Different extracellular domains of neural cell adhesion molecule (N-CAM) are involved in different functions

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Different Extracellular Domains of the Neural Cell Adhesion Molecule (N-CAM) Are Involved in Different Functions

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Abstract. The neural cell adhesion molecule (N-CAM) engages in diverse functional roles in neural cell interactions. Its extracellular part consists of five Ig-like domains and two fibronectin type III homologous (type III) repeats. To investigate the functional properties of the different structural domains of the molecule in cell interactions and signal transduction to the cell interior, we have synthesized, in a bacterial expression system, the individual domains and tandem sets of individual domains as protein fragments. These protein fragments were tested for their capacity to influence adhesion and spreading of neuronal cell bodies, promote neurite outgrowth, and influence cellular migration patterns from cerebellar microexplants in vitro. Ig-like domains I and II and the combined type III repeats I–II were most efficient for adhesion of neuronal cell bodies, when coated as substrates. Neurite outgrowth was best on the substrate-coated combined type III repeats I–II, followed by the combined Ig-like domains I–V and Ig-like domain I. Spreading of neuronal cell bodies was best on substrate-coated combined type III repeats I–II, followed by Ig-like domain I and the combined Ig-like domains I–V. The cellular migration pattern from cerebellar microexplant cultures plated on a mixture of laminin and poly-L-lysine was modified by Ig-like domains I, III, and IV, while Ig-like domains II and V and the combined type III repeats I–II did not show significant modifications, when added as soluble fragments. Outgrowth of astrocytic processes from the explant core was influenced only by Ig-like domain I. Metabolism of inositol phosphates was strongly increased by Ig-like domain I and less by the Ig-like domains II, III, IV, and V, and not influenced by the combined type III repeats I–II. Intracellular concentrations of Ca^{2+} and pH values were increased only by the Ig-like domains I and II. Intracellular levels of cAMP and GMP were not influenced by any protein fragment. These experiments indicate that different domains of N-CAM subserve different functional roles in cell recognition and signal transduction, and are functionally competent without nervous system–derived carbohydrate structures.

CELL recognition plays an important role during the formation, maintenance, and regeneration of the nervous system. Several recognition molecules have been discovered to mediate cell-to-cell and cell-to-substratum interactions (for reviews see Edelman et al., 1990; Edelman and Crossin, 1991; Schachner, 1991). Among these, the neural cell adhesion molecule (N-CAM)[1] has been implicated in several types of neural cell interactions (Cunningham et al., 1987). N-CAM is involved in neuron-to-neuron and neuron-to-glia interactions in short-term adhesion assays (Keilhauer et al., 1985). It allows neurites to fasciculate (Rutishauser and Edelman, 1980; Fischer et al., 1986) and to interact with their muscle targets in the periphery (Grumet et al., 1982; Covault and Sanes, 1986; Covault et al., 1986). Formation of the retinal and tectal cytoarchitecture has also been attributed, at least in part, to N-CAM (Buskirk et al., 1980; Fraser et al., 1984). N-CAM is expressed early during neural development at the time of neurulation (Crossin et al., 1985). Its functional role in this process, in particular neural induction, has, however, remained obscure (Jacobson and Rutishauser, 1986). It remains expressed by the majority of cell types of neuroectodermal origin during later stages of nervous system development and appears to subserve yet unknown functions in the adult nervous system, where it continues to be expressed, although at lower levels than at early developmental stages. N-CAM is expressed on neural crest cells before migration, ceases to be detectable during migration, and reappears once these cells have arrived at their final position (Thiery et al., 1982). After peripheral nerve transection, the temporal and spatial sequence of N-CAM expression is the same as during development, suggesting that N-CAM also plays a role in regeneration (Nieke and Schachner, 1985; Martini and Schachner, 1988).

N-CAM is a recognition molecule that belongs to the immunoglobulin superfamily, carries one more and one less conserved fibronectin type III homologous repeat (Barthels

1. *Abbreviation used in this paper*: N-CAM, neural cell adhesion molecule.

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et al., 1987) and expresses the L2/HNK-1 carbohydrate (Kruse et al., 1984), which is itself functionally involved in cell interactions (Künemund et al., 1988), in common with other adhesion molecules (Kruse et al., 1985). N-CAM consists of several isoforms which derive from alternative splicing of one gene (Owens et al., 1986; Barbas et al., 1988; Santoni et al., 1989). The three major forms covering molecular masses of 180, 140, and 120 kD are identical in their extracellular part and differ from each other in the disposition of their cytoplasmic domains. The 120-kD form is anchored into the membrane via phosphatidylinositol (He et al., 1986; Sadoul et al., 1986), whereas the 140- and 180-kD forms are integral membrane glycoproteins, with the 180-kD component (N-CAM 180) having the largest cytoplasmic domain. Several other isoforms derived by alternative splicing of the extracellular part have been recognized in the mouse, with an additional π-exon in the fourth Ig-like domain and an a-exon within the fibronectin type III homologous repeats (Santoni et al., 1989). The lower molecular weight isoforms appear to be preferentially expressed by glial cells (Keilhauer et al., 1985; Seilheimer and Schachner, 1988), whereas the larger isoforms are predominantly, but not exclusively, expressed by neurons (Keilhauer et al., 1985; He et al., 1986; Sadoul et al., 1986). N-CAM 180 tends to be expressed at later developmental stages in the mouse nervous system and is concentrated at sites of cell contact (Pollerberg et al., 1985). This isoform has a reduced lateral mobility within the plasma membrane of neuronal cells and appears to be stabilized at sites of cell contact by interaction with the membrane cytoskeleton linker protein brain spectrin (fodrin) (Pollerberg et al., 1986, 1987). N-CAM 180 is accumulated in the postsynaptic densities of some, but not all synapses in hippocampus and cerebellum, where it has been suggested to play a role in modulating synaptic efficacy (Persohn et al., 1989). Accumulation of N-CAM 180 at sites of contact with partner ligands can be induced by beads coated with extracellular matrix molecules (Pollerberg et al., 1990*a*). L1 shows a similar, although not as distinct, localization at sites of cell contact, a similar reduced lateral mobility, and a similar accumulation at the contact sites between cells and between coated beads and cells (Pollerberg et al., 1990*a*,*b*). A molecular association between N-CAM and L1 within the plasma membrane of neurons could indeed be demonstrated by chemical crosslinking (Simon et al., 1991), underscoring the possibility that N-CAM and L1 are able to function by a mechanism termed assisted homophilic binding (Kadmon et al., 1990*a*). Association of N-CAM and L1 within the plasma membrane appears to be dependent on particular carbohydrates (Kadmon et al., 1990*b*), with the structure of such carbohydrate moieties having yet to be determined. Whether the highly sialylated, so-called embryonic and less adhesive form of N-CAM (Hoffman and Edelman, 1983; Sadoul et al., 1983) or heparin which has been found to bind to N-CAM (Cole and Akeson, 1989; Cole and Glaser, 1986), play a role in the interaction between L1 and N-CAM remains to be seen.

N-CAM not only engages in cell recognition, but is also capable of transducing recognition events into intracellular consequences. In different cell types, N-CAM stimulates changes in second messenger systems, such as inositol phosphates, Ca^{2+} and pH, independently of and interdependently with L1 (Schuch et al., 1989; von Bohlen und Halbach, F., J. Taylor, and M. Schachner, manuscript submitted for publication). Furthermore, L1 and N-CAM have been shown to reduce tyrosine phosphorylation of α and β tubulin in growth cone membranes, thus, increasing the state of tubulin polymerization (Atashi et al., 1992).

The fact that N-CAM engages in diverse functional roles raises the question as to the functional roles of the different structural domains of the molecule. In other words, are particular domains specialized for certain functions, are some domains functionally active and others inactive, or are the same functional domains of N-CAM involved in the total set of diverse functional roles. To investigate these structure–function relationships, we have synthesized protein fragments from the extracellular domains of N-CAM from a cDNA clone which does not contain the π- or a-exons (Santoni et al., 1989) and used these fragments in several assay systems, including the triggering of intracellular second messengers. Here, we report that the different domains of the molecule subserve different functional roles in cell adhesion, neurite outgrowth, fasciculation of neurites, and orientation of inhibitory GABAergic interneurons in the cerebellar cortex. Furthermore, we present evidence that different domains of N-CAM are specialized to trigger different second messenger systems.

Materials and Methods

Analytical Procedures

Bacterial lysates and renatured protein fragments were separated by SDS-PAGE on 12% slab gels (Laemmli, 1970) either under reducing or nonreducing conditions. Gels were stained with Coomassie blue, or transferred to nitrocellulose filters (type HAHY 00010; Millipore, Bedford, MA) for immunoblot analysis according to Faissner et al. (1985). Polyclonal and monoclonal antibodies to N-CAM were used at dilutions of 1:200 and 1:50, respectively. Protein determinations were performed as described (Bradford, 1976) or by comparing staining intensities of protein bands with protein marker standards after SDS-PAGE and staining with Coomassie blue.

Production of Protein Fragments of N-CAM in a Prokaryotic Expression System

Nine different constructs were derived from the mouse N-CAM cDNA clone DW3 LE which encodes the five Ig-like domains (Barthels et al., 1987) and the pM1.3 clone which encodes the two fibronectin type III domains (Goridis et al., 1985). The different cDNA fragments were excised from the two clones with appropriate restriction enzymes (see Fig. 1), blunt ended by T4 DNA polymerase, and subcloned into the blunt ended BamHI restriction site of the pET vector system (Rosenberg et al., 1987) according to standard procedures (Maniatis et al., 1982). All protein fragments expressed by the pET vector system contain 11 amino acids contributed by the translation initiation site and 3–10 amino acids contributed by the translation stop site of the vector. Thus, the proteins carrying the individual domains have larger apparent molecular weights than the protein encompassing all Ig-like domains. Recombinant clones with correct orientation were identified by restriction analysis of recombinant plasmid DNA. Expression of the nine recombinant cDNA constructs and a control construct of a plasmid without an inserted cDNA fragment in *E. coli* strain BL21(DE3) (Studier and Moffatt, 1986) was carried out as described (Rosenberg et al., 1987). A crude bacterial lysate contained 6–8 mg recombinant protein per 200 ml as determined by SDS-PAGE.

Purification and Renaturation of Protein Fragments

Recombinant plasmid containing *E. coli* cells were treated with 0.4 mM isopropylthio-β-D-galactoside (IPTG) for 4 h to induce transcription of recombinant mRNA. Cells were then collected by centrifugation at 4°C and 4,000 *g* for 15 min and the viscous pellet was sonicated (Branson sonifier B15) in PBS saline, pH 7.3, at maximum intensity for 3 min on ice without

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interruption. Inclusion bodies resulting from all constructs including that without a cDNA insert were collected from the lysate by centrifugation at 9,000 *g* for 30 min at 4°C. The pellet was stored in PBS at −20°C. Denaturation and renaturation of protein fragments contained in the inclusion bodies were carried out as described (Buchner and Rudolphe, 1991). Briefly, the pellet was solubilized by incubation at room temperature for 2 h in 300 mM dithioerythrol (DTE), 6 M urea, 2 mM EDTA, 100 mM Tris-HCl, pH 8.0, and reoxidized for 36 h in 10 mM oxidized glutathione, 0.3 M L-arginine in 100-fold excess volume to allow refolding of the molecules at 4°C. The protein solution was then concentrated by filtration through Amicon filters (YM 10) or filtration through centricon 10 microconcentrator tubes (catalog no. 4205; Amicon, Beverly, MA) and dialyzed against 50 mM Tris-HCl, pH 8.5. The concentrated samples contained 1–3 mg/ml of protein as determined according to Bradford (1976). The purity of fragments in the inclusion bodies was 90% for Ig I, 88% for Ig II, 85% for Ig III, 72% for Ig IV, 86% for Ig V, 91% for Ig I-V, 88% for FN I, 85% for FN II, and 89% for FN I-II as determined by SDS-PAGE. The insoluble pellet from the bacterial lysate containing the control construct was denatured and renatured as the other protein fragments. The supernatant from the bacterial lysate containing this construct was not further treated.

