

Expression of NCAM Containing VASE in Neurons Can Account for a Developmental Loss in Their Neurite Outgrowth Response to NCAM in a Cellular Substratum

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Abstract. Binding of the neural cell adhesion molecule (NCAM) in neurons to NCAM on non-neuronal cells can stimulate axonal growth. A developmentally regulated loss of this response is associated with the insertion of 10 amino acids (called VASE) into the fourth Ig domain in up to 50% of the NCAM receptors in neurons. In the present study we have transfected PC12 cells with the major neuronal isoforms of human NCAM and tested cells expressing these isoforms for their ability to respond to NCAM in a cellular substratum. Whereas both the 140- and 180-kD isoforms of NCAM can act as functional receptors for

neurite outgrowth, the presence of the VASE sequence in a minority of the receptors specifically inhibited this response. A synthetic peptide containing the VASE sequence inhibits neurite outgrowth from PC12 cells and primary neurons stimulated by NCAM. The same peptide has no effect on integrin dependent neurite outgrowth or neurite outgrowth stimulated by N-cadherin or L1. We discuss the possibility that the VASE peptide inhibits the NCAM response by preventing NCAM from binding to the FGF receptor in the plasma membrane.

DURING development the ability of neurons to elaborate axons and dendrites in a highly ordered manner is essential for the establishment of appropriate patterns of synaptic connections. The extension of neurites requires the interaction of receptor molecules on the neuronal growth cone with growth-promoting molecules present within the extracellular matrix or on the surface of other cells (Rathjen and Jessell, 1991; Reichardt and Tomaselli, 1991; Doherty and Walsh, 1992). A general loss of morphological plasticity in the adult central nervous system (CNS)¹ might be explained by a number of mechanisms including a developmental loss of the above growth-promoting molecules (Vidal-Sanz et al., 1987) and/or a temporal increase in the expression of molecules that directly inhibit growth cone motility (Schwab, 1990). However, recent studies have shown that embryonic neurons transplanted into the adult rat CNS can extend axons in a robust and precise manner with termination of growth in appropriate target fields (Wictorin et al., 1992). Thus the adult CNS can be a good environment

for axonal growth, and developmental changes in neuronal responsiveness to growth-promoting cues are likely to contribute to the poor regenerative capacity of adult CNS neurons.

There are three well characterized receptor molecules present on essentially all neuronal growth cones that function during cell-contact dependent axonal growth and these are the neural cell adhesion molecules (CAMs) NCAM (Walsh and Doherty, 1991), L1, which is functionally homologous to G4 in the chicken (Rathjen and Jessell, 1991), and neural or N-cadherin (Takeichi, 1991). A cocktail of antibodies that block the function of β 1-integrins (which act as receptors for a variety of extracellular matrix molecules) and the above receptors prevents neurons from extending neurites over a variety of non-neuronal cell types such as muscle cells, astrocytes, and Schwann cells (e.g., see Bixby et al., 1987; Neugebauer et al., 1988; Seilheimer and Schachner, 1988; for review see Doherty and Walsh, 1989). In addition, neurons extend longer neurites when grown over genetically modified fibroblasts that express transfected N-cadherin (Matsunaga et al., 1988; Doherty et al., 1991a), NCAM (Doherty et al., 1989, 1990a; Liu et al., 1993), or L1 (Williams et al., 1992). In all three instances the homophilic binding of the transfected CAM in the substratum to the same CAM acting as a neuronal receptor stimulates neurite outgrowth by activating a second messenger pathway that culminates in calcium influx into neurons (Doherty et al., 1991b; Saffell et al., 1992; Williams et al., 1992, 1994).

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1. *Abbreviations used in this paper:* CNS, central nervous system; ELISA, enzyme-linked immunosorbent assay; NCAMs, neural cell adhesion molecules.

Neurons show considerably more variability in their responsiveness to NCAM in a cellular substratum as compared to N-cadherin or L1. For example, the ability of three populations of central neurons (chick retinal ganglion cells and granule cells from the rat cerebellum and hippocampus) to extend neurites in response to NCAM is lost over relatively short developmental periods independently of changes in responsiveness to L1 and/or N-cadherin (Doherty et al., 1990b; 1992a,b; Williams et al., 1992). Loss of responsiveness cannot be explained by loss of receptor (i.e., NCAM) from the neurons, suggesting that changes in receptor structure as a consequence of differential splicing and/or post-translational processing might account for this phenomenon.

Whereas the effects of alternative splicing on NCAM's ability to act as a substrate molecule for neurite outgrowth are well characterized (Doherty et al., 1990a; 1992b,c) little is known concerning the consequences of alternative splicing for NCAM's ability to act as a neuronal receptor molecule. This is particularly important because when acting as a neuronal receptor NCAM must be capable both of binding to NCAM in the substratum and of initiating the transduction of this homophilic recognition event into a complex cell response via activation of a second messenger pathway. The initial step in this pathway has been postulated to involve CAM-induced clustering of a receptor tyrosine kinase (Williams et al., 1994). Downstream events include activation of a pertussis toxin sensitive G-protein and calcium influx into neurons via N- and L- type calcium channels (Doherty et al., 1991b).

In general, neurons express only transmembrane forms of NCAM (e.g., see Nybroe et al., 1988), and the most conspicuous differences between NCAM isoforms in neurons are the use of the VASE exon which adds 10 amino acids to the fourth Ig domain (Small et al., 1988) and in the use of exon 18 which encodes for isoforms with a larger cytoplasmic domain (Walsh and Doherty, 1991). Like essentially all primary neurons, the PC12 neuronal cell line expresses both 140 and 180-kD isoforms of NCAM (Mann et al., 1989) and can respond to NCAM in a cellular substratum by extending longer neurites (Doherty et al., 1991b). NCAM transcripts containing VASE are not found in neurons early in development nor in PC12 cells (Small and Akesson, 1990). In contrast, up to 50% of all NCAM transcripts in the adult CNS contain VASE with the temporal pattern of upregulation of VASE in cerebellar and hippocampal neurons broadly correlating with a loss of their responsiveness to NCAM in the substratum (Walsh et al., 1992). In the present study we have transfected cDNA constructs encoding the 180- and 140-kD (\pm VASE) isoforms of human NCAM (hNCAM) into PC12 cells and isolated a number of clones that stably express these isoforms at the cell surface. Our results suggest that both the 140 and 180-kD isoforms of NCAM can act as functional receptors for neurite outgrowth. In contrast, the expression of VASE containing isoforms results in a loss of neuronal responsiveness to NCAM even though the neurons continue to predominantly express isoforms lacking in VASE. These results show for the first time that alternative splicing of a CAM in neurons can directly lead to loss of a neurite outgrowth response. A peptide containing the VASE sequence also specifically inhibits NCAM-dependent neurite outgrowth, but not NCAM-dependent adhesion. A search of protein data banks revealed that a highly conserved sequence

homologous to the VASE sequence is present in all members of the FGF receptor family and we discuss the possibility that VASE inhibits NCAM-dependent neurite outgrowth by preventing the FGF receptor from directly or indirectly associating with NCAM via this homologous region.

