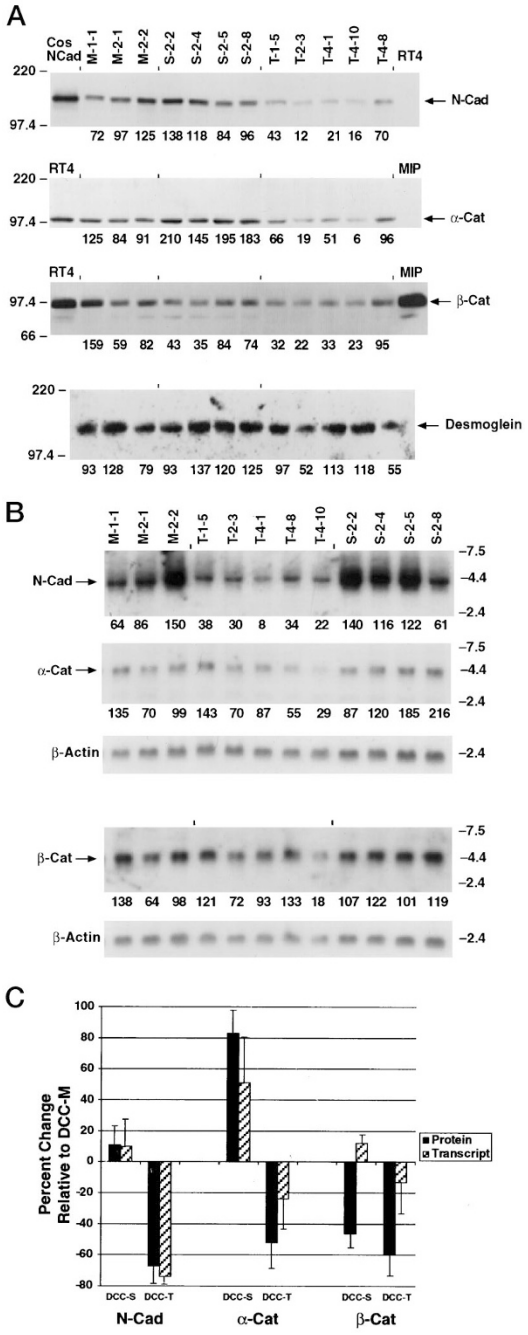

Figure 5.

Expression of N-cadherin and α- and β-catenin in DCC transfected SJNB-8 neuroblastoma cells. A, Immunoblot: protein-equivalent lysates (N-cadherin (*N-cad*), 12 ug/lane; α- and β-catenin (α-/β-*cat*), 30 ug/lane; *desmoglein*, 150 ug/lane) were examined. Protein equivalence was also confirmed by examination of a parallel Coomassie stained gel (not shown). Arbitrary densitometry units are included below each blot, and molecular size markers (in kilodaltons) are shown. Lysates from the following control cell lines were included: *CosNCad*, Cos cells transiently transfected with N-cadherin; *RT4*, N-cadherin negative, α-/β-catenin positive human bladder cell line; MIP101 (*MIP*), α-catenin negative, β-catenin positive human bladder cell line. The specificity of the β-catenin and desmoglein immunoreactivity was further confirmed by comparison with negative control antibody reagents. B, Northern blot analysis: total RNA-equivalent lysates (5 ug/lane) were examined. The expression of β-actin was assessed to control for variation in RNA loading and integrity. Arbitrary densitometry units normalized for β-actin expression are included below each blot, and molecular size markers are indicated (in kilodaltons). C, Densitometric summary of expression in the full-length (*S*) and truncated (*T*) relative to the negative control (*M*) DCC transfectants (mean ± SEM).

