

# NCAM Polysialic Acid Can Regulate both Cell–Cell and Cell–Substrate Interactions

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**Abstract.** We have proposed previously that the polysialic acid (PSA) moiety of NCAM can influence membrane–membrane apposition, and thereby serve as a selective regulator of a variety of contact-dependent cell interactions. In this study, cell and tissue culture models are used to obtain direct evidence that the presence of PSA on the surface membrane can affect both cell–cell and cell–substrate interactions. Using a neuroblastoma/sensory neuron cell hybrid, it was found that removal of PSA with a specific neuraminidase (endo-N) augments cell–cell aggregation mediated

by the L1 cell adhesion molecule as well as cell attachment to a variety of tissue culture substrates. In studies of embryonic spinal cord axon bundling, which involves both cell–cell and cell–substrate interactions, the pronounced defasciculation produced by removal of PSA is most easily explained by an increase in cell–substrate interaction. The fact that in both studies NCAM's intrinsic adhesion function was found not to be an important variable further illustrates that regulation of the cell surface by PSA can extend beyond binding mediated by the NCAM polypeptide.

THE neural cell adhesion molecule NCAM is an abundant integral membrane glycoprotein that can promote cell-to-cell adhesion through a homophilic binding mechanism (Rutishauser et al., 1983; Hall et al., 1990; Doherty et al., 1990b). The kinetics of this adhesion vary with the concentration of the ligand, and inversely with the degree of glycosylation (Hoffman and Edelman, 1983; Rutishauser et al., 1985; Doherty et al., 1990b). Though NCAM has multiple carbohydrate attachment sites (Crossin et al., 1984; Kruse et al., 1984; Frelinger and Rutishauser, 1986; Hemperly et al., 1986; Watanabe et al., 1986; Cole and Schachner, 1987), modulation of adhesion specifically arises from differences in the length of linear homopolymers of alpha-2, 8-linked neuraminic acid units (polysialic acid or PSA) linked to NCAM via a core carbohydrate (Finne, 1982; Cunningham et al., 1983; Finne et al., 1983; Finne and Makela, 1985).

The molecular mechanism whereby polysialic acid (PSA)<sup>1</sup> on NCAM can modulate cell adhesion remains an open question. Several studies using cell membranes are consistent with a direct effect on NCAM-mediated adhesion. That is, enzymatic removal of PSA increases binding between NCAM-bearing liposomes and neuroblastoma cells (Sadoul et al., 1983), and the rate of NCAM-dependent aggregation among membrane vesicles in vitro (Hoffman and Edelman, 1983; Rutishauser et al., 1985). However, in studies of the purified molecule, homophilic interactions are only slightly if at all increased when PSA is removed (Hoffman et al., 1982; Hall

and Rutishauser, 1987; Hall et al., 1990). On the other hand, specific loss of PSA from NCAM increases the relative degree of overall membrane–membrane apposition between cells (Rutishauser et al., 1988). These and other studies on a variety of cell–cell interactions have led to the alternative or supplemental hypothesis that the increase in the extent, duration or intimacy of surface–surface contact upon removal of PSA allows greater proximity and function of membrane-associated ligands, including but not limited to NCAM (Rutishauser et al., 1988). Thus the model makes the striking prediction that PSA could affect not only NCAM function, but also other ligands not directly involved in NCAM-mediated adhesion. Moreover, such a physical change should in principle include adhesive contacts with any surface, for example a tissue culture substrate.

To explore the ability of PSA to affect ligands other than NCAM, in both cell–cell and cell–substrate adhesion, the present study largely focuses on the role of PSA in modulating the function of two cell surface proteins on neurons, neuronal receptors for laminin (LN) (Edgar et al., 1984, 1988; Bozyczko and Horwitz, 1986; Tomaselli et al., 1986, 1987; Aumailley et al., 1987; Cohen et al., 1987; Hall et al., 1987) and L1-like cell–cell adhesion molecules (Rathjen and Schachner, 1984; Grumet and Edelman, 1984; Rathjen and Rutishauser, 1984; Bock et al., 1985; Stallcup and Beasley, 1985; Lagenauer and Lemmon, 1987; Moos et al., 1988). Two experimental systems are used: cell–cell aggregation and cell–substrate attachment assays with F11 sensory neuron/neuroblastoma cell hybrids (Platika et al., 1985), and the pattern of neurite–neurite fasciculation produced by spi-

1. *Abbreviations used in this paper:* LN, laminin; P, polyornithine; PSA, polysialic acid; SpC, spinal cord.

nal cord axons on an LN substratum. The F11 cells provide the ability to examine cell and substrate interactions separately, whereas these parameters are combined in the more biologically intact spinal cord system. In both studies the data are most consistent with a model in which PSA affects overall membrane-membrane or membrane-substrate apposition.

## Materials and Methods

### Reagents for Analysis of NCAM, L1, and PSA

Both NCAM and L1-like adhesion molecules were detected using polyclonal rabbit anti-chicken or polyclonal rabbit anti-mouse reagents. Each antibody was generated against an affinity-purified antigen, using mAbs 5E (chick NCAM; Frelinger and Rutishauser, 1986), H28 (mouse NCAM; Gennarini et al., 1984), G4 (chick L1-like molecule; Rathjen et al., 1987) and L1 (Rathjen and Schachner, 1984). Fab fragments were prepared by pepsin digestion, reduction, and alkylation, followed by extensive dialysis. PSA was identified using one of three mouse mAbs: mAb 22B (IgM) ascites (Rougon et al., 1986; gift of W. Zollinger), mAb 5A5 (IgM) culture supernatant (gift of J. Dodd and T. M. Jessell), or mAb 735 purified IgG (Frosch et al., 1985; gift of D. Bitter-Suermann).

PSA on NCAM was specifically removed using purified endo-N, isolated as described elsewhere (Vimr et al., 1984; Rutishauser et al., 1985; Hallenbeck et al., 1987). This enzyme works optimally at neutral pH, and specifically cleaves homopolymers of alpha-2,8-linked sialic acid in linear chains of at least five units. It therefore has no effect on other sialic acid-containing structures.