Further purification of the first Ig-like domain of N-CAM solubilized from the inclusion bodies was achieved by chromatography on a Sephadex G-50 fine column (Pharmacia LKB) (1.5 × 100 cm) under denaturing conditions (4 M urea in PBS), yielding a major peak with an approximate molecular mass of 13 kD which contained the protein fragment clearly separated from protein contaminants at higher molecular weights. The peak fraction was renatured and concentrated as described in the previous paragraph and had a purity of 96% as determined by SDS-PAGE (see Fig. 2).

Adhesion Molecules

N-CAM and L1 were immunoaffinity purified from detergent extracts of crude membrane fractions from adult mouse brain using monoclonal antibody columns (Hirn et al., 1983; Faissner et al., 1984; Rathjen and Schachner, 1984). Laminin from murine EHS sarcoma and fibronectin from human serum were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Antibodies

Polyclonal rabbit antibodies to murine N-CAM and L1 (Faissner et al., 1984; Rathjen and Schachner, 1984; Gennarini et al., 1984) and monoclonal rat antibody to murine N-CAM (H28.123) (Goridis et al., 1983; Hirn et al., 1981) have been described. The polyclonal antibodies were purified using a protein A-Sepharose column (Pharmacia LKB), according to Ey et al. (1978). The polyclonal antibodies to murine N-CAM recognized only some epitopes on the N-CAM molecule as revealed by Western blot analysis. The mAb H28.123 was purified using a protein G-Sepharose 4B column (Pharmacia LKB, Piscataway, NJ) according to Åkerström et al. (1985). Polyclonal rabbit antibodies to glial fibrillary acidic protein from bovine brain were purchased from Dakopatts (Denmark). FITC-conjugated goat anti-rabbit Ig antibodies were obtained from The Jackson Laboratory (West Grove, PA).

Indirect Immunofluorescence

Immunolabeling for glial fibrillary acidic protein, a marker for mature astrocytes (Bignami et al., 1972), was performed as described (Schnitzer and Schachner, 1981) with some modifications. Briefly, microexplant cultures were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and unsaturated binding sites were blocked with 10% horse serum and 1% BSA in PBS. Cells were then permeabilized with the same buffer containing 0.3% Triton X-100 and washed three times with PBS. Cultures were incubated for 1 h at room temperature with antibodies to GFAP at a dilution of 1:250, followed by FITC-conjugated secondary antibodies for 1 h at room temperature at a dilution of 1:100. Cultures were examined with an Axiophot fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

Cell Adhesion Test

The adhesion test was carried out according to Bachmann et al. (Bachmann, M., J. F. Conscience, R. Probstmeier, S. Carbonetto, and M. Schachner, manuscript submitted for publication). In brief, 96-multiwell plates (catalog no. 1-43761 A; Nunclon Delta, Denmark) were coated with adhesion molecules or protein fragments at equimolar concentrations (50 nM) in 100 μl PBS for 3 h at 37°C, except for laminin which was coated overnight at 37°C and 20 μg/ml in basal medium Eagle's. Concentrations of proteins were determined by comparing staining intensities of protein bands with protein marker standards after SDS-PAGE. For the N-CAM peptides, the protein content was estimated for the major band containing the peptide. For the bacterial proteins, molar concentrations were calculated based on the average molecular mass of 60 kD. Coating efficiencies were measured by protein determination using a modification of the Bradford assay (Fahrig et al., 1987) and found to be between 55 and 65% of the input for all proteins and protein concentrations used. Unsaturated binding sites seen at all protein concentrations were blocked with heat-inactivated (5 min at 70°C) fatty acid–free BSA (Boehringer Mannheim Biochemicals) for 1 h at room temperature, single cell suspensions of small cerebellar neurons from 6–7-d-old ICR mice (Keilhauer et al., 1985) were seeded in 100 μl serum-free neuron culture medium (Fischer, 1982) at a density of 3×10^5 cells per well. Single cell suspension of cerebellar neurons was obtained by dissociation in the presence of 0.1% trypsin, resulting in neurons expressing N-CAM at the cell surface as shown by Faissner et al. (1984). After incubation for 3 h at 37°C in a CO_2 incubator, cells were treated with 2.5% glutaraldehyde in PBS for 30 min. Cells were stained with crystal violet (0.5% crystal violet [Merck] in 10% ethanol, 3% formaldehyde, 0.15% NaCl) for 10 min at room temperature and washed with PBS. The optical density associated with the adherent cells was measured at 595 nm with an ELISA-reader (Titertek multiskan plus; Flow Laboratories, Inc., McLean, VA). The data from four independent experiments carried out in duplicates were analyzed for statistical significance by using the *t* test.

Determination of Neurite Outgrowth and Cell Spreading

Neurite outgrowth of small cerebellar neurons was determined according to Conscience et al. (Conscience, J. F., F. Appel, A. Faissner, F. van Bohlen und Halbach, J. Holm, and M. Schachner, manuscript in preparation). In brief, glass coverslips (1.1 cm in diameter) were coated overnight at 37°C with poly-L-lysine (200 μg/ml; Sigma Chemical Co., St. Louis, MO) in 150 mM Na-borate, pH 8.4, and washed three times with 100 μl PBS. Purified N-CAM and protein fragments (0.5 μM) in PBS were then added and incubated for 3 h at 37°C. Laminin (20 μg/ml) was incubated overnight at 37°C. Single cell suspensions of small cerebellar neurons from 6–7-d-old ICR mice (Keilhauer et al., 1985) were seeded at a density of 1×10^5 cells per glass coverslip and maintained for 20 h at 37°C in a CO_2 incubator.

Cells were then treated with 2.5% glutaraldehyde in PBS and stained with crystal violet for 5 min as outlined under Cell Adhesion Test. Neurons with processes were analyzed by evaluating the lengths of neurites per cell and the surface area of the cell body by using an Ai Tectron (VIDS IV software) image analysis system (Seilheimer et al., 1989). Neurites were evaluated only when they did not contact other cells or neurites and when their length was longer than the diameter of the cell body (Lemmon et al., 1989). Approximately 50 neurites were measured for each value. The Mann-Whitney U rank test was used to determine whether different substrates produced a significantly different extent of neurite outgrowth and cell spreading, with a P value of <0.05 being considered significant.

Microexplant Cultures

Cerebellar microexplant cultures were prepared as described (Fischer et al., 1986; Hekmat et al., 1989). Briefly, cerebella from 6-d-old ICR mice were freed of meninges, white matter, and deep cerebellar nuclei, pressed through a Nitrex net with a pore diameter of 300 μm, and cultured in serum-free neuron culture medium (Fischer et al., 1982) on poly-L-lysine- and laminin-coated glass coverslips. After incubation for 4 h at 37°C in a CO_2 incubator, purified N-CAM or protein fragments (1 μM) were added. Cultures were taken for staining with crystal violet for 5 min as outlined under Cell Adhesion Test or for indirect immunofluorescence after different times of maintenance in culture (72–96 h). The data are from four independent experiments carried out in duplicates and are blinded.

Determination of Inositol Phosphates, Cyclic Nucleotides, Intracellular Ca^{2+}, and pH

Intracellular levels of inositol phosphates, cyclic nucleotides, Ca^{2+}, and pH were determined in small cerebellar neurons from 6–7-d-old ICR mice (Keihauer et al., 1985) maintained in culture on laminin. Measurements were performed as originally described by Schuch et al. (1989) as modified for cerebellar neurons by von Bohlen und Halbach, F., J. Taylor, and M. Schachner (manuscript submitted for publication): For IP turnover measurements, the extraction of cells was not done with chloroform/methanol

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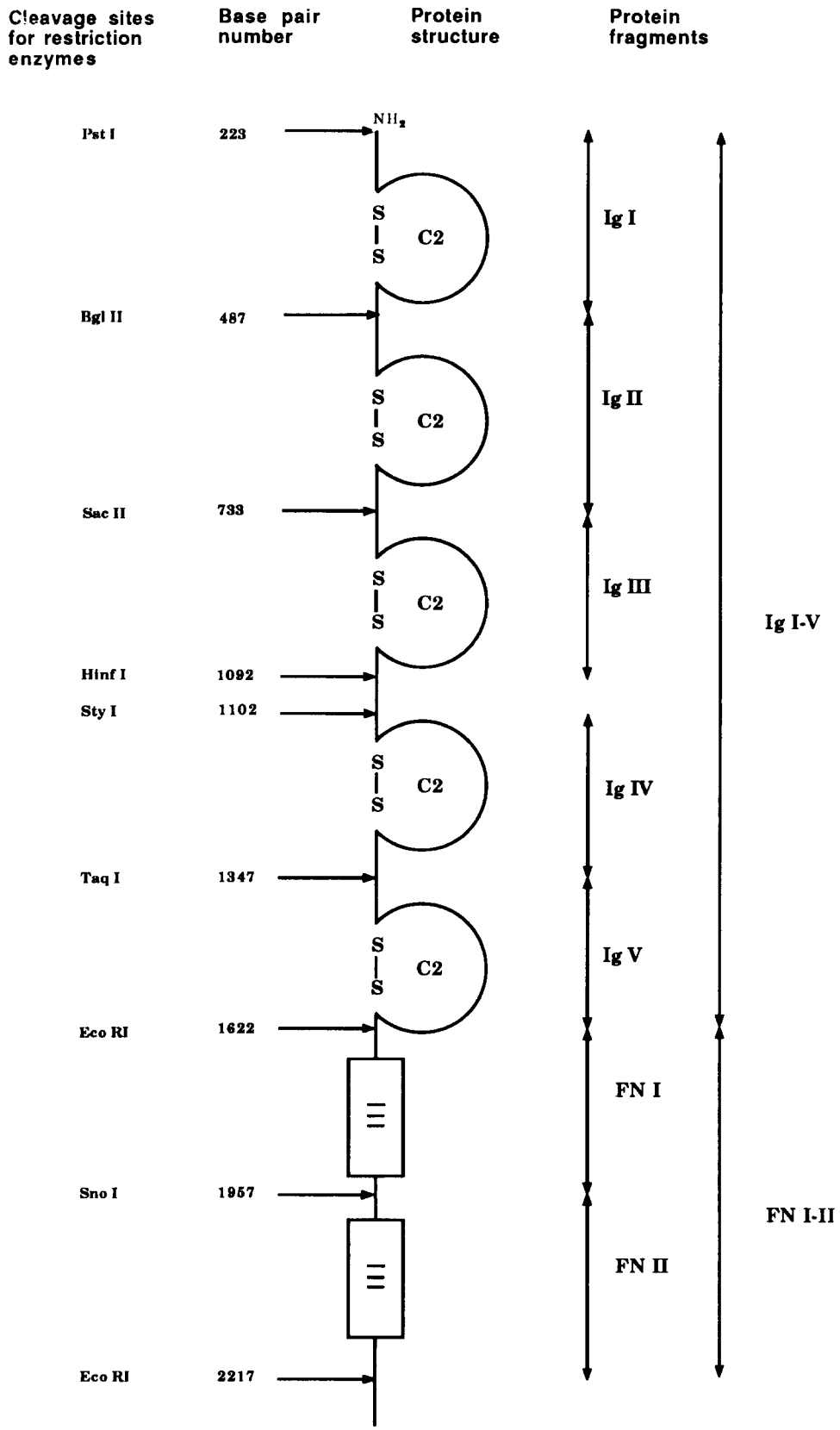