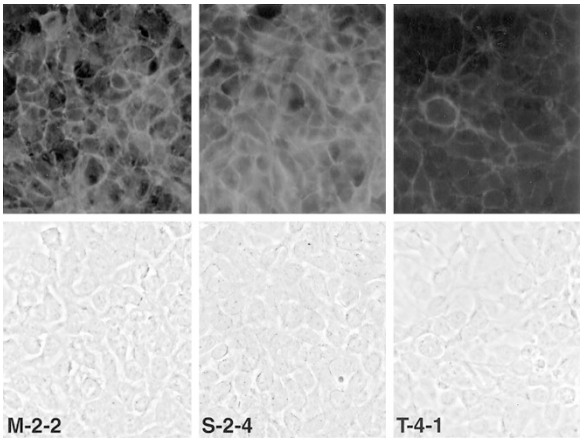

Figure 6.

N-cadherin immunofluorescence and corresponding phase contrast photomicrographs of confluent DCC transfected SJNB-8 neuroblastoma cells. Subsets of unusually confluent DCC-T cells were selected in this case so that any effect of cell-cell contact on N-cadherin expression would be equivalent among the different DCC transfectants. Negative control (*M*), full-length (*S*) and truncated (*T*) transfectants are shown. Original magnification, ×40.

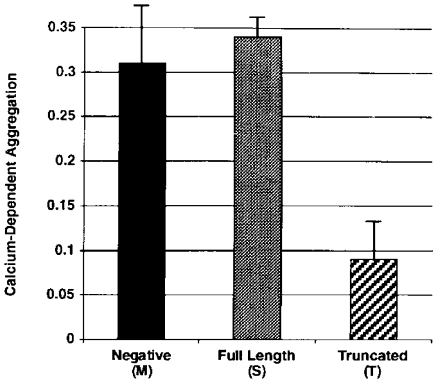

Figure 7.

Calcium-dependent cell-cell aggregation in DCC transfected SJNB-8 neuroblastoma cells. Each clonal line was examined in triplicate (mean ± SEM).

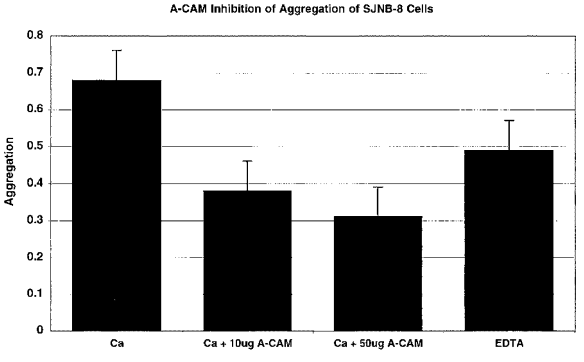

Figure 8.

Adhesion assay showing inhibition of SJNB-8 cell aggregation by N-cadherin antibody. Each experiment was performed in triplicate and repeated two to four times.

existence of other DCC ligands (Chan et al, 1996). The components of the pathway(s) immediately downstream await characterization of DCC cytoplasmic domain protein interactions, an area of still-limited

investigation. However, a recent study has demonstrated binding of the DCC cytoplasmic domain to proteins encoded by mammalian homologs of the *Drosophila seven in absentia (sina)* gene (Hu et al, 1997).

It is a novel finding of significant interest that truncated DCC can modulate expression of N-cadherin and α- and β-catenin at the level of the transcript, although a full understanding of this effect awaits the delineation of transcriptional versus post-transcriptional mechanisms. In the present study, N-cadherin and α-catenin expression were coordinately regulated because protein and transcript levels were both diminished in the truncated transfectants and conversely increased in the full-length DCC transfectants. The effect of DCC on N-cadherin expression seems to occur largely at the transcript level, as evidenced by the concordance between protein and transcript in the direction and degree of change. Although a substantial proportion of the changes in α-catenin protein levels could be accounted for by modulation of transcript, the present data do not rule out an independent effect on translation or protein stability. The effect of DCC on β-catenin expression was clearly distinct from its effect on N-cadherin and α-catenin expression. Although both Western and Northern blot analyses demonstrated diminished β-catenin levels in the truncated transfectants, most of the protein decrease could not be accounted for by effects on the transcript. Moreover, β-catenin protein levels were decreased in the full-length DCC transfectants, whereas transcript levels were slightly increased. This protein-transcript discordance is not surprising because β-catenin is known to be regulated through targeted protein degradation (Munemitsu et al, 1995). The reduction in β-catenin protein levels in the full-length DCC transfectants is interesting in view of β-catenin's dual role in adherens junction function and wnt/wingless signaling (Behrens et al, 1996; Morin et al, 1997). This reduction in β-catenin protein in the absence of the morphologic and adhesion changes of the truncated transfectants suggests that a possible effect of DCC on β-catenin–mediated signal transduction should be explored. There is some previous basis for this hypothesis because the aberrant migration of Q neuroblasts in *C. elegans DCC/unc-40* mutants has been linked to wnt/wingless signaling (Chan et al, 1996).