Materials and Methods

Generation of PC12 Cell Lines Expressing hNCAM

Expression vectors, under control of the β -actin promoter and containing a neomycin resistance gene and full length cDNAs encoding the 140 \pm VASE and 180-kD isoforms of hNCAM have been described in detail elsewhere (Barton et al., 1988; Gower et al., 1988; Doherty et al., 1992b). These were introduced into exponentially growing PC12 cells by electroporation using a BTX (San Diego, CA) Transfector-300 set at 230 V and a capacitance of 450 μ F. Briefly, 10^7 PC12 cells suspended in 0.4 ml Hepes buffer (20 mM Hepes, pH 7.05; 137 mM NaCl; 5 mM KCl; 0.7 mM Na_2HPO_4 ; 6 mM dextrose) were placed in a BTX disposable 2 mm gap cuvette with 20 μ g vector DNA and 125 μ g salmon sperm carrier DNA, electroporated, and then left to stand for 10 min at room temperature before plating onto a poly-L-lysine-coated plastic culture dish in SATO media containing G418 (0.5 mg/ml). After 7–10 d G418 selection was complete but G418 resistant PC12 clones were not isolated for characterization for an additional three weeks when the number of cells per clone was high enough to ensure survival on dissociation and replating in individual wells of a poly-L-lysine-coated 24-well plate. Clonality was checked by staining for the transgene product (see below).

Cell Culture

Parental and CAM-expressing 3T3 cells (the 140-kD isoform of hNCAM, N-cadherin or the L1 glycoprotein, for details see Doherty et al., 1990a, 1991a; Williams et al., 1992) were maintained on plastic culture dishes in DMEM containing 10% FCS. Parental and hNCAM-expressing PC12 cells were grown on poly-L-lysine (Sigma Chem. Co., St. Louis, MO)-coated plastic culture dishes in defined SATO medium (Doherty et al., 1988). All cells were grown at 37°C in 8% CO_2 . For establishment of cocultures parental or CAM expressing 3T3 cells were seeded at 8×10^4 cells per chamber of an eight-chamber slide (Lab-Tek, Naperville, IL) coated sequentially with poly-L-lysine and collagen and allowed to form confluent monolayers overnight. PC12 cells were then seeded as a single cell suspension onto the monolayer at a density of 1.5×10^3 cells per chamber (Doherty et al., 1991b). Cocultures were maintained for 40–46 h in SATO medium before being fixed for 30 min with 4% paraformaldehyde and immunostained for Thy-1 to delineate PC12 cell morphology (see below). In some experiments 3×10^3 dissociated neurons from the post-natal day 4 rat cerebellum were cocultured over monolayers of parental and CAM expressing 3T3 cells for 16 h before being fixed and stained for GAP43 (for details see Williams et al., 1992). To assess cell surface expression of hNCAM in transfected PC12 cells 2×10^3 cells from each clone were introduced to individual chambers of an eight chamber slide (Lab-Tek) previously coated with collagen. The PC12 cells were differentiated for 48 h in the presence of NGF (50 ng/ml), and then immunostained for hNCAM.

Immunostaining

For neurite outgrowth analysis fixed cocultures were washed and non-specific protein binding sites blocked with 0.5% gelatin (45 min, room temperature). PC12 cells were then visualized by the sequential application (1 h each, room temperature) of the OX-7 mAb which recognizes the Thy-1 antigen followed by biotinylated anti-mouse immunoglobulin and Texas red-conjugated streptavidin (both Amersham International, diluted 1:500). Cerebellar neurons were immunostained for GAP 43 (e.g., see Williams et al., 1992). For immunolocalization of hNCAM, PC12 cells were incubated for 1 h at 4°C with the Leu 243 mAb (diluted 1:1,000) which recognizes the extracellular domain of human NCAM, (Patel et al., 1989), and then sequentially with biotinylated anti-mouse immunoglobulin and Texas red-conjugated streptavidin (both 1 h at 4°C). Cultures were then fixed for 30 min with paraformaldehyde before mounting for analysis.

Image Analysis

Fluorescent images were detected by fluorescence microscopy using a low

light sensitive video camera (model 4722-5000) and analyzed using an Image manager (Sight Systems, Newbury, England). Mounted cultures were scanned systematically over the whole slide and the length of the longest neurite per PC12 cell or cerebellar neuron was measured as previously described (Doherty et al., 1991b). For computational reasons cells with no visible neurite were assigned a length of 2 μm ; this did not significantly affect the results.

Western Blot Analysis

Western blot analysis was carried out on SDS extracts of parental and hNCAM-transfected PC12 clones as described in Moore et al. (1987) and modified in Doherty et al. (1991a). The primary antibody used was Leu 243 mAb (diluted 1:1,000) which specifically recognizes hNCAM.

Determination of the Relative Levels of NCAM in Parental and Transfected PC12 Cells

The relative levels of NCAM expression in transfected and parental PC12 cells were determined by quantitative enzyme-linked immunosorbent assay (ELISA) using three characterized antibody reagents that react specifically with human and/or rat NCAM. These were the Leu 243 mAb that reacts exclusively with human NCAM (e.g., see Doherty et al., 1990a), a rabbit polyclonal antibody that reacts with rat but not human NCAM (e.g., see Mann et al., 1989), and a rabbit polyclonal antibody raised against a fusion protein derived from the common exon 18 shared between our construct encoding the 180-kD isoform of human NCAM and endogenous rat NCAM (see Doherty et al., 1982).

Briefly, $\sim 5,000$ cells from clones of parental and transfected PC12 cells were plated into individual wells of a poly-lysine-coated 96-well microtitre plate and allowed to attach overnight before being fixed with 4% paraformaldehyde. The relative level of expression of the transfected hNCAM was then determined using Leu 243 by standard ELISA. The effects of expression of hNCAM on the level of endogenous NCAM was determined by standard ELISA using the antibody that reacts only with rat NCAM. The effect of expression of the 180-kD isoform of hNCAM on the total level of the 180-kD NCAM in PC12 cells was determined by standard ELISA using the antibody that reacts with the product of exon 18 (that is common to both human and rat NCAM). All values were normalized to cell numbers which were determined by counting representative areas of the microwells. Control experiments showed a linear relationship between cell number and antibody binding, and similar results are obtained using ELISA assay as compared with the direct binding of iodinated Fab fragments of a mAb to human NCAM (e.g., see Doherty et al., 1990a; 1992b).