Figure 1. Schematic representation of the extracellular domain of mouse N-CAM at the cDNA and protein levels and derivation of cDNA clones for expression of protein fragments. The nine cDNA clones encode the following protein fragments: Ig I, first Ig-like domain (bp 223–487); Ig II, second Ig-like domain (bp 487–733); Ig III, third Ig-like domain (bp 733–1092); Ig IV, fourth Ig-like domain (bp 1102–1347); Ig V, fifth Ig-like domain (bp 1347–1622); FN I, first fibronectin type III homologous domain (bp 1622–1957); FN II, second fibronectin type II homologous domain (bp 1957–2217). Ig I–V, all five Ig-like domains (223–1622), FN I–II, the two fibronectin type III domains (1622–2217). The cDNA fragments were subcloned into the pET expression vector after digestion with the appropriate restriction enzymes as indicated by arrows. The numbering of base pairs corresponds to that used by Barthels et al., (1987), where number 1 refers to the transcription initiation site.

as originally described (Schuch et al., 1989) but instead with 20% TCA and the probes were separated on an automated Pharmacia FPLC-system equipped with an anion exchange column (Mono Q HR 5/5; Pharmacia Fine Chemicals) using a step gradient from O to 1.5 M ammonium acetate, pH 6.4. 1-ml fractions were collected, mixed with 4 ml scintillation cocktail (Ultima-Gold XR, Canberra Packard), and counted for 1 min in a beta scintillation counter (Packard 1900 TR). To measure the influence of N-CAM and L1 from adult mouse brain, fibronectin, and N-CAM fragments on second messengers, the proteins were added in PBS to the cultures to give a concentration of 0.8 μM. The IgG fractions of polyclonal antibodies to

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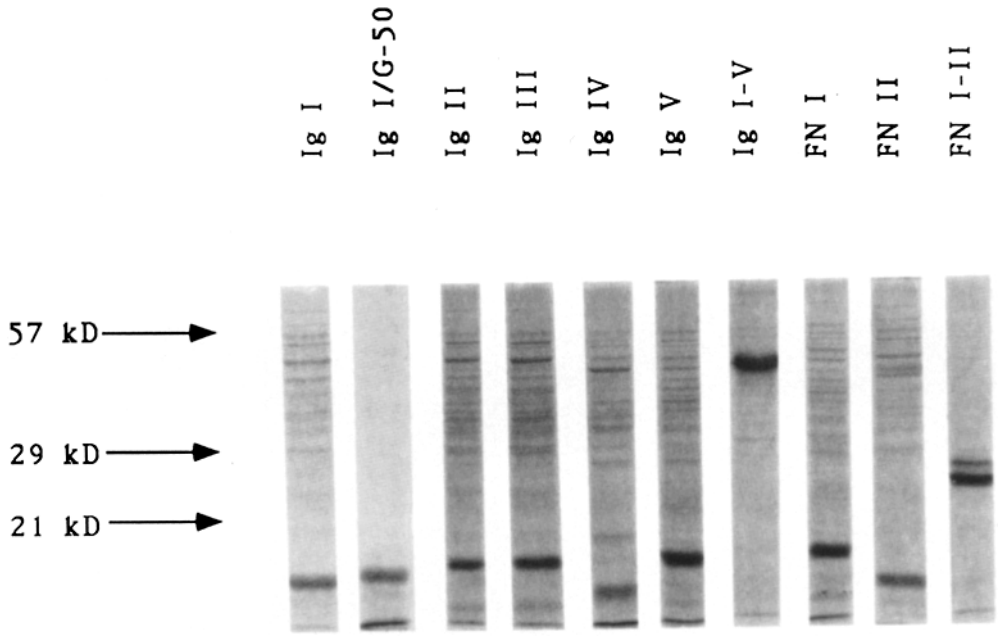

Figure 2. Analysis of molecular weights and protein contaminants of the N-CAM protein fragments synthesized in the pET expression system. Inclusion bodies containing the proteins (Ig I through Ig V, Ig I–V, FN I, FN II, FN I–II, Ig I–V + FN I–II, for abbreviations see legend to Fig. 1) were solubilized and renatured. Ig I G-50 is Ig I from inclusion bodies further purified by gel filtration over a Sephadex G-50 column. Proteins (10 μg per lane) were separated by SDS-PAGE and visualized by Coomassie blue staining. The bromophenol blue front is seen at the bottom of each lane. Molecular mass markers are indicated in kilodaltons (*kD*) at the left margin.

N-CAM and L1 were added to give a final concentration of 500 μg/ml. Lithium was added to prevent the degradation of inositol phosphate to inositol and monophosphate by inhibition of the IPI-phosphatase (Berridge et al., 1982). Thus, IP1 is the final product of the inositol phosphate turnover pathway and thereby an indicator for the observed stimulation. The data from three independent experiments were analyzed for statistical significance by using the *t* test.

Results

To assess the functional roles of the different domains of N-CAM, we chose to synthesize, in a bacterial expression system, the five Ig-like domains and the two fibronectin type III homologous (FN) repeats individually and as sets of domains, consisting of Ig-like domains I to V (Ig I–V) and type III domains I to II (FN I–II) (Fig. 1). In the pET vector expression system, proteins were collected as inclusion bodies, which were solubilized in urea under reducing conditions and slowly oxidized under controlled oxygen tension using a protocol designed for the renaturation of bacterially produced immunoglobulins into a functionally active state (Buchner and Rudolphe, 1991). Using this protocol, protein fragments remained soluble in PBS. The protein fragments thus obtained and their protein contaminants are shown in Fig. 2 under reducing conditions. The protein fragments showed the expected apparent molecular masses of 13 kD for Ig, I, 14 kD for Ig II, 14 kD for Ig III, 12 kD for Ig IV, 13 kD for Ig V, 52 kD for Ig I–V, 14 kD for FN I, 12 kD for FN II, and 26 kD for FN I–II. Under nonreducing conditions, all protein fragments, except for the fragment containing the total set of Ig-like domains I–V, gave one band (not shown), thus, underscoring that a single configuration is attained. The Ig I–V fragment was seen as several distinct bands under nonreducing conditions, probably representing the different conformations of the partially renatured molecule. The protein fragment of Ig I was further purified by gel filtration using a Sephadex G-50 column to remove the high molecular weight protein contaminants.

The functional activity of these protein fragments was tested in several assay systems. First, the adhesion of small cerebellar neurons to the substrate-coated fragments was tested. Then, neurite outgrowth and spreading of cell bodies of small cerebellar neurons was tested on these fragments adsorbed onto poly-L-lysine–coated substrates. The ability of soluble protein fragments to affect cellular migration patterns was tested in cerebellar microexplant cultures plated onto a mixed laminin/poly-L-lysine substrate. Finally, the

Table I. Summary of the Functional Properties of N-CAM Protein Fragments in Cell Culture

	Cell adhesion								
	BSA	PLL	Neurite outgrowth	Cell spreading	Neurite fasciculation	IP	Ca^{2+}	pH	cAMP cGMP
Ig I	+++	+++	+	+++	decrease	+++	+++	+++	–
Ig II	++	++	–	+	–	++	+++	+++	–
Ig III	+	–	–	–	increase	+	–	–	–
Ig IV	+	–	–	–	increase	+	–	–	–
Ig V	+	–	–	–	–	+	–	–	–
Ig I-V	+	–	++	++	–	+++	+++	+++	–
FN I-II	+	+	+++	+++	–	–	–	–	–
brain N-CAM	+	–	+++	++	ND	+++	+++	+++	–

The efficacy with which a fragment induces the functional effects is schematically represented as +++, maximal effect; ++, intermediate effect; +, small effect; –, no effect. Cell adhesion, neurite outgrowth, and spreading were tested with substrate-coated fragments. The values for cell adhesion are based both on BSA or PLL as control. The values for neurite outgrowth and cell spreading are based on poly-L-lysine as control. All other tests were carried out with soluble fragments added to the cultures. ND, not determined, since brain N-CAM contains 0.1% deoxycholate which damages the explant cultures.

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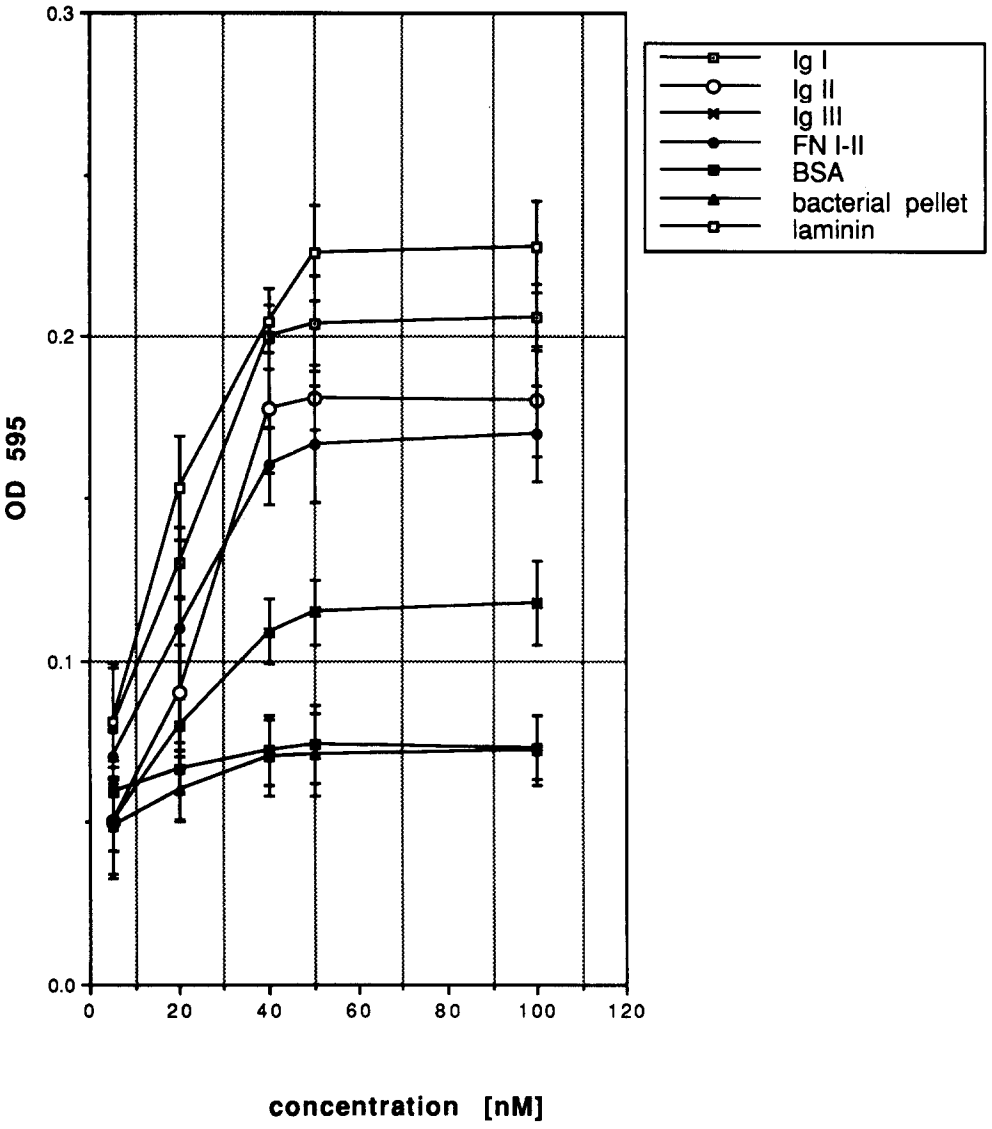