### SDS-PAGE and Immunoblot Analysis of Proteins

Samples for immunoblot analysis were obtained from freshly dissected tissue, immunoaffinity-purified PSA-containing proteins, or cultures of cells or explants as described below. Membrane proteins of tissues or cells were solubilized at 4°C by sonication in 14 vol of PBS (pH 7.4) containing 0.5% NP-40, 1 mM EDTA, and 100 kallikrein inhibitor units/ml aprotinin. The extract was centrifuged for 10 min at 13,000 g and the supernatant was saved. When indicated, tissue sample supernatants were treated with endo-N at 100 U/ml for 90 min at 4°C.

For immunoaffinity isolation of PSA-containing material, protein from E7 chicken SpC was extracted as above, and adsorbed to Sepharose beads coupled with mAb 22B, for 90 min at 4°C. After washing the beads, the adsorbed material was released by addition of one bed volume of 78 mM diethylamine, pH 11.5 (Hoffman et al., 1982), for 5 min on ice. The eluate was neutralized by addition of 1 M K<sub>2</sub>HPO<sub>4</sub>. When indicated, immunoaffinity purified samples were treated with 250 U/ml endo-N for 90 min at 4°C.

In all cases, freshly prepared samples were solubilized by addition of SDS-PAGE sample buffer (Laemmli, 1970), followed by 3 min heating at 100°C. Proteins were separated by SDS-PAGE (Laemmli, 1970), using a 7.5% separating gel (7.0% where indicated) with a 4% stacking gel. Sample loads were equal for all lanes within each comparison (see figure legends). Separated proteins were either stained for total protein by the silver method (Oakley et al., 1980), or electrophoretically transferred from the gel to nitrocellulose for immunoblot analysis (Towbin et al., 1979).

For immunoblotting, primary antibody was reacted with the transferred protein, bound antibody was detected using peroxidase-conjugated goat anti-rabbit, anti-mouse IgM, or IgG secondary antibodies (Cooper Biomedical, Inc., Malvern, PA) (Johnson et al., 1984), and the enzyme reaction developed using 0.5% 4-chloro-1-naphthol and 0.01% H<sub>2</sub>O<sub>2</sub> as substrates (Hawkes et al., 1982). In some experiments, biotinylated secondary antibodies were used, followed by streptavidin-alkaline phosphatase and development of the enzyme reaction using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates.

### Immunofluorescence

Cultured cells were fixed in freshly prepared 4% paraformaldehyde for 20 min at room temperature. Nonspecific binding was blocked by incubation (60 min) with H/G10 (HBSS with 35 mg NaHCO<sub>3</sub>/100 ml and 10% normal goat serum). Cells were then incubated with the primary antibody diluted in H/G10 overnight at 4°C. To stain for the PSA moiety, mAb 735

(IgG, culture supernatant 1:500), 22B (IgM, ascites 1:200), and 5A5 (IgM, culture supernatant undiluted) were used. Polyclonal anti-NCAM and anti-L1 IgG were used at 10 µg/ml final concentration. After washing, biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) directed to the specie and Ig class defined by first antibodies were added at 7.5 µg/ml in H/G10, and incubated either 2 h at room temperature, or overnight at 4°C. After a second washing, Texas Red-streptavidin (Amersham Corp., Arlington Heights, IL) was added at 5 µl/ml in H/G10, and incubated 30 min at room temperature.

### F11 Cells

F11 cells are a hybrid of E17 mouse primary sensory ganglion neurons and rat neuroblastoma cells formed by fusing the two cell types (Platika et al., 1985). F11 cells were grown in L15-CO<sub>2</sub> medium containing 10% NuSerum IV (Collaborative Research, Lexington, MA) or in DME containing 10% FCS (DME/FCS).

### Tissue Culture Substrates

Three types of substrates were used in these studies, representing a sequential addition of molecules to the tissue culture plastic: the plastic alone, plastic treated with polyornithine, and finally plastic treated, first with polyornithine (P) and then with laminin (P/LN). The different substrata were prepared using 35-mm-diam tissue culture dishes (Falcon 3000 series, Falcon Labware, Oxnard, CA), or 25-cm<sup>2</sup> tissue culture flasks (Corning 25100, Corning Medical, Corning, NY), which were incubated overnight at 4°C with polyornithine solution (Helfand et al., 1986), washed twice with sterile PBS, and incubated overnight at 4°C with a solution of 3–4 µg/ml LN (Collaborative Research) in PBS. The amount of laminin used was in excess of available binding sites, and the use of polyornithine results in a greater than threefold increase in the amount of bound LN (Edgar et al., 1984).