The present evidence suggesting a functional link between DCC, an NCAM family molecule, and cadherins/catenins is significant regarding relationships between adhesion molecule families. The coexistence and coordinated interplay of adhesion molecule systems that mediate cell-cell and cell-substratum interactions has long suggested these functional interrelationships, although there is relatively limited experimental evidence. Regulatory effects of NCAM on other cell-cell interactions have been suggested by demonstrations that abrogation of NCAM-mediated adhesion can block or delay the formation of neuromuscular and gap junctions (Grumet et al, 1982; Keane et al, 1988; Rutishauser et al, 1983), and Michalides et al (1994) have shown that NCAM can augment adherens junction formation in mesenchymal cells by redistributing cadherins to sites of cell-cell contact. Protein zero (Doyle et al, 1995) and Ep-CAM (Litvinov et al, 1997), immunoglobulin superfamily proteins that mediate calcium-independent cell adhesion, have also been shown to modulate cadherin function. The demonstration that overexpression of truncated DCC can modulate N-cadherin/catenin expression and possibly adhesion function in neuroblastoma cells suggests that an important role of the DCC protein may be to mediate such adhesion molecule "cross talk." This would seem to be consistent with the known function of DCC in the complex and dynamic process of pathway guidance in development (Chan et al, 1996; Keino-Masu et al, 1996; Kolodziej et al, 1996).

The present studies may also have implications for understanding neural crest development. As neural crest cells segregate from the neural tube, they demonstrate morphologic changes, diminished N-cadherin expression, and loss of intercellular junctions that constitute a prototypic epithelial-mesenchymal transition. This is followed by distant migrations along stereotyped pathways to form a variety of tissues (Duband et al, 1995). The cessation of migration is often accompanied by the regroupment of cells into clusters and re-expression of N-cadherin (Akitaya and Bronner-Fraser, 1992; Duband et al, 1988). The initial epithelial-mesenchymal transition of neural crest morphogenesis bears striking similarity to the in vitro truncated DCC phenotype and suggests that DCC may play a similar role in vivo. It is also reasonable to speculate that DCC may play a role in the distant migrations of neural crest cells in light of its known role in developmental neuroblast migrations (Chan et al, 1996). It is clear that the potential role of the DCC protein in neural crest development requires examination.

In conclusion, we have provided the first evidence that DCC can modulate the expression of N-cadherin and α- and β-catenin, and also calcium-dependent cell adhesion, demonstrating that there is cross talk between members of the NCAM and cadherin families of adhesion molecules. These studies suggest that one function of the DCC protein in neuroblastoma cells is the regulation of adherens junction function. This is quite consistent with the findings of earlier studies that implicated DCC loss in neuroblastoma dissemination (Kong et al, 1997; Reale et al, 1996) and the role of cadherins in suppressing tumor invasion (Birchmeier, 1995). The DCC–N-cadherin link and the less-epithelioid morphology of the truncated DCC phenotype also suggest a role for the DCC protein in neural crest development.