Figure 3. Adhesion of small cerebellar neurons to substrate-coated N-CAM protein fragments as a function of the coated protein concentration. Adhesion of neurons to protein fragments Ig I, Ig II, Ig III, FN I–II, bacterial pellet, laminin, and to BSA (for abbreviations see legends to Figs. 1 and 2) was evaluated after 3 h of maintenance on the substrate and staining with crystal violet as determined by measuring the optical density at 595 nm. Mean values ± SD are from four independent experiments carried out in duplicate.

ability of the protein fragments to modulate intracellular messenger systems in small cerebellar neurons was monitored. Inositol phosphates, cyclic nucleotides, and intracellular levels of Ca^{2+} and pH were determined. Table I summarizes the effects of N-CAM protein fragments in these different assays.

Adhesion of Neurons to Substrate-coated Protein Fragments

Small cerebellar neurons were allowed to adhere for 3 h to the protein fragments which had been coated onto the plastic surface of wells of plastic microtiter plates at equimolar concentrations. In dose-response curves, maximal effects were reached, when proteins were coated at 50 nM concentrations (Fig. 3). Attachment of cell bodies was best on laminin (2.1-fold compared to poly-L-lysine and 3.0-fold compared to BSA), followed by Ig I (1.9-fold compared to poly-L-lysine and 2.7-fold compared to BSA), Ig II (1.7-fold compared to poly-L-lysine and 2.4-fold compared to BSA), and FN I–II (1.5-fold compared to poly-L-lysine and 2.2-fold compared to BSA) (Fig. 4). The adhesion of neurons to Ig II, but not to other fragments was reduced in the presence of heparin (not shown). The other protein fragments (Ig III, IV, V, Ig I–V, and FN I and II) were not significantly different in stimulating adhesion when compared to poly-L-lysine (ranging from 1.0 to 1.3-fold for the Ig domains and 1.3–1.8-fold for the FN domains). When compared to adhesion of neurons to BSA, adhesion to these protein fragments was significantly increased (ranging from 1.3- to 2.7-fold for the Ig domains and 1.6- to 2.2-fold for the FN domains). Both N-CAM isolated from adult mouse brain by immunoaffinity chromatography and the mixture of Ig I–V and FN I–II showed some enhancement of neuronal adhesion, when compared to poly-L-lysine (ranging from 1.2- to 1.3-fold) but this was not significant. Thus, the individual Ig I and Ig II fragments were more effective in promoting neuronal attachment than the protein fragment encompassing all five Ig-like domains and N-CAM from brain. Similarly, FN I–II was a more effective substrate for neuronal adhesion than N-CAM from brain. In contrast, the two FN domains contained together in one fragment were better adhesive substrates than the single FN domains.

When Ig I purified by gel filtration was taken as a substrate and compared to Ig I taken directly from the inclusion bodies (see Fig. 2), no differences in the efficiency of cell adhesion

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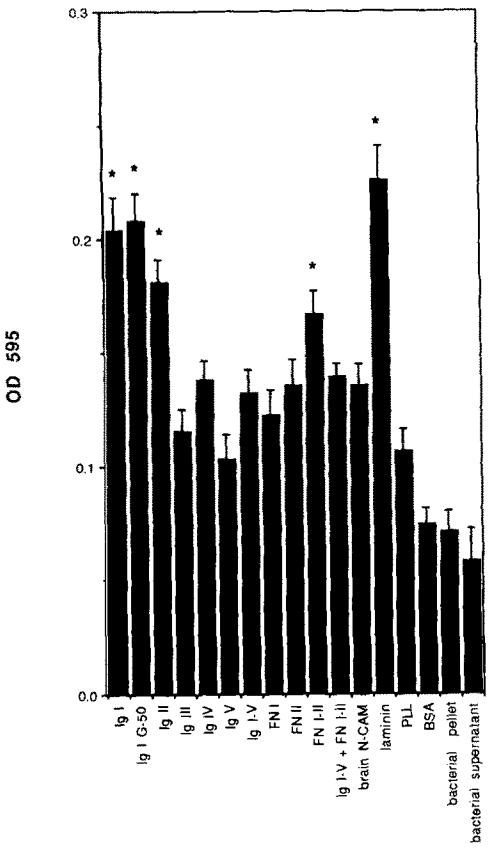

Figure 4. Adhesion of small cerebellar neurons to substrate-coated N-CAM protein fragments at 50 nM concentrations. Adhesion of neurons to protein fragments Ig I to Ig V, Ig I–V, FN I, FN II, FN I–II, and Ig I–V + FN I–II (for abbreviations see legends to Figs. 1 and 2), brain N-CAM, immunoaffinity purified from adult mouse brain (N-CAM), laminin, poly-L-lysine (PLL), BSA, and bacterial proteins from the insoluble pellet which were denatured and renatured as described (bacterial pellet) and from the supernatant (bacterial supernatant) was evaluated after 3 h of maintenance on the substrate and staining with crystal violet as determined by measuring optical density at 595 nm. Mean values ± SD are from four independent experiments carried out in duplicate. Bars marked by * are significantly different ($P < 0.001$) from the control in which PLL was used as a substrate.

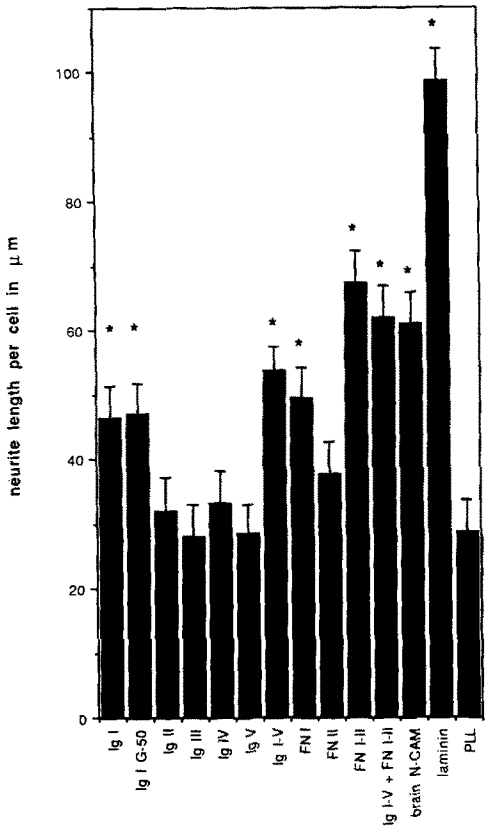

Figure 5. Determination of neurite length of small cerebellar neurons maintained on N-CAM protein fragments. Cells were maintained on protein fragments Ig I to Ig V, Ig I–V, FN I, FN II, and Ig I–V + FN I–II (for abbreviations see legends to Figs. 1 and 2), brain N-CAM, immunoaffinity purified from adult mouse brain (N-CAM), and laminin adsorbed to poly-L-lysine–coated glass coverslips for 20 h, fixed and stained with crystal violet. Lengths of neurites were determined per cell. Mean values ± SD are from ~50 neurons from two independent experiments carried out in duplicate. Bars marked by * are significantly different ($P < 0.05$) from the control (PLL alone as a substrate).

were observed, when the protein concentrations of the Ig I fragments were estimated from the intensity of the protein bands in polyacrylamide gels. To evaluate the contribution of the bacterial protein contaminants, proteins from the inclusion bodies and supernatants of bacterial lysates from the control clone were used as substrates. There was no significant difference in adhesion to these proteins when compared to BSA.

Neurite Length and Cell Body Spreading of Neurons on Substrate-coated Protein Fragments

To determine the influence of the molecular domains on neurite length and the spreading of cell bodies, protein fragments, N-CAM from brain, and laminin, were adsorbed onto poly-L-lysine that had been coated onto glass coverslips. Small cerebellar neurons were maintained on the substrates for 20 h and neurite length and cell body area monitored by semi-automated image analysis. Titration of protein concentrations for substrate coating revealed that 0.5 μM was required for coating in order to see a maximal effect on neurite outgrowth and neuronal cell body spreading (not shown). In the following, all comparisons are based on these maximal effects.

Neurite length was highest on laminin (98.7 μm per neuron and 3.4-fold higher than on poly-L-lysine alone, on which neurites were only 28.8 μm long; Fig. 5). Of the N-CAM protein fragments, FN I–II caused highest increase in neurite outgrowth when compared to poly-L-lysine alone (67.3 μm and 2.3-fold), followed by Ig I–V (53.6 μm and 1.8-fold), FN I (49.5 μm and 1.7-fold), and Ig I (46.4 μm and 1.6-fold). The other protein fragments (Ig II, III, IV, and V and FN II) did not give significantly different values than those obtained on poly-L-lysine alone (ranging from 1.0- to 1.3-fold). N-CAM from brain or the substrate mixture of Ig I–V and FN I–II also gave enhanced neurite outgrowth when compared to poly-L-lysine alone (2.1-fold for both), and were almost as potent as the FN I–II. Thus, the protein fragments encompassing the total extracellular domains and N-CAM from brain were almost as efficient in promoting neurite outgrowth as the most potent protein fragments.

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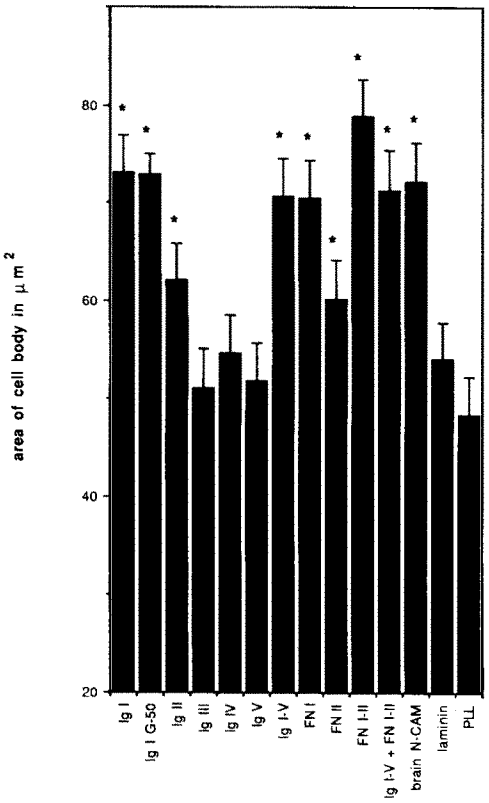

Figure 6. Determination of spreading of cell bodies of small cerebellar neurons maintained on N-CAM protein fragments. Cells were maintained on the protein fragments Ig I to Ig V, Ig I–V, FN I, FN II, Ig I–V + FN I–II (for abbreviations see legends to Fig. 1), brain N-CAM, immunoaffinity purified from adult mouse brain (N-CAM), and laminin adsorbed to poly-L-lysine–coated coverslips for 20 h, fixed, and stained with crystal violet. The area covered by one neuronal cell body was determined. Mean values ± SD are from ~50 neurons from two independent experiments carried out in duplicate. Bars marked by * are significantly different ($P < 0.05$) from the control (PLL alone as a substrate).