VASE Peptides

A synthetic peptide corresponding to the VASE sequence was synthesized with an additional lysine at the amino terminal to increase solubility, and a cysteine at the carboxy terminal for coupling experiments (KASWTR-PEKQEC). Structure-function relationships clearly showed that the cysteine and lysine residues did not influence the functional activity of the peptide. A scrambled VASE peptide, (KEWQTAPKRSE) was used as a control. Both were prepared by standard F-moc chemistry using a 431A peptide synthesizer (Appl. Biosystems, Inc., Foster City, CA).

Results

Characterization of Transfected PC12 Clones Expressing Human NCAM

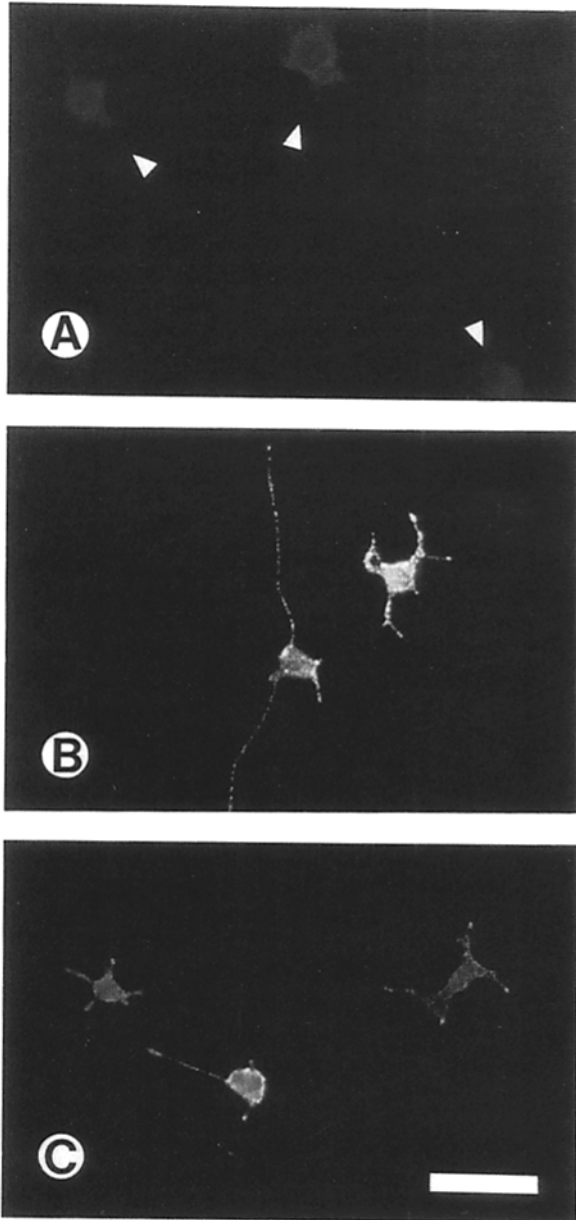
PC12 cells were transfected with cDNAs encoding three major neuronal isoforms of hNCAM (140, 140 VASE, and 180 kD). Clones selected for G418 resistance were screened for hNCAM expression by live cell staining with a mAb which recognizes the extracellular domain of human but not rat NCAM. Clones expressing NCAM gave a punctate staining pattern consistent with cell surface expression. Fig. 1 *a* shows hNCAM immunoreactivity on soma and neurites of transfectants, regardless of which isoform was expressed while parental PC12 cells were not immunoreactive. A similar staining pattern was found for endogenous NCAM in pa-

rental cells using an antibody reacting with rat NCAM (not shown). The mAb to human NCAM was used to determine the relative level of expression of hNCAM between the clones by ELISA which gives the same results as measurement of the binding of a saturating concentration of an ^{125}I -labeled monovalent Fab (Doherty et al., 1990a; Doherty et al., 1992b). These results show that the clones transfected with the three transmembrane isoforms express similar levels to each other (Table I). The relative level of endogenous NCAM can also be measured by ELISA using an antibody that recognizes both the 140- and 180-kD isoforms of rodent but not human NCAM (e.g., see Mann et al., 1989). In three independent experiments we found no significant difference in the level of endogenous NCAM in parental PC12 cells and in the three clones expressing the highest level of each individual isoform of transfected NCAM (data not shown). In contrast, an antibody raised against a region common to our 180-kD construct of human NCAM and to rodent NCAM, that reacts exclusively with the 180-kD isoform of human and rat NCAM (Doherty et al., 1992c and unpublished observations), differed only in its binding to the 180-kD transfectants. For example this antibody showed a highly significant ($P < 0.001$) $43 \pm 1.2\%$ (mean \pm SEM from three independent experiments) increase in binding to cells expressing the 180-kD isoform of human NCAM (clone A) as compared to parental PC12 cells and the above clones expressing the highest level of the other isoforms of human NCAM (a pooled value being taken for the latter clones). This suggests that the human 180-kD isoform is expressed at 40–50% of the level of the endogenous 180-kD isoform. Western blot analysis of extracts of transfected cells from the highest and lowest expressing clones show that the hNCAM is expressed at the appropriate molecular weight (Fig. 1 *b*). Endogenous NCAM in PC12 cells also runs as discrete bands of 180 and 140 kD (Mann et al., 1989) with both isoforms expressed at similar levels (Saffell, J., unpublished observation).

Effect of Transfected hNCAM Isoforms on the NCAM Response of PC12 Cells

Parental or transfected PC12 cells expressing hNCAM 140 VASE (three clones), hNCAM 140 (two clones), or hNCAM 180 (three clones), were cultured on confluent monolayers of control or NCAM-expressing 3T3 cells. After 40–46 h, the cocultures were fixed and labeled with an antibody to Thy-1 which specifically recognizes PC12 cells. Parental PC12 cells express NCAM and after 40–46 h the majority of parental PC12 cells cultured on NCAM expressing 3T3 monolayers responded by extending longer neurites. This response can be fully blocked by species specific antibodies that bind only to NCAM in the neuron (see below and also Doherty et al., 1991b) or by antibodies that bind only to transfected human NCAM in the substratum (Doherty et al., 1991b). Thus it is most likely that a trans-homophilic-binding mechanism stimulates neurite outgrowth. In contrast, PC12 cells cultured on control 3T3 monolayers showed limited neurite growth (see Doherty et al., 1991b). In a representative experiment the mean length of the longest neurite (hereafter called mean neurite length) rose from $18.2 \pm 1.2 \mu\text{m}$ ($n = 270$) on control 3T3 monolayers to $32.5 \pm 2.0 \mu\text{m}$ ($n = 179$) on NCAM expressing monolayers (values for n give the number of PC12 cells sampled). The % increases