### Spinal Cord (SpC) Explant Cultures

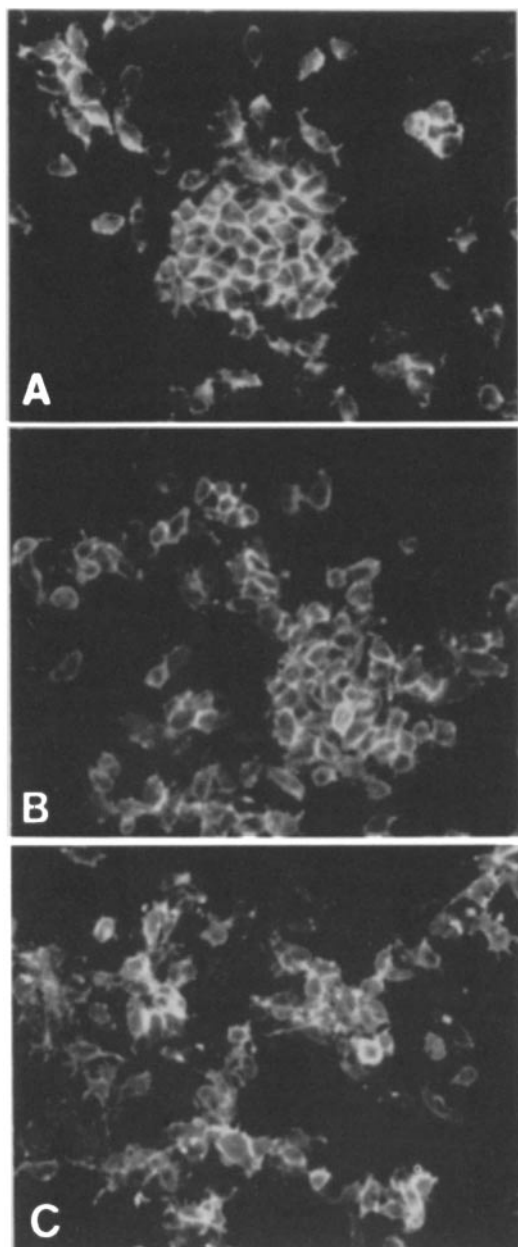
Spinal cords from E7 chickens were dissected in sterile PBS, freed of meninges and dorsal root ganglia, and cut into pieces of 1–2 mm<sup>3</sup>. The explants were cultured in 1.5 ml/dish or 3 ml/flask of F14 medium (Vogel et al., 1972) containing 10% horse serum (F14/H10) and 20 µl/ml muscle extract (Dohrmann et al., 1986) that had been titrated to allow maximal neurite outgrowth with minimal flat cell outgrowth. 15–25 explants per dish or all explants from one SpC per flask were added in a minimum volume of medium (1 ml/dish or 2 ml/flask) to facilitate initial adhesion of explants. Antibodies were added to cultures after explant attachment. For functional inhibition of NCAM in cultures, Fab fragments were prepared from polyclonal rabbit anti-chicken NCAM IgG fractions (Mage, 1980) and used at 0.5 mg/ml. Polyclonal rabbit antibodies to LN were obtained from two sources: Gibco Laboratories' serum (Grand Island, NY; formerly the BRL reagent) that required dialysis to remove azide, and two different lots of IgG fractions from Collaborative Research, Inc. Before examination by light microscopy, explants were fixed by addition of 1.5 ml of 1.0% glutaraldehyde (0.5% fixative final) 40–48 h after the initial plating.

### Dissociated SpC Cells

One or two E7 chicken SpCs were dissected free of meninges and dorsal root ganglia and then digested with 0.05% trypsin for 30 min at 37°C (Dohrmann et al., 1986). DNAase was then added to 0.2% final concentration, and after 2 min at 25°C, the enzymes were removed by two 5-ml washes with F14/H10 and the tissue was triturated five times with a fire-polished Pasteur pipette. Debris and cells were separated by layering over 5 ml of 3.5% BSA in F14 and centrifugation at 100 g for 15 min. The pellet was gently resuspended in 1 ml F14/H10, and 50,000 cells/dish were plated in 1.5 ml of medium.

### Endo-N Treatment of Cells in Culture

For treatment of SpC explants, endo-N was added directly to the medium at a final concentration of 20 U/ml. F11 cells were detached from tissue culture flasks or dishes by a 2-min exposure to 0.025% trypsin plus 0.265 mM EDTA. They were then treated with 20 U/ml endo-N in suspension at 37°C (in medium) for 2 h. Removal of PSA from NCAM was confirmed for both types of cells using both immunoblots and immunocytochemistry (see Results).



**Figure 1.** Presence of NCAM, PSA and L1 on F11 cells: effect of endo-N treatment. F11 cells grown on tissue culture plastic form loose aggregates that are not well attached to the substrate. There is very little spontaneous neurite growth. Unpermeabilized cells show positive immunostaining for NCAM (A), L1 (B), and PSA (22B mAb; C). All cells are positively stained for all three antigens.

### Cell Aggregation Assay

F11 cells at 50% confluence were detached from dishes by gentle mechanical pipetting and then pelleted at 100 g through a cushion of 3.5% BSA in HBS plus 10 mM  $\text{Ca}^{2+}$  + 2% DNase. The pellet was resuspended in L15 + 10% NuSerum and cells were incubated with or without endo-N (see above) for 2 h at 37°C. After this incubation period, cells were repelleted as above and resuspended in 15–20 times the pellet volume in HBS/DNase/BSA plus 2 mM EDTA. Cells were then preincubated with Fab fragments (0.5 mg/ml final concentration) for 20 min at 4°C. Cells plus Fab fragments were then diluted 1:7 into HBS/DNase/BSA and 1 mM EDTA that had been prewarmed to 37°C, and allowed to aggregate with rotation at 70 rpm

for 0 or 30 min at 37°C. Aliquots were taken and fixed in 1% glutaraldehyde at each of these time points, then evaluated for number of free particles of 10–100  $\mu\text{m}$  using a Coulter counter. Data are expressed as percent decrease in free particle number after 30 min.

### Substrate Attachment Assay

F11 cells suspended in DMEM/FCS treated with or without endo-N (as above) were washed with fresh medium twice to remove excess endo-N. 100  $\mu\text{l}$  of cell suspension (containing  $42,400 \pm 500$  cells) was added to 35-mm dishes containing 1 ml PBS and which were either untreated (TC plastic), treated with polyornithine (P), or treated with polyornithine and laminin (P/LN) (see above). The contents of the dish were mixed carefully in a cross-shaped motion for 10 s. After appropriate attachment times (between 0 and 10 min at room temperature), the liquid was aspirated out of the dish, the dishes rinsed gently with 1 ml PBS, and the attached cells fixed with 1% glutaraldehyde in PBS. In some experiments, attachment times were extended up to 1 h; in these cases Hepes-buffered saline was used instead of PBS to maintain cell viability.

Using the mixing conditions specified above, the distribution of attached cells was uniform across the dish. Thus the total number of attached cells could be determined reliably by counting the number of cells present in 10–30 1-mm<sup>2</sup> fields (amounting to 1–3% of the total area of the dish) using an inverted phase-contrast microscope. The absolute number of cells counted in this manner ranged from 30 to 1,000, depending on the substrate. Data are expressed as mean number of cells attached per dish ( $\pm$  S.E.M.). In some experiments, antibodies against LN (Gibco Laboratories; 50  $\mu\text{l}$ /dish in 1 ml) or NCAM (500  $\mu\text{g}$ /ml as Fab) were added to the washed P or P/LN dishes, incubated at 4°C overnight, and left in the dishes during the assay.