The spreading of cell bodies of small cerebellar neurons as estimated by the area covered by the cell body was measured in the same experiments that were performed to determine neurite length (Fig. 6). FN I–II were best in inducing cell body spreading followed by Ig I, and Ig I–V, FN I, Ig II, and FN II. Ig III, IV, and V, and laminin did not significantly differ from the poly-L-lysine substrate alone. N-CAM from brain and the substrate mixture of Ig I–V and FN I–II were almost as efficient in promoting spreading of cell bodies as FN I–II and Ig I.

The percentage of neuronal cell bodies extending neurites was not quantitated because it was not strikingly different for any of the domains of N-CAM.

Cellular Outgrowth Patterns from Cerebellar Microexplants in the Presence of Soluble Protein Fragments

The outward migration pattern of neuronal and astrocytic cell bodies and processes from microexplants taken from early postnatal mouse cerebellum was taken as another sensitive assay parameter to monitor the impact of N-CAM protein fragments on cellular behavior. In this assay system, small tissue pieces of cerebellum are plated onto a mixture of laminin and poly-L-lysine as substrates and the protein fragments are added in soluble form after the explants have attached to the substrate. Titration of protein concentrations, as determined by comparing staining intensities of protein bands after SDS-PAGE revealed that 1 μM was required to induce a maximal, visible effect. The outgrowth pattern was observed 72 or 96 h after plating the explants and the effects were essentially the same at these two time points.

The normal outgrowth pattern of these microexplants has been described (Hekmat et al., 1989). In particular, cells are observed with an orientation of processes largely perpendicular (Figs. 7 *A*, 8 *A*, and 9 *A*) to the direction of the fasciculating neurites of granule cells extending radially away from the explant core. These cells first have a bipolar morphology and then elaborate a rich dendritic arbor-like structure distal from a long, thin axon-like process. As these cells take up GABA, have small cell bodies, and amount to 10% of all small cerebellar neurons, they represent the small inhibitory interneurons of the cerebellum, the stellate, and basket cells.

In the presence of Ig I, the pattern of neurite outgrowth and cell body migration was significantly different from that in control cultures (Figs. 7 *B*, 8 *B*, and 9 *B*). Neurites were less fasciculated and outward migration and clustering of cell bodies less pronounced than in the control cultures (Figs. 7 *A*, 8 *A*, and 9 *A*). Of the few cell bodies that migrated beyond the area occupied by GFAP positive astrocytes (Fig. 10 *B*), the inhibitory interneurons were clearly distinguishable, although less frequent than in the control, by the perpendicular orientation of their neurites. The outgrowth of GFAP positive astrocytic processes was reduced by Ig I. These effects could be reversed when 60 h after plating the Ig I was removed by replacing the culture medium. 72 h afterwards the inhibitory effect was completely reversible and the culture looked healthy and not damaged. The Ig III and IV (Figs. 7 *D*, 8 *D*, 9 *D*, and Figs. 7 *E*, 8 *E*, and 9 *E*, respectively) also produced an outgrowth pattern different from the control cultures (Figs. 7 *A*, 8 *A*, and 9 *A*). Neurites were more fasciculated and clusters of cell bodies tended to be larger than in the control cultures and only a few, misaligned perpendicular cells were seen. The outgrowth of astrocytic processes from the explant core was not affected by Ig III and IV (Fig. 10, *D* and *E*). No differences in outgrowth patterns were seen for Ig II, V, and FN I–II, when compared to the untreated controls. To evaluate the contribution of the bacterial protein contaminants, proteins from the inclusion bodies and supernatants of bacterial lysates from the control clone were used. There was no significant difference in the outward migration pattern of these proteins when compared to the untreated control (not shown).

Second Messenger Systems of Neurons in the Presence of Soluble Protein Fragments

The influence of the different protein fragments on intracellular second messenger systems was investigated using small cerebellar neurons maintained on laminin and by addition of the soluble fragments. In dose-response curves, maximal effects were reached when proteins were added at 0.8 μM concentrations. Levels of inositol phosphates (IP1, IP2, and IP3) were determined after incubation of small cerebellar

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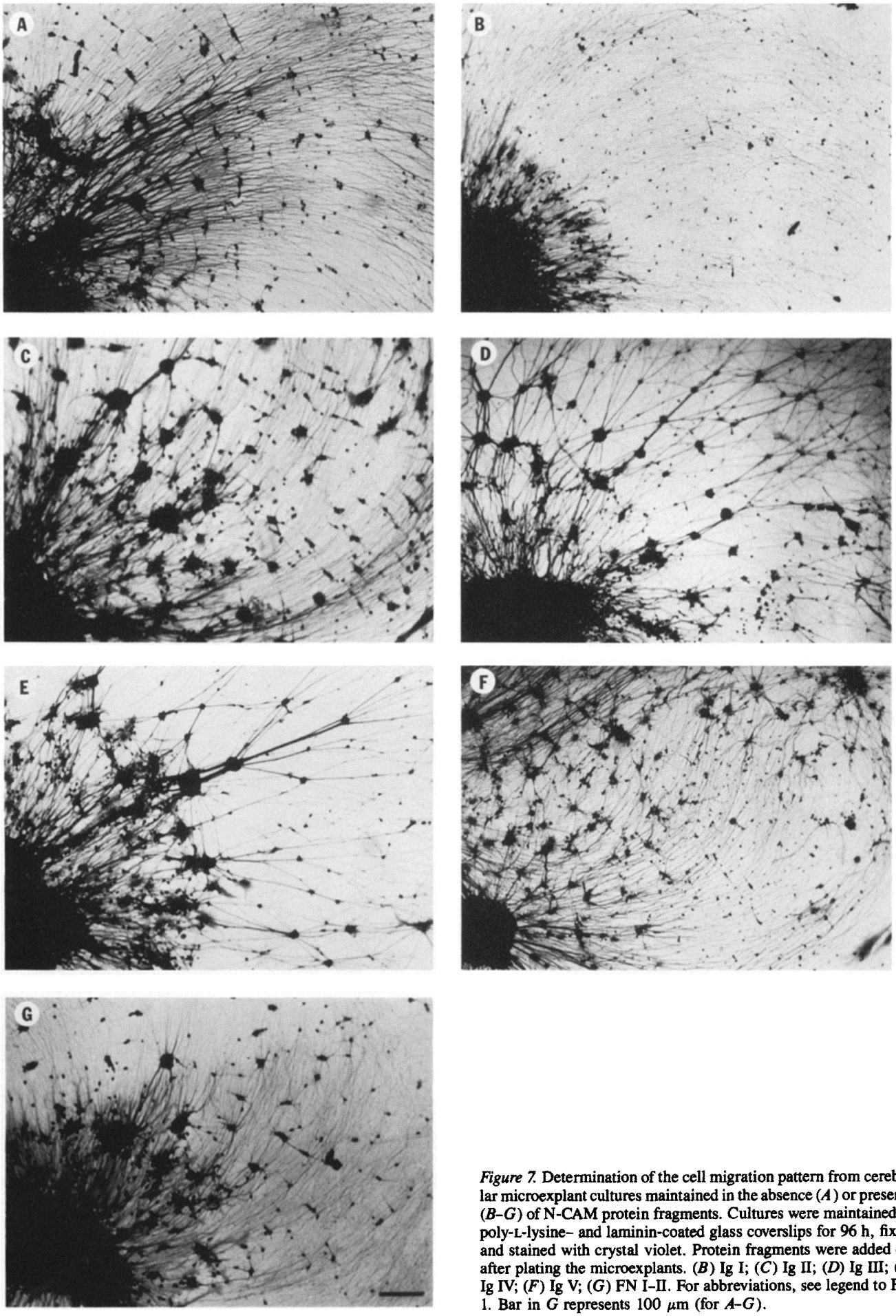

Figure 7. Determination of the cell migration pattern from cerebellar microexplant cultures maintained in the absence (*A*) or presence (*B–G*) of N-CAM protein fragments. Cultures were maintained on poly-L-lysine– and laminin-coated glass coverslips for 96 h, fixed, and stained with crystal violet. Protein fragments were added 4 h after plating the microexplants. (*B*) Ig I; (*C*) Ig II; (*D*) Ig III; (*E*) Ig IV; (*F*) Ig V; (*G*) FN I–II. For abbreviations, see legend to Fig. 1. Bar in *G* represents 100 μm (for *A–G*).

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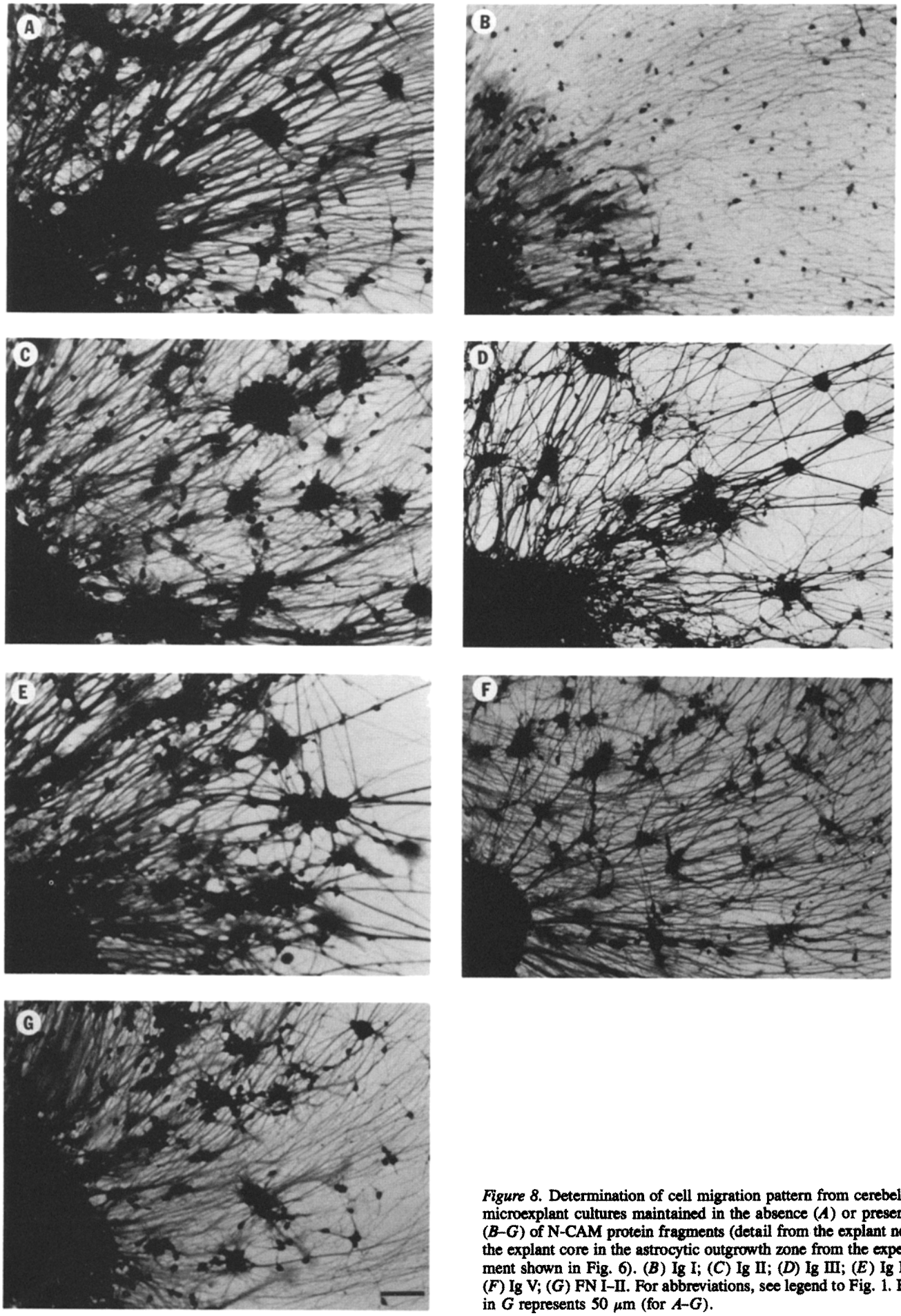