a



b

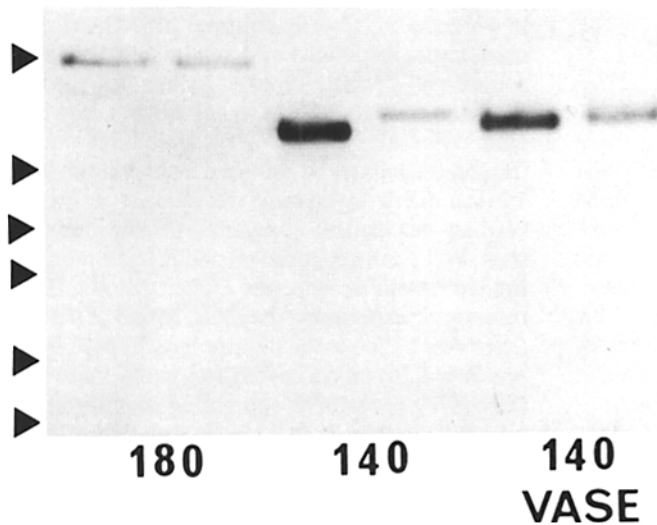


Figure 1. Expression of transfected hNCAM by PC12 cells. (a) Cultures of PC12 cells were grown on a collagen coated substratum for 48 h in the presence of 50 ng/ml NGF and stained live with a monoclonal antibody which recognizes the extracellular domain only of hNCAM. Parental PC12 cells were not immunoreactive (position of cells indicated by arrows) (upper panel, A) while positive punctate staining was seen on PC12 cells transfected with cDNA encoding the 140- (middle panel, B) and 180-kD (lower panel, C) isoforms of hNCAM. Bar, 50 μ m. Expression of VASE did not alter the staining pattern (data not shown), and NGF had no effect on the level of human NCAM in PC12 cells (data not shown). (b) The antibody used to stain PC12 cells in Fig. 1 a was used for Western blotting of extracts from the same cells. The antibody recognizes bands at 180 and 140 kD on a Western blot of SDS extracts of PC12 cells transfected with cDNA encoding the 180, 140, and 140 VASE isoforms of hNCAM (see Materials and Methods for details). Results for the clones expressing the highest and lowest levels of each isoform (see Table I) are shown. Lysates from parental PC12 cells were not immunoreactive with this species specific antibody. Arrows denote molecular weights of 198, 120, 88, 70, 56, and 38 kD. For each sample 15 μ g of protein was loaded and resolved on a 7.5% polyacrylamide gel in buffer containing SDS.

Table 1. Relative Level of Human NCAM Expression in Transfected PC12 Cells

Clone	NCAM Isoform (kDa)		
	140	140 VASE	180
A	95	100	51
B	49	60	44
C	—	44	42

Approximately 5,000 cells for the given clones of transfected PC12 cells were plated into a total of eight wells of a 96-well microtitre plate and the binding of an antibody that reacts only with human NCAM determined by standard ELISA (see Materials and Methods). Antibody binding was normalized to the number of cells present in the wells at the end of the assay (as determined by direct cell counts), and the absolute level of antibody binding per cell then expressed in arbitrary units relative to the highest value (100 U). The standard errors on cell counts were typically from 5 to 8% of the measured value and errors on antibody binding from 1 to 5% of the measured value. The above show the results obtained in one representative experiment, however similar results were obtained in two further experiments. There was no significant binding to parental PC12 cells.

in neurite outgrowth on NCAM expressing monolayers (hereafter called % NCAM response) in a number of experiments were pooled to give a response of $69.4 \pm 4.3\%$ ($n = 19$ independent experiments) (Fig. 2).

The three clones expressing hNCAM 140 VASE were unresponsive to NCAM in the monolayer (see Fig. 2). Expression of hNCAM 140 VASE in PC12 cells clearly prevents the endogenous NCAM from promoting neurite outgrowth. However this effect was not observed with other hNCAM isoforms. Two clones of PC12 cells expressing hNCAM 140 were tested for their ability to extend neurites on NCAM expressing monolayers and both gave robust responses with mean neurite length in a representative experiment with one clone rising from 19.7 ± 1.7 ($n = 200$) μm on 3T3 monolayers to 31.8 ± 2.2 ($n = 161$) μm on NCAM expressing monolayers. Despite differences in transfected hNCAM 140 expression levels between the two clones (see Table I), they gave almost identical NCAM responses (data not shown). Results from individual experiments with both clones were pooled to give a % NCAM response of $74.7 \pm 7.3\%$ ($n = 10$ independent experiments). In the same way, three clones expressing hNCAM 180 all extended neurites in response to NCAM expressed by 3T3 cells and the results were pooled to give a % NCAM response of $71.2 \pm 6.1\%$ ($n = 9$ independent experiments). It is noteworthy that despite elevated cell surface expression of additional 140 or 180 kD isoforms of transfected NCAM on PC12 cells, their NCAM response is no greater than that of their parental cells (Fig. 2). These data demonstrate the considerable influence of the VASE exon on the ability of neurons to respond to substrate associated NCAM.

Polyclonal Antiserum to Rat NCAM Prevents the NCAM Response of Parental PC12 Cells But Not PC12 Cells Expressing hNCAM 140 or hNCAM 180

The expression of hNCAM 140 or hNCAM 180 had no effect on the PC12 NCAM response (Fig. 2). This implies either that these isoforms are not functional or that the PC12 cell NCAM response is maximal and cannot be enhanced by elevating NCAM expression via transfection. To test between these possibilities the NCAM response of parental and hNCAM 140 and hNCAM 180 expressing PC12 cells was determined