### Neurite Outgrowth from Single SpC cells

To score outgrowth, single processes from 200 cells in each dish, covering  $\sim 1 \text{ cm}^2$ , were measured on a straight line from soma center to tip of growth cone on the longest neurite. For inclusion in the count, a cell had to meet the following criteria: appear separate from other soma, refract brightly with phase contrast optics, and extend neurites more than five cell body diameters long.

### Quantification of Fasciculation

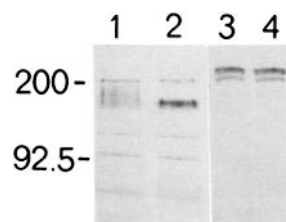
For video cinematography, explants in tissue culture flasks prepared as described above were grown for 12–24 h, and then observed in a 37°C room using an Olympus IMT inverted stage phase contrast microscope with a video attachment (RCA TC 2000 video camera and a Panasonic AG-6050 time-lapsed 1/2" tape recorder). After viewing all explants, fields were selected with good initial outgrowth. Recordings were made at 1:240. Of all recordings made, only those that had growth typical of the entire flask were studied further. All growth cones in a field were scored within a 6-h real-time window. Each identifiable growth cone was numbered and observed from time of initial visualization until it either grew out of the field, became lost in other growth, or the end of the time window was reached. A growth cone was considered in one of two categories: growing "on" other neurites, or growing "off," free of others upon open substrate. During observation, time spent in each category was noted, then all were tallied, and a percentage of observed time in the "on" category calculated for each growth cone. Wilcoxon Rank Sum Test statistics were used to evaluate the data.

## Results

### Removal of NCAM PSA Increases F11 Cell Attachment to Different Substrates

Although F11 cells have NCAM and L1 on their surface (Figs. 1 and 2), they appear in culture as separate (or only loosely associated) phase-bright cells with only a weak attachment to tissue culture plastic. Because their NCAM was found to have relatively high levels of PSA (Figs. 1 C and 2), the question arose as to whether their poor adhesion to each other and to the underlying substrate reflected the presence of this carbohydrate on their surfaces.





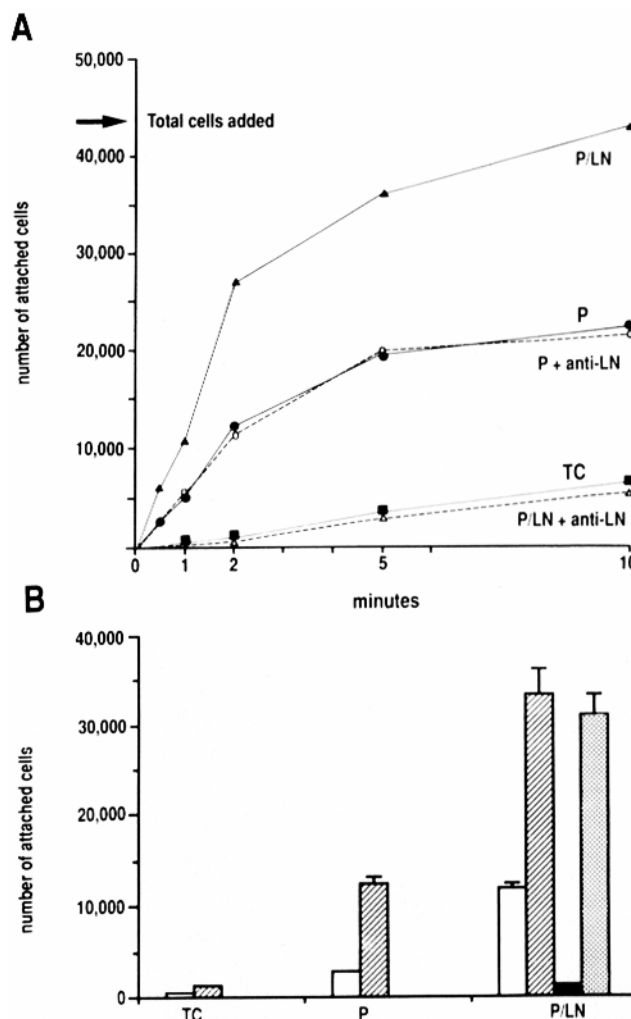
**Figure 2.** Endo-N treatment removes PSA from NCAM without altering L1. F11 cell extracts were immunoblotted using anti-NCAM (lanes 1 and 2) and anti-L1 (lanes 3 and 4). Untreated control cells show a diffuse pattern of NCAM staining ~200 kD (lane 1), which resolves into two distinct bands

after endo-N treatment (lane 2). Samples of the same two extracts show the pattern of L1 immunoreactivity in untreated cells (lane 3), which is not affected by endo-N treatment (lane 4). The position of  $M_r$  standards is indicated on the left.

To determine whether NCAM PSA could modulate cell-substrate interactions of F11 cells, we examined the ability of these cells to attach to several substrates in a short-term (1–10 min) assay at room temperature. The choice of substrates (TC plastic, P, and P/L) was dictated by two major concerns: a range of measurable attachment levels representing a variety of different interactions (Fig. 3 A), and inclusion of an appropriate substrate for fasciculation studies (P/L). Laminin alone on plastic was not used because the lower amounts of bound laminin obtained without polyornithine pretreatment (Edgar et al., 1984) produces an unsatisfactory amount and uniformity of moderately-fasciculated neurites from spinal cord explants (Dohrman et al., 1986). The choice of room temperature and no shear forces was dictated by the extremes of attachment: lower temperature to slow down attachment to P/LN and no shear to allow measurable attachment to TC plastic. These conditions facilitated quantitation but did not qualitatively change the results obtained at 37°C or with mixing (see below).

F11 cells with their PSA intact attached poorly to TC plastic, markedly better to P, and most rapidly to P/LN (Fig. 3 A). The attachment to P/LN was strongly inhibited (to TC plastic levels) by anti-LN; however, attachment to P was unaffected by this antibody (Fig. 3 A). Thus P and P/LN represent demonstrably different mechanisms of attachment. The different levels of attachment did not appear to reflect different subpopulations of cells, in that 80% of the total cells added attached to P/LN, P, or TC plastic substrates after 5, 30, and 60 min, respectively. At 37°C these levels were achieved after 3, 15, and 30 min, suggesting that the lower temperature used in the assay did not alter the observed hierarchy of attachment among these substrates.