Materials and Methods

DCC-Transfected Cell Lines

The parental SJNB-8 human neuroblastoma cell line (Reale et al, 1994) was passaged in RPMI medium with 10% FBS and penicillin/streptomycin. The DCC constructs included a mutant (M) negative control construct that contains a nonsense mutation within the first immunoglobulin domain, a full-length construct in the sense (S) orientation, and a truncated (T) construct lacking 293 of the 331 carboxy terminal amino acids of

the cytoplasmic domain (Pierceall et al, 1994a) (Fig. 1). The generation of stable transfectants with these DCC cDNA constructs in the pCMV-neo constitutive expression vector has been previously described (Pierceall et al, 1994a). Briefly, SJNB-8 cells were transfected with a mixture of 1 μg of plasmid DNA and the Lipofectamine reagent (Gibco-BRL, Gaithersburg, Maryland) in reduced serum medium (OptiMEM; Gibco-BRL). G418-resistant colonies were ring cloned and established as lines.

Antibody Reagents

DCC reagents included the rabbit affinity purified antisera 641, 645, and 723 (Reale et al, 1994). Antisera 723 and 641 are directed to the DCC cytoplasmic domain and 645 to the extracellular domain. The truncated DCC protein is not recognized by 723, whereas 641 is directed to peptide sequence retained in the truncated form. Cadherin reagents included a pan-cadherin (CH-19; Sigma, St. Louis, Missouri) and N-cadherin monoclonal antibodies: (13A9, Sacco et al, 1995); GC4, Sigma; and A-CAM, Accurate Chemical and Scientific, Westbury, New York). α-catenin reagents included a rabbit antisera (YR4) (Pierceall et al, 1995) and a monoclonal antibody (clone 5; Transduction Laboratories, Lexington, Kentucky). β-catenin reagents also included a rabbit antisera (5h; Pierceall et al, 1995) and a monoclonal antibody (clone 14, Transduction Laboratories). The monoclonal antibody to desmoglein was obtained from Transduction Laboratories.

Immunoblot Analysis

Subconfluent monolayers were pelleted and then solubilized in Tris buffered saline (25 mM Tris, pH 8) with 1% Triton X-100 and a protease inhibitor cocktail for DCC and desmoglein immunoblots (Reale et al, 1994). For N-cadherin and α- and β-catenin immunoblots cells were lysed in the dish in sample buffer at 95° C (Rieger et al, 1995). Protein concentrations were determined with the BCA protein assay (Pierce, Rockford, Illinois). Lysates underwent SDS-PAGE and Transblot semi-dry transfer (BioRad, Hercules, California) to an Immobilon (Millipore, Bedford, Massachusetts) membrane. The primary antibody reagents were used at the following concentrations or dilutions: DCC 723 (0.1 ug/ml), DCC 641 (0.05 ug/ml), pan-cadherin CH-19 (1:100 ascites), N-cadherin 13A9 (1:50 supernatant), N-cadherin GC4 (1:100 ascites), α-catenin antisera YR4 (1:5,000), α-catenin clone 5 (1:250 ascites), β-catenin antisera 5h (1:2,000), β-catenin clone 14 (1:500 ascites), desmoglein (0.5 ug/ml). The secondary goat-antirabbit/mouse:horseradish peroxidase antibody (Pierce) was used at 1:20,000 dilution. Detection was by enhanced chemiluminescence and exposure to Hyperfilm (Amersham, Arlington Heights, Illinois). Films were scanned and densitometric analysis performed with the National Institutes of Health Image software program.

Cell Surface Biotinylation

Subconfluent monolayers in 75 cm^2 tissue culture flasks were washed twice with PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Three milliliters of biotinylation buffer (10 mM sodium borate, 150 mM NaCl, pH 8.8) and 15 μl of 10 mg/ml D-biotinyl-epsilon-amidocaproic acid N-hydroxysuccinimide ester (made fresh in dimethyl sulfoxide) (Boehringer Mannheim, Indianapolis, Indiana) were added to the flask. The flask was gently rocked at room temperature for 15 minutes, and 30 μl of 1 M NH_4Cl was then added to terminate the reaction (Meier et al, 1992). Cells were washed twice with PBS (with $CaCl_2$ and $MgCl_2$) and lysates were prepared as described above. Immunoprecipitations were carried out with affinity purified rabbit DCC antisera 645 and protein A agarose beads (ImmunoPure Plus; Pierce) as described (Harlow and Lane, 1988). Biotinylated DCC was detected with an avidin-biotin-horseradish peroxidase complex (1:10 in tris buffered saline) (Pierce) and enhanced chemiluminescence as above (Pierceall et al, 1994a).