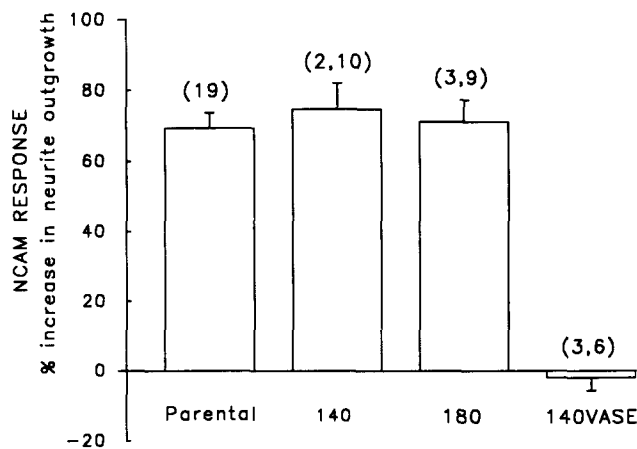


Figure 2. Effect of transfected NCAM isoforms on PC12 cell NCAM response. Parental and transfected PC12 cells expressing the given isoforms of hNCAM were cultured on monolayers of control or NCAM expressing 3T3 cells for 40–46 h before being fixed and stained with a mouse anti-rat Thy-1 mAb specific for PC12 cells and the length of the longest neurite was determined. The NCAM response is expressed as % increase in neurite outgrowth on NCAM expressing monolayers over background growth on 3T3 monolayers (% NCAM response). Each value is the mean \pm SEM pooled for 6–18 independent experiments in which 150–200 PC12 cells were sampled. The first of the two numbers above each column denotes the number of clones tested and the second the number of experiments pooled in total. The mean neurite length for parental PC12 cells on control 3T3 monolayers was 21.1 ± 1.2 μm ($n = 18$) and was not obviously different for transfected PC12 cells, e.g., 17.8 ± 0.9 μm ($n = 9$) for hNCAM 180 expressing PC12 clones.

in the presence of polyclonal anti-serum that reacts with rat NCAM in the PC12 cells but not hNCAM in the transfected PC12 and 3T3 cells. Under these conditions parental PC12 cells do not respond to NCAM in the monolayer while the responses of clones expressing hNCAM 140 or hNCAM 180 were maintained at $62 \pm 7\%$ and $65 \pm 3\%$, respectively (both values mean of eight independent experiments) (Fig. 3). This indicates that the transfected hNCAM 140 and hNCAM 180 molecules are fully functional receptors capable of initiating transduction of the NCAM binding signal into a complex cellular response.

To confirm that the antiserum used was specific for NCAM, its effect on the N-cadherin response of parental and hNCAM transfected PC12 cells was investigated. The results of a representative experiment (see Fig. 4) show the N-cadherin response of both parental and human 180 kD NCAM expressing PC12 cells to be unaffected by the antiserum. Background growth on control 3T3 monolayers was similarly unaffected (data not shown).

Expression of hNCAM 140 VASE in PC12 Cells Does Not Impair Their Ability to Respond to Other CAMs and Other Agents

We have shown that PC12 cells expressing hNCAM 140 VASE cannot extend neurites in response to NCAM in the monolayer (Fig. 2). It is possible that this failure is a symptom of the inability of these clones to differentiate per se. The three PC12 clones expressing the 140 VASE isoform were therefore tested for their ability to respond to N-cadherin in

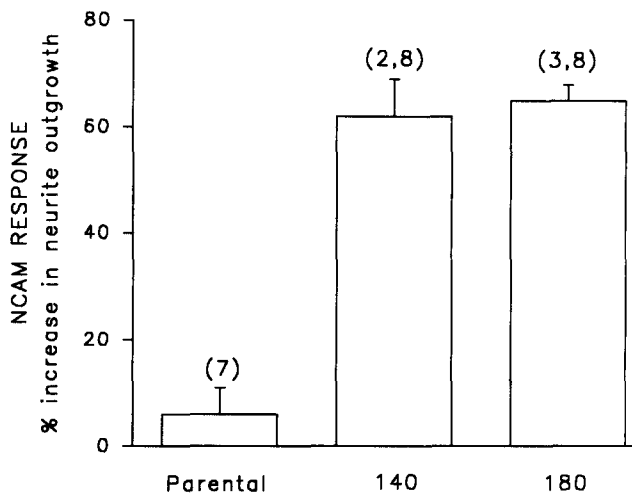


Figure 3. Comparison of the effect of antiserum to rat NCAM on the NCAM response of parental and hNCAM expressing PC12 cells. Parental PC12 cells and PC12 clones expressing hNCAM 140 and hNCAM 180 were cultured on monolayers of control or NCAM expressing 3T3 cells for a 40–46 h in the presence of a rabbit polyclonal antiserum raised against rat NCAM (diluted 1:100) and fixed for determination of mean neurite length. The results are expressed as % NCAM response (see Fig. 2) and each value is the mean \pm SEM pooled for 7–8 independent experiments. The first number above each column denotes the number of clones tested and the second the total number of experiments pooled. The rabbit antiserum was shown to react with rat and not human NCAM by a variety of methods that included live cell staining of transfected 3T3 cells and Western blotting.

the monolayer. N-cadherin stimulates neurite outgrowth by activating the same second messenger pathway as NCAM in PC12 cells and primary neurons. PC12 clones expressing the 140 VASE isoforms gave robust N-cadherin responses of $88.0 \pm 10\%$ ($n = 6$ independent experiments), which was indistinguishable from the parental PC12 response (Fig. 5). We can therefore conclude that expression of 140 VASE NCAM in PC12 cells can specifically inhibit the function of endogenous NCAM without affecting the cells' ability to respond to other CAMs.

The possibility that the VASE isoforms inhibit neurite outgrowth by binding to NCAM in the substratum and generating inhibitory signals as has been suggested for other molecules (e.g., see Tiveron et al., 1992) has also been tested. PC12 cells can extend neurites in response to a number of other agents including NGF, cholera toxin (which acts via increased cAMP), or in response to K^+ depolarization (which directly activates the CAM pathway) (Doherty et al., 1991; Saffell et al., 1992). In the present study PC12 cell clones expressing the 140 VASE isoform of hNCAM responded equally as well as parental PC12 cells to direct activation of the CAM pathway by K^+ depolarization when cultured on both parental or NCAM expressing 3T3 fibroblasts (Table II). Similar results were obtained when neurite outgrowth was stimulated by NGF or cholera toxin (data not shown). Thus the 140 VASE isoform does not block neurite outgrowth by generating inhibitory signals.

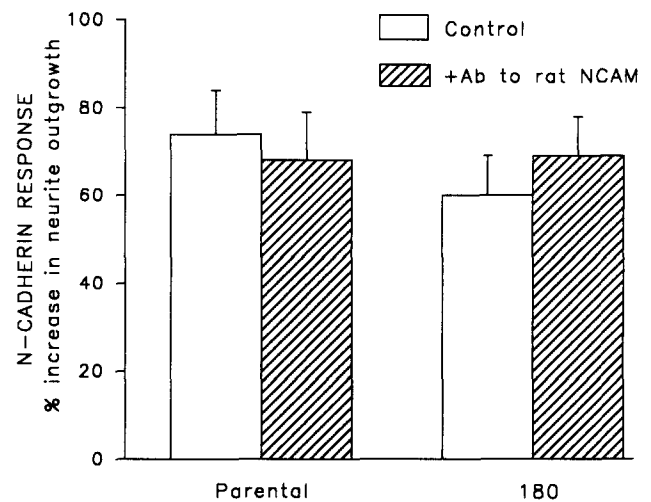


Figure 4. Antiserum to rat NCAM has no effect on the N-cadherin response. Parental and hNCAM 180 expressing PC12 cells were cultured on control and N-cadherin expressing 3T3 monolayers in the presence and absence of the anti-rat NCAM antiserum (diluted 1:100) for 40–46 h before being fixed and immunostained for determination of mean neurite length. Each value is expressed as % increase in mean neurite length on N-cadherin expressing monolayers over background growth on 3T3 monolayers. 150–200 PC12 cells were sampled and bar shows \pm SEM.