When PSA was removed from F11 cells using endo-N, the cells displayed enhanced attachment to all three substrates. Using an early time point (1 min) (Fig. 3 A), there was a three- to fourfold increase in the number of cells bound (Fig. 3 B). Since 75% of the cells attached to P/LN within this time, the effect of PSA removal was probably underestimated for this substrate. As with the untreated cells (Fig. 3 A), attachment of the endo-N-treated cells to P/LN was strongly inhibited (to TC plastic levels) by antibodies against laminin. In contrast, antibodies against the NCAM polypeptide had no effect (Fig. 3 A), suggesting that it functions essentially as a carrier for PSA in this study. Again, the particular conditions used in the assay (temperature and shear forces) were not a qualitative factor in the observed effects. Attachment to both TC plastic and P was enhanced three- to fourfold at 37°C with no shear, as well as for P/LN at 37°C with 70 rpm gyroscopic mixing (data not shown).



**Figure 3.** Attachment of F11 cells to tissue culture substrates: modulation by PSA. (A) Time course of F11 cell attachment to TC plastic (TC; filled squares), polyornithine (P; filled circles) or polyornithine/laminin (P/LN; filled triangles). The number of untreated F11 cells (having high levels of PSA) attached to each of the three substrates was determined after 0–10 min at room temperature. In some dishes, the substrate was pretreated with anti-laminin antibodies (open symbols) before the attachment assay was performed. Data are the means of duplicate dishes from a representative experiment. The total number of cells added to each dish is this experiment was 44,700 (indicated by arrow). This experimental protocol was replicated three times with qualitatively similar results. (B) Effect of removal of PSA on F11 cell attachment. The number of F11 cells treated with endo-N (hatched bars; low PSA) or untreated (open bars; high PSA) which had attached after 1 min at room temperature was determined. Values are the means of  $\pm$  SEM of data obtained from six dishes in three independent experiments. The total number of cells added to each dish was  $42,500 \pm 570$ . Some laminin-coated dishes were preincubated with anti-laminin antibodies (filled bar) or anti-NCAM antibodies (stippled bar) before and during the attachment assay. In all cases, there was a three- to fourfold increase in the number of cells attached after 1 min when PSA was removed. This increase could not be blocked by anti-NCAM, but was completely inhibited by anti-laminin.

#### Removal of NCAM PSA Enhances L1-mediated Aggregation of F11 Cells, while Decreasing NCAM-mediated Aggregation

The cell-substrate attachment studies suggest that PSA can

**Table I. Removal of NCAM PSA Selectively Augments Function of L1 in F11 Cell Aggregation**

	Extent of aggregation*		
	Control	+ Anti-NCAM <sup>‡</sup>	+ Anti-L1 <sup>‡</sup>
+PSA <sup>§</sup>	17.6 ± 1.2	3.6 ± 1.2 (80%)	13.1 ± 1.0 (26%)
–PSA <sup>  </sup>	42.5 ± 1.6	26.6 ± 2.0 (37%)	14.3 ± 1.0 (66%)

\* Extent of aggregation is expressed as the percent decrease in cell number after rotation at 70 rpm for 30 min at 37°C. Data are means ± SEM of 9–12 values from 3 independent experiments. Values in parentheses are the percent inhibition of aggregation relative to the aggregation obtained without antibody.  
<sup>‡</sup> Cells were preincubated for 15 min at 4°C with 0.5 mg/ml anti-NCAM or anti-L1 Fab fragments, then aggregation assays were carried out as described in the continued presence of Fab.

<sup>§</sup> +PSA NCAM refers to untreated cells.

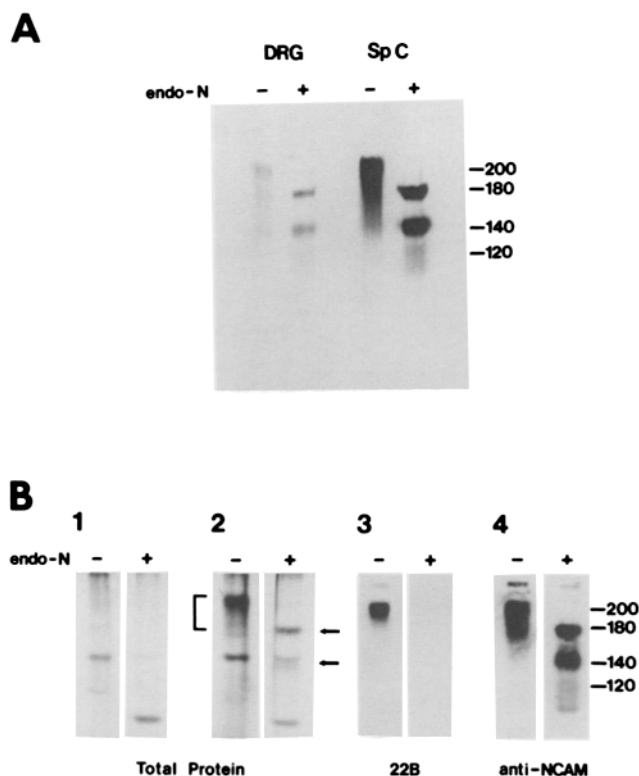
<sup>||</sup> –PSA NCAM refers to cells treated with endo-N for 2 h at 37°C. Removal of PSA from NCAM was confirmed by immunoblots in each experiment.

modulate adhesion-related events that do not directly involve NCAM's own binding activity. This hypothesis was also tested for cell–cell aggregation involving more than one adhesion molecule. In particular, the relative contribution of NCAM and L1 was evaluated in simple suspension–aggregation assays with F11 cells in the absence of calcium. These studies were carried out in the presence or absence of anti-NCAM Fab and anti-L1 Fab, and with or without endo-N treatment. In contrast to NCAM, endo-N treatment removes PSA from F11 cells without altering the amount or electrophoretic profile of L1 (Fig. 2). With PSA present, F11 cells aggregated slowly, with over three-quarters of this aggregation being inhibited by anti-NCAM Fab, and about one-quarter by anti-L1 Fab (Table I). When endo-N was used to remove PSA from NCAM, the extent of F11 cell aggregation increased 2.5-fold. However, about two thirds of the resultant adhesion was now inhibited by anti-L1 Fab and only one third by anti-NCAM Fab (Table I). Thus, removal of PSA from NCAM appeared to enhance the role of L1-mediated adhesion, while actually diminishing the relative contribution of NCAM-mediated adhesion to the overall aggregation.