Northern Blot Analysis

Total RNA was extracted with the Trizol reagent (Life Technologies, Grand Island, New York) following the manufacturer's protocol. Samples of 5 ug of RNA per lane were separated on 1.2% agarose/4.4% formaldehyde gels. The RNA was transferred to Hybond N nylon membranes (Amersham) and hybridized in 1% BSA, 0.5 M $NaPO_4$, 1 mM EDTA, 7% SDS at 65° C for 16 to 48 hours with probes labeled by random priming. Blots were washed twice at 65° C with 0.04 M $NaPO_4$, 5% SDS, 1 mM EDTA and twice at 65° C with 0.04 M $NaPO_4$, 1% SDS, 1 mM EDTA. Blots were exposed to Hyperfilm at −80° C. Probes were the following: 493 bp *Hin*dIII-*Cla*I α-catenin fragment, 651 bp MscI β-catenin fragment, 1.6 kb *Acc*I N-cadherin fragment, and a 300 bp β-actin fragment produced by polymerase chain reaction and confirmed by sequencing. Densitometry was performed as above.

Immunofluorescence

Cells were fixed in cold methanol (−20° C) for 5 minutes, followed by cold acetone (−20° C) for 10 seconds. The cells were then washed in PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$. The primary antibodies were the N-cadherin (13A9; neat supernatant) and pan-cadherin (CH-19; 1:500) monoclonal antibodies, and the DCC affinity purified rabbit antisera 645 (2 ug/ml). Negative controls were media alone and normal mouse/rabbit immunoglobulin, respectively. The secondary reagent was a fluorescein-conjugated goat antimouse/rabbit immunoglobulin antibody (1:300; Sigma). Coverslips were mounted onto glass slides and examined on a Nikon Microphot microscope.

Cell Aggregation

The degree of aggregation was measured according to the method of Takeichi (1977) with slight modification.

Briefly, cells were detached from 100-mm tissue culture plates by placing the plates on a gyratory shaker (150 rpm) for 15 minutes at 37° C in 10 ml of HEPES-buffered HBSS (pH 7.4) containing 5 mM $CaCl_2$ and 0.01% trypsin. The detached cells were washed free of calcium and adjusted to a density of 10^5 cells/ml in the HEPES-buffered HBSS containing 1% BSA with or without 5 mM $CaCl_2$. The cells (0.5 ml) were plated in 24-well plates and placed on the gyratory shaker (150 rpm) for 30 minutes at 37° C. The total number of particles per well was counted using a 16-square reticle, and three separate fields were counted for each well. All transfectants were examined in triplicate. The degree of calcium-dependent aggregation was calculated with the following formula:

$$[(N_0-N_t)/N_0]_{(+)\text{calcium}} - [(N_0-N_t)/N_0]_{(-)\text{calcium}},$$

where N_0 is the total number of particles per well at the start of incubation and N_t is the total number of particles per well after time *t*. A similar procedure was used to assess the aggregation of the JSNB parental cell in the presence of A-CAM at 10 and 50 μg, and 5 or 10 mM $CaCl_2$.

Statistical Analysis

The significance of differences in cell aggregation between the transfectants was examined by the Student's *t* test. $p < 0.05$ was considered significant.

Acknowledgements

We would like to thank Kimberly Rieger-Christ and Karina L. Brierley for their excellent technical assistance. We would also like to thank Eric R. Fearon for helpful discussions, and Scott Thomas and David Fenton for their help in the generation of the transfected cell lines.

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