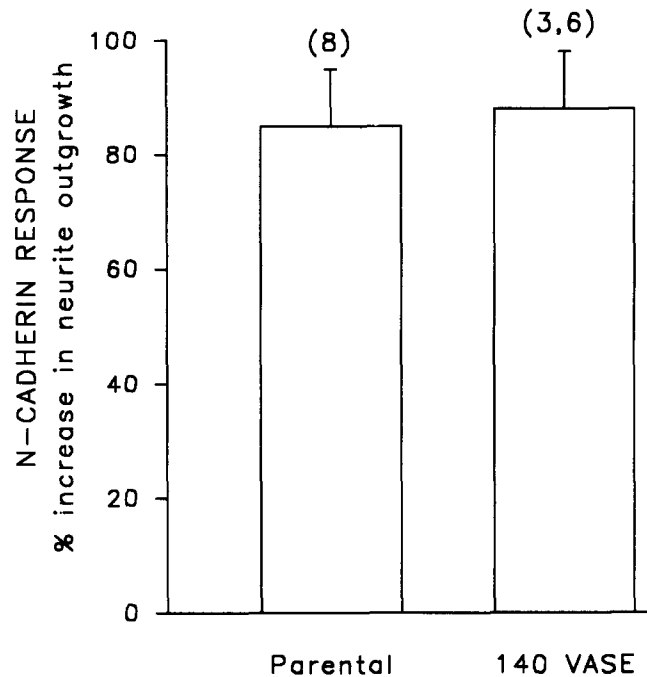


Figure 5. PC12 cells expressing hNCAM 140 VASE exhibit a normal responsiveness to N-cadherin. Parental and transfected PC12 cells expressing hNCAM 140 VASE were cultured on control and N-cadherin expressing 3T3 monolayers for 40–46 h before being fixed for determination of mean neurite length. The results are expressed as % N-cadherin response for each PC12 cell as shown, each value being the mean \pm SEM of 6–8 independent experiments.

Table II. PC12 Cell Clones Expressing 140 VASE Respond Normally to K⁺ Depolarization

Monolayer	Parental PC12	PC12 + hNCAM 140 VASE
a) 3T3	12.4 ± 0.8 (222)	14.4 ± 0.8 (170)
NCAM	28.3 ± 1.3 (227)	16.0 ± 1.1 (149) n.s.
b) 3T3+K ⁺	38.8 ± 2.0 (205)*	32.1 ± 1.9 (176)*
NCAM+K ⁺	37.4 ± 2.1 (219)*	37.6 ± 2.0 (173)*

Parental and transfected PC12 cells were cultured on confluent monolayers of control of NCAM expressing 3T3 cells in control media (a) or media further supplemented with 40 mM KCl (b). After 40 h the cultures were fixed and the mean length of the longest neurite determined. The results show this value (± SEM) for the given numbers of PC12 cells sampled in replicate cultures. Similar results were obtained in a second independent experiment. * $P < 0.005$; n.s., not significantly different for growth in treated cultures as compared to growth over parental 3T3 monolayer in control media.

The VASE Peptide Specifically Inhibits Neurite Outgrowth Stimulated by NCAM

The VASE sequence could either introduce an additional recognition site into NCAM, or alternatively it could disrupt a recognition site. The highly conserved nature of this sequence, with a single amino acid difference between xenopus and human, clearly supports the former possibility. To test for a recognition function, the corresponding peptide (the VASE peptide, see Materials and Methods) was added to parental PC12 cells cultured over monolayers of control or CAM expressing 3T3 cells. The VASE peptide completely inhibited neurite outgrowth stimulated by NCAM in the monolayer with a significant effect detected at ~20 µg/ml and a half-maximal inhibition at ~50 µg/ml (Fig. 6) but had

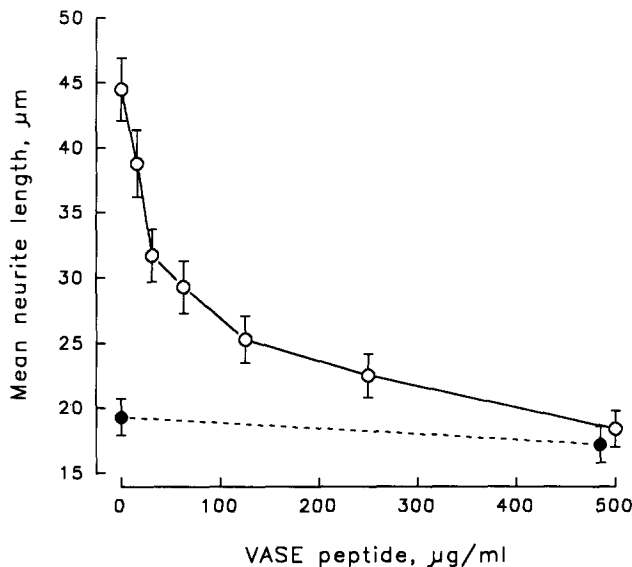


Figure 6. VASE peptide inhibits the NCAM response. PC12 cells were cultured on control (●) or NCAM expressing 3T3 monolayers (○) in the presence of VASE peptide (0–500 µg/ml) for 44 h before being fixed and stained for determination of neurite length. The results show the mean length of the longest neurite per cell, each value being the mean of 107–170 PC12 cells sampled. Bars show ± SEM. A similar dose-response curve was obtained in a second independent experiment.

no effect on basal neurite outgrowth over the parental 3T3 cells. A scrambled version of the VASE peptide had no effect on NCAM dependent neurite outgrowth, the measured NCAM response being 93 ± 13% of the control value in the presence of 500 µg/ml of the scramble peptide, mean ± SEM $n = 3$. The VASE peptide did not however reverse the inhibitory effects associated with expressing VASE containing isoforms of NCAM in PC12 cells (data not shown).