Calcium was removed from the assay to simplify the analysis and interpretation by eliminating the simultaneous action of cadherins. Studies with calcium present were also conducted and gave results for NCAM and L1 that are consistent with those described above, but also superimposed on more complex phenomena that are likely to reflect synergistic actions among the different CAMs (see Rutishauser et al., 1988).

### Characterization and Removal of PSA from E7 Chicken Spinal Cord

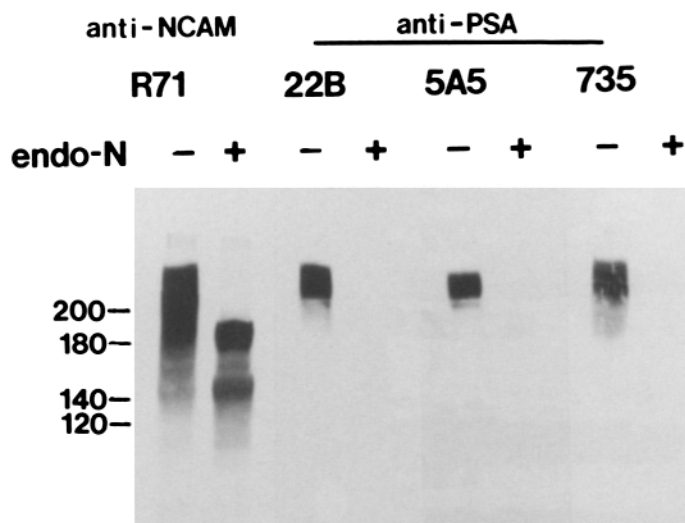
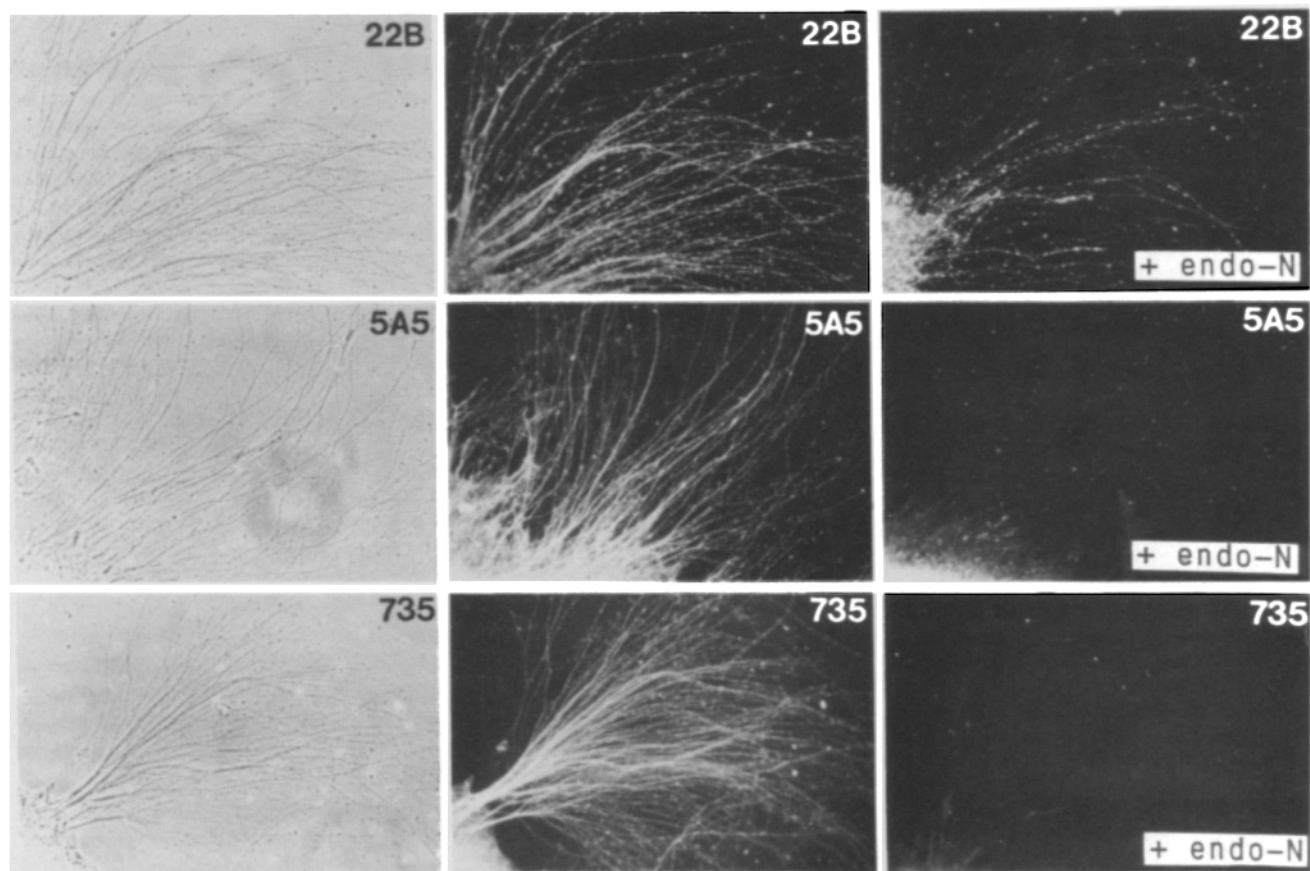
A classical system for observing the interplay of cell–cell and cell–substrate interactions is the bundling and branching patterns of growing neurites. In moving our studies into this more biologically intact but also more complex system, it was necessary to begin with a thorough characterization of the NCAM and PSA present, and the effectiveness of endo-N in removal of PSA from live explants. NCAM from E7 chicken spinal cord (SpC) demonstrated a low mobility in SDS-PAGE (Fig. 4 A, lane SpC –) that increased after removal of PSA by endo-N (lane SpC +). This behavior is characteristic for heavily sialylated NCAM (Rutishauser et al., 1985; Sunshine