Figure 8. Determination of cell migration pattern from cerebellar microexplant cultures maintained in the absence (*A*) or presence (*B–G*) of N-CAM protein fragments (detail from the explant near the explant core in the astrocytic outgrowth zone from the experiment shown in Fig. 6). (*B*) Ig I; (*C*) Ig II; (*D*) Ig III; (*E*) Ig IV; (*F*) Ig V; (*G*) FN I–II. For abbreviations, see legend to Fig. 1. Bar in *G* represents 50 μm (for *A–G*).

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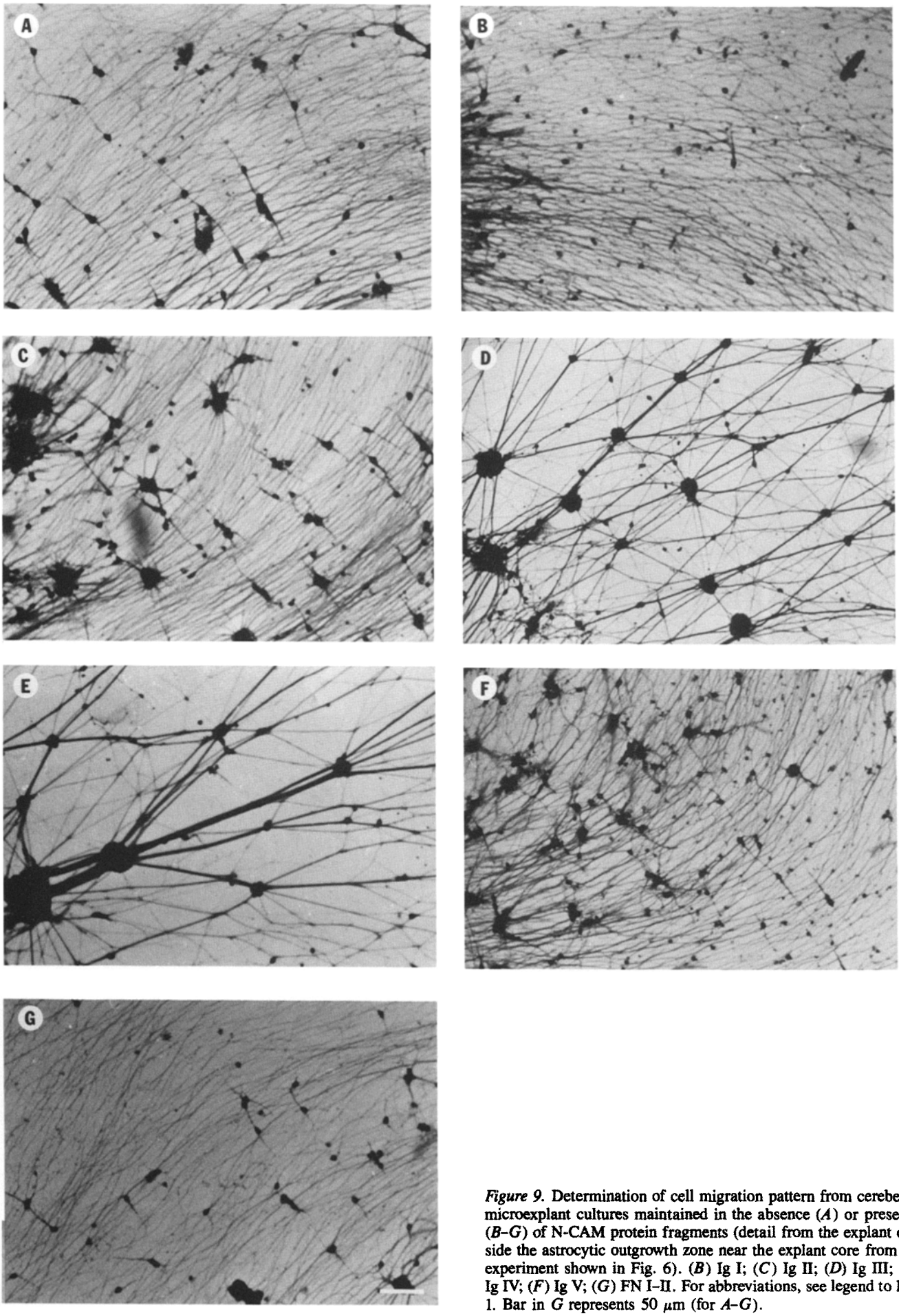

Figure 9. Determination of cell migration pattern from cerebellar microexplant cultures maintained in the absence (*A*) or presence (*B–G*) of N-CAM protein fragments (detail from the explant outside the astrocytic outgrowth zone near the explant core from the experiment shown in Fig. 6). (*B*) Ig I; (*C*) Ig II; (*D*) Ig III; (*E*) Ig IV; (*F*) Ig V; (*G*) FN I–II. For abbreviations, see legend to Fig. 1. Bar in *G* represents 50 μm (for *A–G*).

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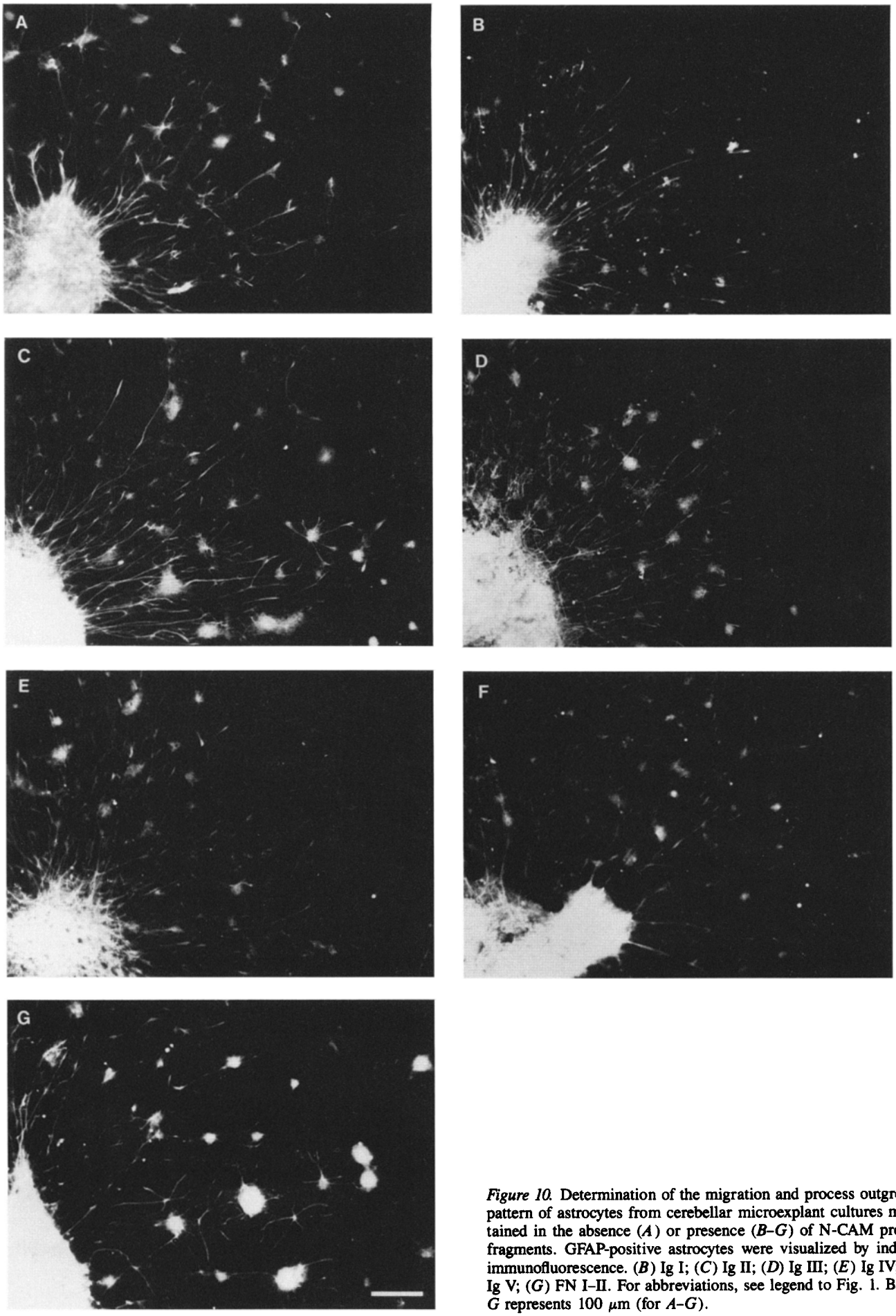

Figure 10. Determination of the migration and process outgrowth pattern of astrocytes from cerebellar microexplant cultures maintained in the absence (*A*) or presence (*B–G*) of N-CAM protein fragments. GFAP-positive astrocytes were visualized by indirect immunofluorescence. (*B*) Ig I; (*C*) Ig II; (*D*) Ig III; (*E*) Ig IV; (*F*) Ig V; (*G*) FN I–II. For abbreviations, see legend to Fig. 1. Bar in *G* represents 100 μm (for *A–G*).