After expression in 3T3 cells, N-cadherin and L1 can also stimulate neurite outgrowth from PC12 cells and a variety of primary neurons (see Introduction). To determine if the VASE peptide was specifically inhibiting NCAM function, PC12 cells and postnatal day 4 cerebellar neurons were cultured on parental and CAM expressing 3T3 cells (NCAM, N-cadherin, and L1) in control media or media containing the VASE peptide (500 µg/ml). Results pooled from a number of independent experiments are shown for PC12 cells in Fig. 7 and cerebellar neurons in Fig. 8. Over a 40-h culture period the VASE peptide inhibited the NCAM response from PC12 cells by 95 ± 4% ($n = 8$), but had little effect on the N-cadherin or L1 responses (13 ± 3 [$n = 6$] and 3 ± 14 [$n = 4$] % inhibition, respectively, all values mean ± SEM determined from the given number of independent experiments). Over a 16-h period of culture the transfected CAMs

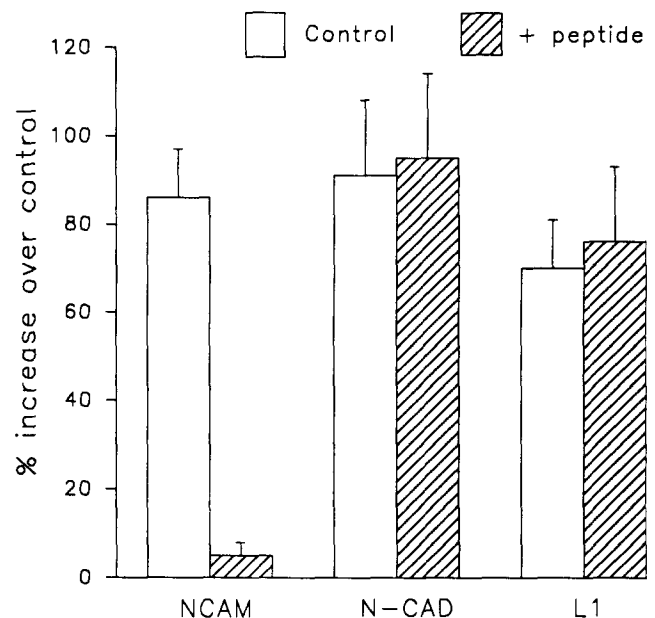


Figure 7. The VASE peptide has no effect on N-cadherin and L1 stimulated neurite outgrowth from PC12 cells. PC12 cells were cultured on monolayers of parental 3T3 cells or 3T3 cells expressing NCAM, N-cadherin, or L1 (as indicated) in control media or media supplemented with 500 µg/ml of the VASE peptide. After 40 h the cultures were fixed and the mean length of the longest neurite determined by sampling 100–170 PC12 cells. The results show the mean percentage increase in mean neurite length for PC12 cells cultured on CAM expressing monolayers relative to the value measured on control 3T3 cells. Each value is the mean ± SEM determined for 4–8 independent experiments. The VASE peptide had little effect on basal neurite outgrowth over 3T3 monolayers with the value in presence of the peptide being 91 ± 2% of the control (mean ± SEM determined in ten independent experiments).

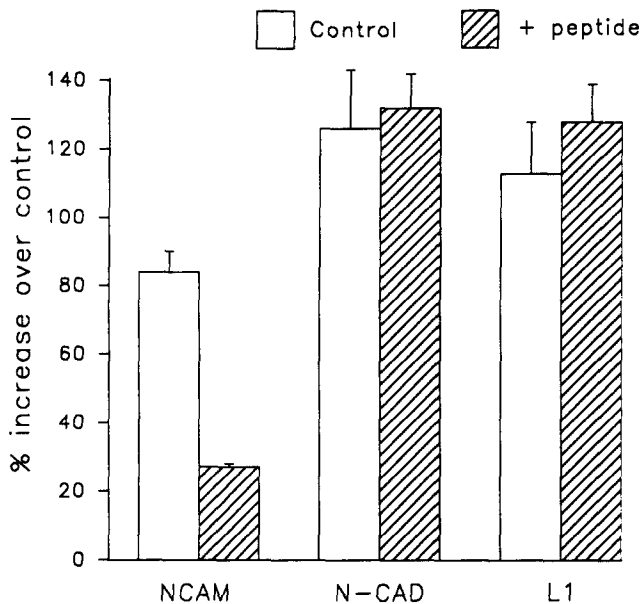


Figure 8. The VASE peptide specifically inhibits NCAM stimulated neurite outgrowth from cerebellar neurons. Postnatal day 4 cerebellar neurons were cultured on monolayers of parental 3T3 cells or 3T3 cells expressing NCAM, N-cadherin, or L1 (as indicated) in control media or supplemented with 500 $\mu\text{g}/\text{ml}$ of the VASE peptide. After 16 h the cultures were fixed and the mean length of the longest neurite determined by sampling 100–150 neurons. The results show the mean percentage increase in mean neurite length for neurons cultured on CAM expressing monolayers relative to the value measured on control 3T3 cells. Each value is the mean \pm SEM determined from three independent experiments for each CAM. The VASE peptide had no significant effect on basal neurite outgrowth over 3T3 monolayers. For example, in a representative experiment the mean length of the longest neurite on control 3T3 fibroblasts was $38.6 \pm 3.3 \mu\text{m}$ in control media and $36.6 \pm 3.0 \mu\text{m}$ in peptide containing media, mean \pm SEM, $n = 120$ –140.

approximately double the rate of neurite outgrowth from cerebellar neurons (Williams et al., 1992). The results in Fig. 8 again show that the VASE peptide can substantially inhibit neurite outgrowth stimulated by NCAM, but has no effect on basal neurite outgrowth which requires neuronal integrin receptor function, or neurite outgrowth stimulated by N-cadherin or L1. We can conclude that the VASE peptide is a specific inhibitor of NCAM stimulated neurite outgrowth.