**Figure 4.** PSA is present in E7 chicken spinal cord. (A) Immunoblot comparison of NCAM between E7 chicken DRG and SpC. Lumbar DRG, or SpC (cervical through sacral) were solubilized, and incubated either in the absence (–), or presence (+) of endo-N. Endo-N specifically removed the PSA, revealing the degree of sialylation and the NCAM polypeptide components. Each lane received the same amount of tissue as estimated by wet weights. Primary antibody was polyclonal anti-NCAM. Calculated  $M_r$  are indicated on the right. (B) PSA from E7 chicken SpC analyzed by SDS-PAGE. Equal amounts of material isolated by affinity chromatography using mAb 22B (anti-PSA) coupled to Sepharose 4b were incubated either in the absence (–) or presence (+) of endo-N, separated on SDS-PAGE, and examined using silver stain (lanes 1 and 2), mAb against PSA (lane 3) or with polyclonal anti-NCAM (lane 4). In the control isolation (lane 1), no tissue was added to the solubilization buffer. In lanes 2–4, the (–) lanes and the (+) lanes received the identical sample from an isolation of E7 SpC. Calculated  $M_r$  are indicated on the right.

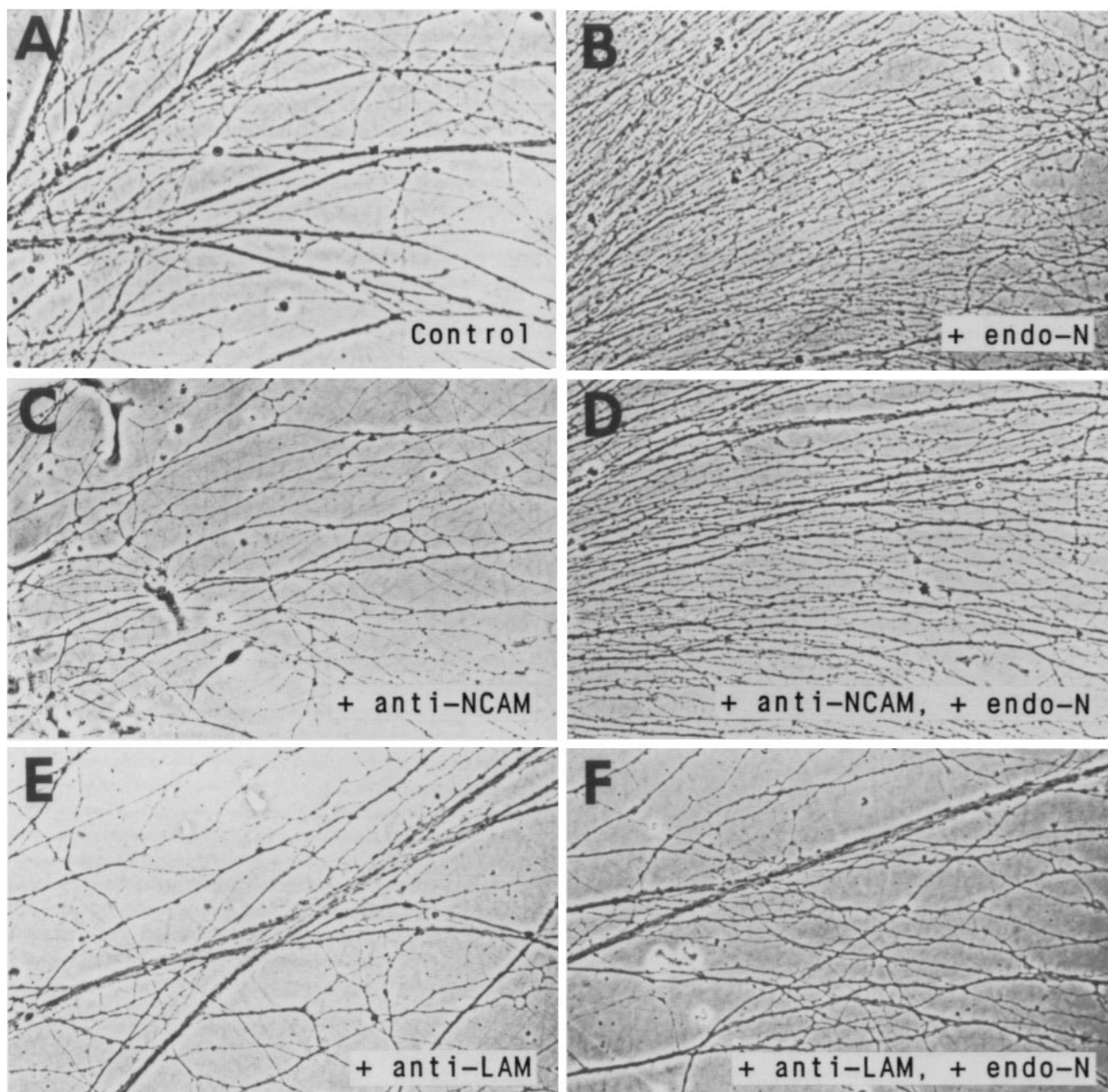
et al., 1987). In fact, SpC NCAM was more highly sialylated than that of the dorsal root ganglia (Fig. 4 A) that had been used in our previous studies on PSA and endo-N (Rutishauser et al., 1985). As also shown in Fig. 4 B, NCAM is the only detectable source of PSA in E7 chicken SpC. That is, immunoaffinity chromatography with mAb 22B, which recognizes PSA, was used to isolate all PSA-associated material in a total NP40 extract of E7 chicken SpC. Endo-N digestion was then used to remove the PSA (lane 3– vs. 3+). After SDS-PAGE separation, the PSA isolate appeared as a diffuse band of low mobility at 180–250 kD (lane 2–), which was not present in the matched control (lane 1–). Endo-N treatment converted this diffuse pattern into two distinct bands at 180 and 140 kD (lane 2+), again not present in the control (lane 1+). As detected by polyclonal anti-NCAM IgG, all specific bands were NCAM positive (lanes 4– and 4+).