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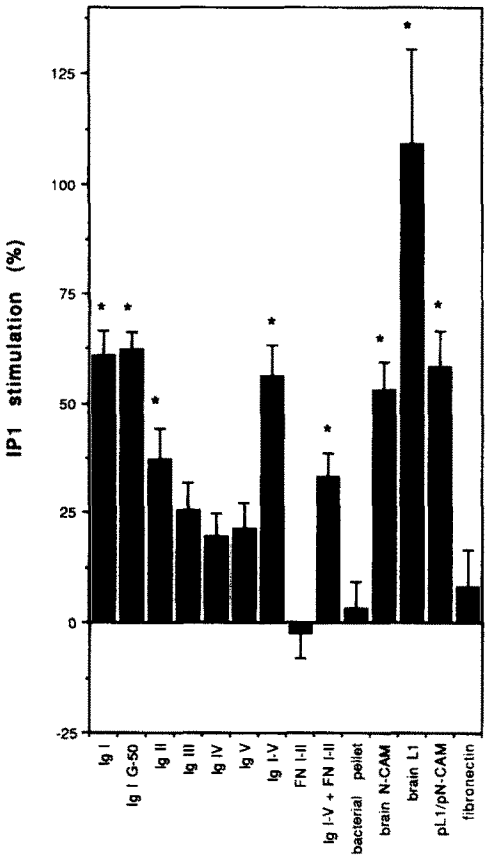

Figure 11. Effect of adhesion molecules, protein fragments and antibodies on intracellular IP1 levels of cultured small cerebellar neurons. After labeling with [^{3}H]inositol, small cerebellar neurons maintained on laminin were incubated for 20 min in the presence of 10 mM Li^+ with adhesion molecules, protein fragments, bacterial pellet (for abbreviations see legends to Figs. 1, 2, and 4), N-CAM and L1 isolated from mouse brain (brain N-CAM and brain L1, respectively) and the mixture of polyclonal antibodies to L1 and N-CAM (pL1/*pN-CAM*). Bars show the percentage increase of [^{3}H]inositol incorporation into IP1 in cells treated with antigens and antibodies compared with control cells and represent mean values ± SD from three independent experiments. Bars marked by * are significantly different ($P < 0.05$) from the control (fibronectin).

neurons with the different protein fragments in the presence of Li^+, allowing the determination of changes in inositol phosphate metabolism with changes in IP1 as an indicator of such changes (von Bohlen und Halbach, F., J. Taylor, and M. Schachner, manuscript submitted for publication). Ig I was the most potent stimulator of inositol phosphate turnover, in that it caused stimulation of IP1 accumulation by ~60% over control values in the absence of protein fragments (Fig. 11). Ig I–V, N-CAM from brain and the mixture of the polyclonal antibodies to L1 and N-CAM gave a similar stimulation when added to the cultures. The highest stimulation was seen with L1 from brain which amounted to ~110% when compared to the untreated control. Smaller stimulatory values were seen with Ig II, III, IV, and V which amounted to ~25% stimulation over control values. Interestingly, FN I–II showed no stimulation over control values. The mixture of Ig I–V and FN I–II was less than additive when compared to the effects caused by the two fragments separately, suggesting that the FN I–II may even be inhibitory in combination with stimulatory fragments. To evaluate the contribution of the bacterial protein contaminants, proteins from the inclusion bodies and supernatants of bacterial lysates from the control clone were used. There was no significant stimulation in IP1 turnover by these proteins.

Measurements of intracellular concentrations of Ca^{2+} ($[Ca^{2+}]_i$) and intracellular pH (pH_i) revealed strikingly parallel effects after addition of soluble protein fragments (Fig. 12). The most prominent increase in $[Ca^{2+}]_i$ and pH_i was seen after addition of Ig I and II, amounting to an increase in $[Ca^{2+}]_i$ by a factor of almost three. This increase was similar to that evoked by Ig I–V, the mixture of Ig I–V and FN I–II, N-CAM from brain, and the mixture of polyclonal antibodies to L1 and N-CAM. Only L1 from brain caused a slightly higher increase in $[Ca^{2+}]_i$. Interestingly, Ig III, IV, and V and FN I–II did not trigger an increase in $[Ca^{2+}]_i$ or pH_i. Evaluation of the kinetics of the response in $[Ca^{2+}]_i$ and pH_i showed that the maximal rise in $[Ca^{2+}]_i$ induced by addition of the protein fragments or antibodies, occurred within the first 15 min after addition of the fragments and was followed by a reduction to lower levels with time of incubation (Fig. 13). This decrease was more pronounced with L1 from brain and the mixture of polyclonal antibodies to L1 and N-CAM and less extensive with Ig I and II and the other stimulatory protein fragments. As observed previously for some cell types (von Bohlen und Halbach, F., J. Taylor, and M. Schachner, manuscript submitted for publication), the increase in pH_i followed the rise in $[Ca^{2+}]_i$ and desensitized less effectively with time of incubation than the response in $[Ca^{2+}]_i$.

None of the protein fragments or antibodies evoked any changes in intracellular levels of cAMP or GMP (not shown).

Discussion

In this study we have shown that in different functional assay systems, the different domains of the extracellular part of N-CAM are implicated in different functions. The results obtained in this study are applicable to the investigated isoform of N-CAM and it might well be that other alternatively spliced isoforms of N-CAM exert different functions. As substrate-coated molecules, the Ig-like domains I and II are most prominently involved in promoting cell adhesion. This is in agreement with a previous observation that the 25-kD fragment of the aminoterminal region of N-CAM, which contains these domains, is involved in cell adhesion (Cole and Glaser, 1986; Frelinger and Rutishauser, 1986). The fibronectin type III homologous repeats are most predominant in promoting neurite outgrowth and cell spreading. When added as soluble fragments into the culture medium, where they may act either as agonists or competitors, Ig-like domain I reduces neurite fasciculation and migration of neuronal cell bodies, while Ig-like domains III and IV enhance neurite fasciculation and clustering of cell bodies and reduce the perpendicular orientation of inhibitory interneurons. The triggering of intracellular messenger systems is also more dependent on some domains than others. For example, the turnover of inositol phosphates is increased by Ig-like domain I more so than by the other Ig-like domains, whereas the type III repeats are not stimulatory. Furthermore, intracellular concentrations of Ca^{2+} and pH levels are increased

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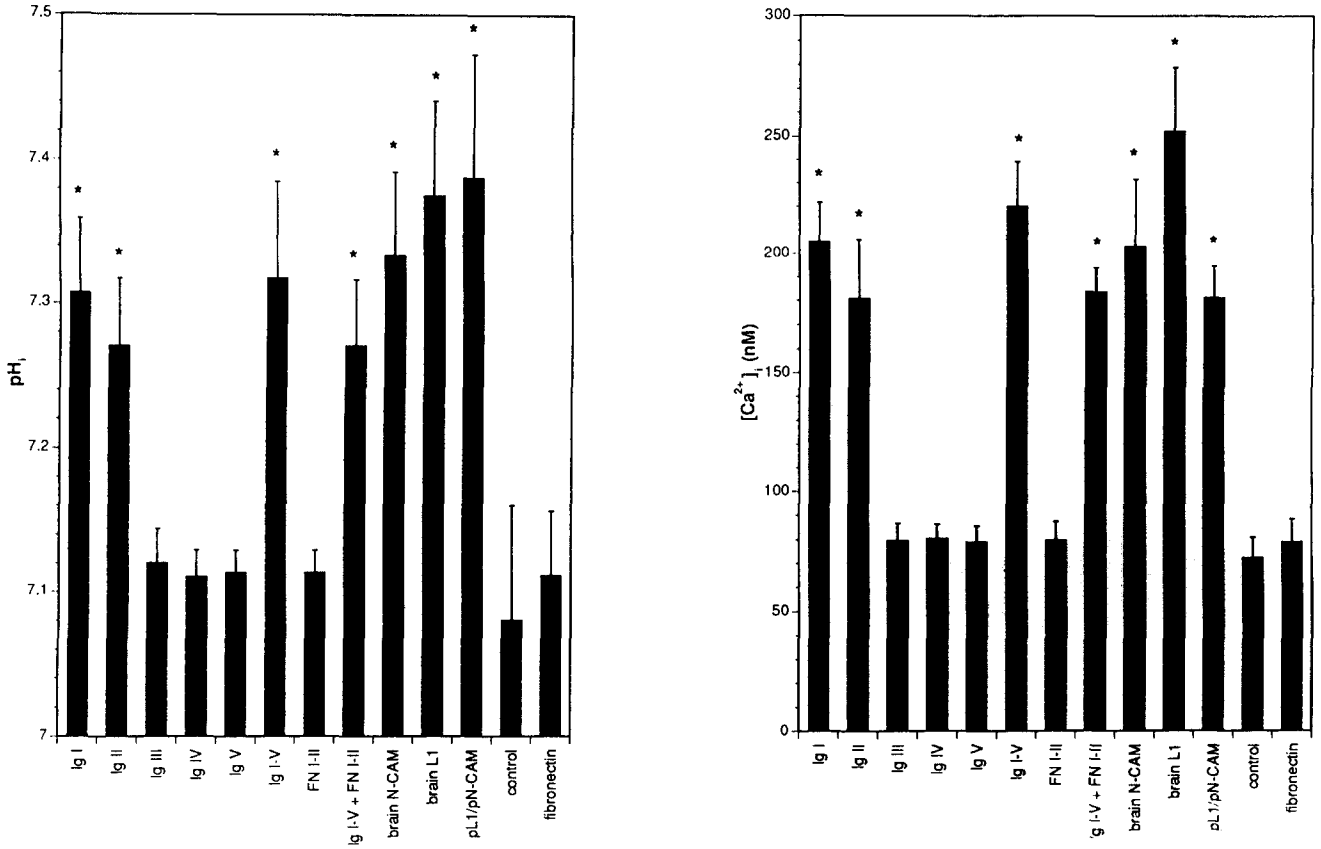

Figure 12. Influence of adhesion molecules, protein fragments, and antibodies on $[Ca^{2+}]_i$ and pH_i of cultured small cerebellar neurons. Small cerebellar neurons were maintained on laminin and incubated without protein fragments (control), with adhesion molecules, protein fragments, and antibodies. Bars show the maximum values observed after addition of proteins within a time period of 60 min and represent mean values ± SD from three independent experiments. Bars marked by * are significantly different ($P < 0.05$) from the control (fibronectin). For abbreviations see legends to Figs. 1 and 11.

only by Ig-like domains I and II, whereas Ig-like domains III, IV, and V and type III domains I–II are completely ineffective.

Two observations argue against the possibility that the observed effects are due to bacterial contaminations which might block the functions of the expressed peptides. First, the fact that all the expressed peptides were active in at least one functional assay indicates that bacterial contaminations are not general blocking agents. Since these peptides are quite small, the contaminating proteins would cover the whole peptide and all possible functional sites would be blocked, hence the peptide could not be active in any of the performed test systems. Second, if a contamination interacts specifically with one peptide, then this contamination must also interact with the construct containing all five Ig-like domains or with the mixture of all Ig-like domains with all fibronectin type III repeats, representing the complete extracellular region of N-CAM. However, this is not the case: The adhesion given by this mixture is as strong as that given by N-CAM from brain which does not contain bacterial contaminations. Another consideration is noteworthy in that the bacterially expressed peptides are capable of assuming their proper configuration, since only one band under nonreducing condition is seen, except for the construct containing all five Ig-like domains. It should be noted that even in the case that several configurations are attained, as seen for all five Ig-like domains, the expressed peptide is functionally active when compared to the peptides encompassing the individual domains and to N-CAM from brain.

In addition, our study has shown that individual domains can subserve distinct functions without the natural decoration by nervous system–derived carbohydrate structures. However, it is likely that the protein backbone may be even more finely tuned to perform its task when modified by attached carbohydrate structures such as the L2/HNK-1 carbohydrate (Kruse et al., 1984; Künemund et al., 1988) which has been localized to the Ig-like domains of N-CAM (Cole and Schachner, 1987). It is interesting in this respect that the Ig-like domain V is least active in all functional tests used in this study and it is therefore conceivable that this particular protein backbone needs its particular carbohydrate structure which could be polysialic acid in the less adhesive, so-called embryonic form of N-CAM (Hoffman and Edelman, 1983; Sadoul et al., 1983; Crossin et al., 1984). It is thus tempting to speculate that certain immunoglobulin-like domains may exert their functions mainly as presenters of functionally important carbohydrate structures.