Discussion

During periods of axonal growth neurons express predominantly the 140- and 180-kD isoforms of NCAM, and in vitro antibodies that block NCAM function partially inhibit neurite regeneration over cells that naturally express NCAM (Bixby et al., 1987; Neugebauer et al., 1988; Seilheimer and Schachner, 1988) or express transfected NCAM (Doherty et al., 1990b). In vivo, antibodies to NCAM can disrupt the pattern of retinotectal innervation (Fraser et al., 1988) and substantially inhibit the side branching of motor axons over muscle surfaces during development (Landmesser et al., 1988, 1990). All of the data point to a role for NCAM in axonal growth during development and regeneration and this is supported by studies on the spatiotemporal pattern of expression of NCAM (Edelman, 1986). However, whereas di-

rect evidence exists that NCAM as a cellular substratum promotes neurite outgrowth, evidence for its receptor function in the responsive cell remains indirect. The present study has shown that expression of the 140- or 180-kD isoforms of hNCAM in PC12 cells does not affect their ability to respond to NCAM in the substratum, implying either that these molecules are non-functional or that the level of endogenous NCAM is sufficient to give a maximal response. Using species specific antibodies against rat NCAM to prevent its interaction with hNCAM in the monolayer we have shown that while the NCAM response of parental PC12 cells is abolished that of clones expressing 140 or 180 isoforms of hNCAM is maintained. We can therefore conclude that both isoforms are able to function as receptors capable of binding to NCAM in the substratum and transducing the interaction into a neurite growth response. This contrasts with previous results that have shown the 180-kD isoforms expressed in 3T3 monolayers is a relatively poor ligand for neurite outgrowth as compared to the 140 or 120-kD isoforms (Doherty et al., 1992c). We have no simple explanation for this phenomenon, but the fact that the isoforms are in different cellular backgrounds raises the possibility that subtle differences in posttranslational processing and/or differences in NCAM's ability to interact with the underlying cytoskeleton might contribute (see Doherty and Walsh, 1992).

Analysis of the developing pattern of expression of the NCAM transcripts containing the VASE exon has led to the postulate that the use of this exon might contribute to a loss of synaptic plasticity. For example, whereas $\sim 3\%$ of NCAM transcripts contain the VASE exon during early brain development, this progressively increases to $\sim 50\%$ of all transcripts in the adult CNS (Small and Akeson, 1990). In addition, VASE is not expressed in the adult olfactory epithelium where neurogenesis and associated axonal growth continue throughout life. Recent studies using cerebellar neurons cultured on 3T3 fibroblasts expressing 140 kD NCAM \pm VASE (Doherty et al., 1992b) and retinal axons growing over two other cellular monolayers expressing 140 kD NCAM \pm VASE (Liu et al., 1993) have shown that the 140-kD isoform containing VASE is relatively poor at promoting neurite outgrowth. In the present study we have obtained three clones of PC12 cells expressing the VASE containing 140 kD isoform of hNCAM at similar levels to those expressing the control 140 kD isoform. However, whereas the latter isoform was capable of acting as a functional receptor (see above), use of the VASE exon dramatically inhibited the ability of PC12 cells to respond to NCAM, but not N-cadherin in the substrate. PCR analysis indicated that VASE containing transcripts accounted for only 20–30% of the total NCAM transcripts in transfected PC12 cells and suggest that this is therefore sufficient to induce loss of responsiveness to NCAM (Saffell, J., unpublished observation). Studies on primary neurons from the developing hippocampus and cerebellum show loss of responsiveness to NCAM (but not N-cadherin or L1) to be associated with an increase in VASE containing transcripts to as little as 20–30% of the total (Walsh et al., 1992). The results of the present study provide the first direct evidence that expression of VASE containing NCAM isoforms in neurons can cause them to become unresponsive (in terms of neurite outgrowth) to NCAM in the substrate even when they continue to express predominantly non-VASE isoforms. Liu et al. (1993) have also recently reported that ex-

pression of the 140-kD isoform of NCAM in a neuronal cell line that has no endogenous NCAM results in significantly greater neurite outgrowth on a NCAM expressing cellular substratum. Again NCAM lacking VASE was found to be more effective than NCAM containing VASE. Thus the failure of adult CNS neurons to regenerate might be dependent less on the permissiveness of the environment than on developmental changes within neurons that cause them to lose responsiveness to growth-promoting cues.

The presence of VASE in NCAM could modulate the homophilic binding of NCAM in either *trans* or *cis*. However, the expression of VASE does not appear to inhibit NCAM mediated adhesion per se (see Doherty et al., 1992; Liu et al., 1993). One possibility is that NCAM contains a complementary binding site for the VASE sequence and that the interaction of VASE (in *cis* and/or *trans*) with this site leads to increased stability of the homophilic interaction. In some circumstances stable adhesion mediated by CAMs can inhibit axonal sprouting (Mayford et al., 1992) and removal of polysialic acid from NCAM, which enhances adhesion (Hoffman and Edelman, 1983) also inhibits neurite outgrowth stimulated by NCAM (Doherty et al., 1990b).

A novel observation in the present study was that a peptide containing the VASE sequence specifically inhibited NCAM stimulated neurite outgrowth from PC12 cells and primary neurons. The peptide had no effect whatsoever on integrin dependent neurite outgrowth or neurite outgrowth stimulated by N-cadherin or L1. As all three CAMs stimulate neurite outgrowth by activating a similar (if not identical) second messenger pathway in neurons we can conclude that the VASE peptide must act at a recognition step upstream from pathway activation. We have previously shown that HAV-containing peptides derived from cadherins (Blaschuk et al., 1990) specifically inhibit neurite outgrowth stimulated by N-cadherin (Doherty et al., 1991a). The HAV peptides are believed to bind to an unidentified site on cadherins, and, by analogy, NCAM might also contain a VASE-binding site. The VASE peptide could conceivably inhibit neurite outgrowth by preventing NCAM-NCAM homophilic binding and/or by preventing the heterophilic interaction between NCAM and other molecules in *cis* and/or *trans*. The homophilic binding of NCAM can be inhibited with peptides derived from the third immunoglobulin domain and does not apparently require NCAM's VASE-containing fourth immunoglobulin domain (Rao et al., 1992). So far we have been unable to demonstrate any inhibitory activity of the VASE peptide in aggregation assays using covaspheres coated with purified NCAM (Waters, H., P. Doherty, and F. S. Walsh, unpublished observations). The second possibility, that the peptide inhibits an NCAM interaction with a second molecule, would require that the latter molecule carries a VASE like sequence and/or a VASE-binding domain. When the NCAM VASE sequence is searched in protein data banks the molecules that show a region with the highest sequence homology are a family of receptor tyrosine kinases that can be activated by FGF (the WTRPDKME sequence in the chick CEK2 receptor is 82.9% similar to the VASE sequence using the Dayhoff MDM-78 matrix test). An intriguing observation is that an adjacent sequence contains the HAV motif which can confer binding to cadherins (Byers et al., 1992). The FGF receptor is widely expressed by post mitotic neurons, and neurons (including PC12 cells) respond to activa-

tion of this receptor by extending longer neurites (e.g., see Walike, 1989; Wanake et al., 1990; Johnson et al., 1990). We have recently provided evidence that NCAM, N-cadherin, and L1 stimulate neurite outgrowth by activating a tyrosine kinase and have postulated that this might in fact be the FGF receptor (Williams et al., 1994). Thus we would suggest that the VASE sequence might inhibit NCAM stimulated neurite outgrowth by preventing NCAM from binding to and activating FGF receptors in neurons.

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