In general, the implication of the individual domains in certain functions is not all or none in the sense that the other domains are rarely completely silent in a particular functional task. However, the predominant effects of certain domains over others is easily seen. It is noteworthy that in sev-

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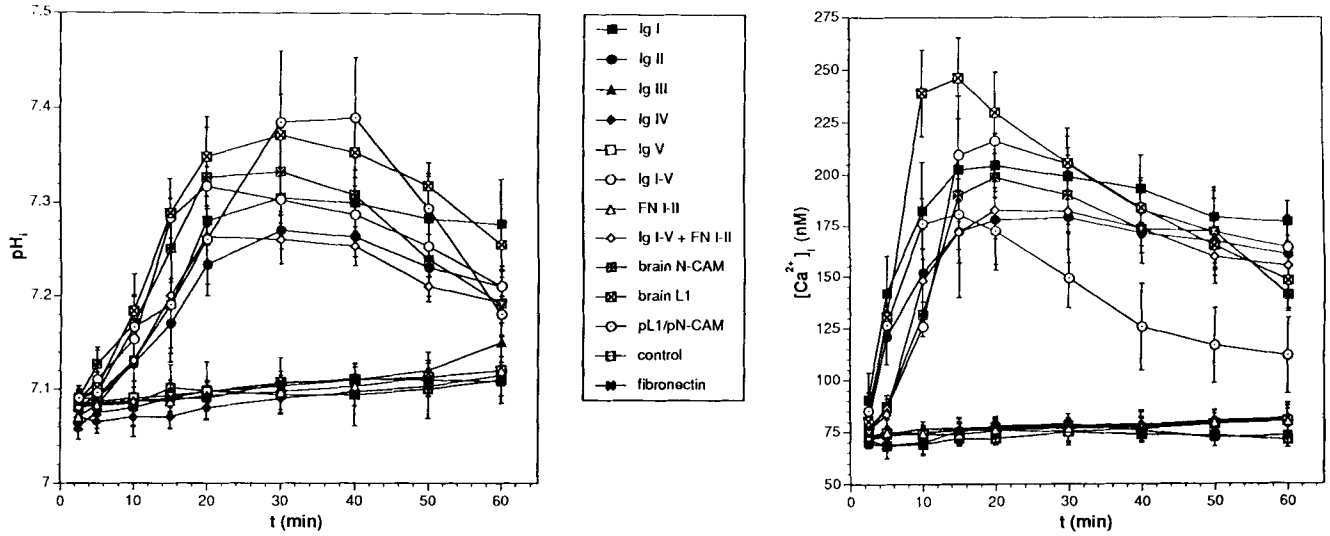

Figure 13. Changes in $[Ca^{2+}]_i$ and pH_i of cultured small cerebellar neurons maintained on laminin as a function of incubation time with adhesion molecules, protein fragments, and antibodies. Small cerebellar neurons were maintained on laminin and incubated with adhesion molecules, protein fragments, and antibodies (pL1/N-CAM). Values recorded after addition of antibodies show changes in $[Ca^{2+}]_i$ and pH_i and are means ± SD from three independent experiments. For abbreviations see legends to Figs. 1 and 11.

eral assay systems, individual domains were more effective in evoking a biological response than N-CAM isolated from adult mouse brain or the combination of the Ig-like domains I–V either alone or in combination with type III repeats I–II. This phenomenon could be explained by the assumption that in the whole molecule the individual domains are not as exposed and thus not as accessible to the corresponding receptor as in the individual fragments. Indeed, the proteolytic fragment containing the Ig-like domain II has been found to bind to heparin more avidly than the whole molecule (Cole and Akeson, 1989). Another example illustrating that a fragment is more active than the whole molecule is parathyroid hormone, where the fragment shows a greater effect in Ca^{2+} excretion than the whole molecule (Rabbani et al., 1990). On the other hand, N-CAM from brain may have been denatured during the isolation procedure and the five Ig-like domains containing five cystine bridges may not have completely regained their functional conformation during the renaturation process, whereas the proportion of molecules with correct conformation may be higher in the preparation of the individual domains. Thus, we have been able to reconstruct the functional features of N-CAM that promote neurite outgrowth which have previously been observed when N-CAM transfected fibroblasts have been used as cellular substrates (Doherty et al., 1989, 1990, 1991), but less so and not as reproducibly when N-CAM isolated from brain was substrate coated (Lagenaur and Lemmon, 1987). Tandem presentation of domains, however, can have advantages over their individual presentation as is the case for the type III repeats I and II which, when contained in one protein fragment, are more effective in promotion of neuronal adhesion, neurite outgrowth, and cell body spreading than the individual domains. Thus, the type III repeats, but to a lesser extent the Ig-like domains, may be susceptible to some conformational reinforcement as repetitive units.

The results obtained in this study justify the strategy in which the structural features of a molecule are dissected into individual motifs or "cassettes," because they appear to be functional entities in themselves. The choice of bacteria as the manufacturers of these cassettes has served the purpose that the function of the protein backbone without the possibly modifying influences of nervous system-derived carbohydrates can be determined. The advantages of our experimental approach are several fold. First, the use of molecular fragments avoids functional tests with cells that have been made to express individual molecular domains at the cell surface by transfection. Such cells are not devoid of recognition molecules, and these endogenous molecules may undergo unknown functional associations with the transfected molecules. Thus, a convincing demonstration of signal transduction triggered by different domains depends on the cleanness of the inducing signal, which is rarely achieved in the context of other cell surface molecules. In addition, the use of isolated protein fragments is much more versatile than cell transfection, since soluble molecules can be readily used in a variety of functional assays that are not practical with cell-bound molecules because of the inability of cells to effect subtle changes in complex cell interactions, such as neuron–glia and nerve–muscle interactions, synaptogenesis, choice of particular substrates, and perpendicular orientation of inhibitory interneurons. Thus, soluble protein fragments can be used to monitor cell interactions in their natural, sophisticated environment in which different cell types possess their original machinery to transduce recognition events at the cell surface into sensible intracellular consequences. Furthermore, protein fragments can be made as cassettes in increasingly smaller sizes to determine the functionally relevant amino acid sequences which are best tested as soluble competitors (Pierschbacher and Ruoslahti, 1984; Yamada and Kennedy, 1984). These can be used with ease, because of their facilitated penetration through the intact tissue, possibly even in the living animal (Boucaut et al., 1984).

Having demonstrated that particular domains subserve certain functions, the question arises as to how these domains induce their particular cellular effects. The N-CAM

fragments may modulate ligand–receptor relationships between the cell surface of neighboring cells, which may be homophilic (Hoffman and Edelman, 1983) or heterophilic (Werz and Schachner, 1988), requiring self or nonself receptors at the cell surface of the recipient cell, respectively. The fragments may also interfere with the molecular association of L1 and N-CAM within the surface membrane of one and the same cell (Kadmon et al., 1990*a*,*b*). An indirect action of the fragments may lie in the modulation of substrate-associated molecules which then exert different effects on their partner cells.

A comparison of structure–function relationships between molecules of the immunoglobulin superfamily (Williams and Barclay, 1988) or molecules of the fibronectin type III family appears warranted. As for the immunoglobulin superfamily, CD4 exhibits receptor activity for the gp120 glycoprotein of HIV and MHC II molecules in its first and second Ig-like domains (Fleury et al., 1991). Furthermore, it was shown that the interaction between CD4 and gp120 is independent of glycosylation on both partner molecules (Fenouillet et al., 1989). MHC I molecules interact with their first two Ig-like domains with the respective antigens of antigen presenting accessory cells (Bjorkman et al., 1987). ICAM-1 was shown to bind to the integrin Mac-1 (CD11b/CD18) with its third Ig-like domain, whereas it binds to the rhinovirus by the first and second Ig-like domains (Staunton et al., 1990) and to the integrin LFA-1 with its first Ig-like domain (Diamond et al., 1991). The third Ig-like domain of the MHC I molecule (α_3) is recognized by the Ig-like domain of the CD8 α-chain (Salter et al., 1990). The interaction of the Ig-like domains of CD2 with the Ig-like domains of its receptor LFA-3 (Seed, 1987) is particularly interesting, since both partner molecules have Ig-like domains of the C2-type as do most recognition molecules in the nervous system. Furthermore it has been shown that the binding sites for mAbs against CD2 fall in three discrete regions: antibodies that stimulate IL-2 release and block erythrocyte adhesion bind to the first region; antibodies that block adhesion bind to the second region; and antibodies that stimulate IL-2 release but do not block adhesion bind to the third region (Peterson and Seed, 1987). Thus, messenger activation and adhesive properties of CD2 seem to be separated as in N-CAM.

However, such specializations of Ig-like domains may not always be implemented, since the neural adhesion molecule P_0, the major cell surface glycoprotein of myelin in peripheral nerves which contains only one V-type domain, has been shown to mediate both homophilic glia–glia and heterophilic neuron–glia recognition (Schneider-Schaulies et al., 1990). It can presently not be assessed whether a particular conformation, either of the V-type as in CD4, CD8 and P_0, or the C2-type as in CD2, LFA-3, N-CAM, the myelin associated glycoprotein, and L1, may subserve different ranges of functional properties. As shown in the present study, the Ig-like domains of N-CAM are not the most effective neurite outgrowth promoters, although they do support neurite outgrowth, as do P_0 (Schneider-Schaulies et al., 1990) and myelin-associated glycoprotein (Johnson et al., 1989). The fibronectin type III homologous repeats are more active promoters of neurite outgrowth and are thus reminiscent of the neurite outgrowth promoting properties of tenascin which localize to the fibronectin type III homologous repeats 10 and 11 in the mouse (Lochter et al., 1991). The fibronectin type III repeats of fibronectin have also been implicated in neurite outgrowth of peripheral nervous system neurons (Humphries et al., 1988). It is therefore tempting to speculate that the neural recognition molecules carrying a greater number and more highly conserved tandem fibronectin type III repeats than N-CAM, such as L1 (Moos et al., 1988), TAG-1 (Furley et al., 1990), F3/F11/contactin (Gennarini et al., 1989; Brümmendorf et al., 1989; Ranscht, 1988), Ng-CAM (Burgoon et al., 1991), and Nr-CAM (Grumet et al., 1991), may be endowed with neurite outgrowth promoting activities proportional to the number of their type III repeats.

The neurite outgrowth promoting activities of the type III repeats do not appear to be mediated by the second messenger systems investigated in this study, since these domains did not evoke any changes in inositol phosphate turnover, intracellular levels of Ca^{2+} and pH, or cAMP, and GMP. However, it is possible that Ig-like domains I and II, which are the most prominent domains in promoting cell adhesion and cell spreading, may trigger and sustain neurite outgrowth via increasing inositol phosphate turnover and intracellular concentrations of Ca^{2+} which then may act in concert with yet unknown signalling systems induced by the fibronectin type III repeats. It is also possible that the experiments in which neurite outgrowth and second messengers systems have been measured cannot be strictly compared, since in the first case protein fragments were substrate coated, while in the second case they were added as soluble compounds. It remains to be seen which structural features are common to the different recognition molecules in specialization for certain functions and how the individual molecular domains exert their functional roles by a combination of recognition at the cell surface and signal transduction from the cell surface to the cell interior.

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