The presence of PSA in control cultures, and its removal

**A****B**

**Figure 5.** Endo-N-mediated removal of PSA in cultures of E7 spinal cord. (A) Immunoblot comparison of sensitivity to endo-N among mAbs which react with PSA. Each pair of lanes received equal amounts of extracted material from SpC cultures grown either without (-) or with (+) endo-N for 1 wk. The primary antibody used in immunoblotting is indicated above each pair of lanes. Polyclonal anti-NCAM (R71) was used to identify all NCAM present. Calculated  $M_r$  are indicated on the left. (B) Staining of cultures with mAbs against PSA. Cultures were grown in the absence (left and middle columns), or presence of endo-N (right column) for 2 d, then were fixed and stained with the anti-PSA mAbs as indicated. Controls without 1st antibody were essentially blank (data not shown). Phase contrast fields (left column) match the untreated fluorescent fields (middle column).

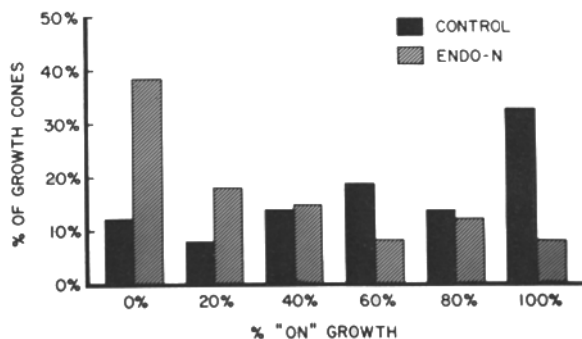




**Figure 6.** Fasciculation pattern of E7 spinal cord neurites grown on a laminin substrate: effect of endo-N treatment. Cultures were grown either without (*A*, *C*, and *E*) or with (*B*, *D*, and *F*) endo-N for 2 d. Addition of polyclonal anti-NCAM (*C* and *D*) reduced baseline fasciculation (*C*), but failed to alter the endo-N effect (*D*). In contrast, polyclonal anti-LAM could be titrated to a concentration that failed to alter baseline fasciculation (*E*), but could reverse endo-N induced defasciculation (*F*).

in cultures with endo-N was established by SDS-PAGE immunoblot analysis with anti-PSA mAbs 22B, 5A5, and 735 (Fig. 5 *A*). All PSA epitopes detected in control cultures were eliminated by the presence of endo-N. The results indicate that there was little if any spontaneous loss of PSA in the control (compare lanes with those in Fig. 4), nor any detectable retention of PSA immunoreactivity in endo-N-treated cultures. To confirm the uniform removal of PSA from all cells and neurites, cultures were also examined by immunofluorescence microscopy using the anti-PSA mAbs. The presence of endo-N markedly reduced staining by mAb 22B (Fig. 5 *B*, first row, right vs. middle), and abolished reaction to mAb

735 and mAb 5A5 (rows 2 and 3, right vs. middle). The persistent 22B fluorescence probably resulted from binding of this less specific antibody to shorter chains of sialic acid (three to four sugars) that endo-N does not remove (Hallenbeck et al., 1987). The complete removal of mAb 735 staining is consistent with the reported minimum epitope chain length (eight residues) for this antibody (Finne et al., 1987). Active digestion of PSA by endo-N persisted in culture for at least 5 d. After 60 h of culture, a 1:1 mixture of the culture supernatant with fresh medium was equally effective in removing all detectable PSA during the next 60 h of a fresh culture, as assessed using immunoblots (data not shown).



**Figure 7.** Quantitative analysis of the influence of endo-N treatment on neurite fasciculation in E7 spinal cord explant cultures. Growth cone preference for other neurites versus the LN substrate was quantified. Time-lapsed videos were analyzed for the percentage of time each growth cone spent extending "on" other fascicles, rather than on unobstructed substrate. In this analysis, the "100% on" bin reflects growth cones that were growing on other axons (fasciculated) throughout the observation period, whereas the "0% on" bin represents growth of an isolated neurite only on substrate, and intermediate values indicate alternation between these choices by an individual growth cone. The percentage of "on" time of the total observed time was calculated for each growth cone. Data were pooled from three control and three endo-N-treated fields. Control growth cones (filled bars) tended to grow "on" fascicles, while endo-N-treated growth cones (hatched bars) preferred open substrate. The statistical significance was calculated to be  $P < 0.00004$ .

#### **Removal of PSA Does Not Alter the Extent of Neurite Elongation on an LN Substrate**

Before examining the role of PSA in neurite fasciculation, it was necessary to determine whether the removal of PSA from SpC cells would affect their ability to extend neurites on an LN substrate. We therefore quantified the extent of neurite elongation from single SpC-derived cells in the presence or absence of PSA. Mean neurite lengths were measured from 400 single SpC cells in dishes with or without endo-N. In control cultures after 48 h, the mean neurite length was  $50 \pm 2 \mu\text{m}$ , whereas with endo-N treatment, the mean was  $48 \pm 2 \mu\text{m}$ . Thus, removal of PSA from NCAM did not change the extent of growth of individual neurites from dissociated SpC cells, implying that any differences in cell-substrate interaction resulting from endo-N treatment lie above that required for optimal growth under culture conditions.

#### **Removal of PSA Alters the Balance of Cell-Cell versus Cell-Substrate Interaction in SpC Explant Cultures**

Although endo-N treatment did not change the extent of growth on LN, it markedly diminished fasciculation of neurites growing from E7 SpC explants (Fig. 6, *A* and *B*). To provide a more quantitative dynamic assessment of this effect, we used time-lapse video recordings to observe individual growth cones with respect to their time spent growing along ("on") another previously extended neurite, versus time spent growing along the LN substrate ("off"). Fig. 7 shows a comparison of this parameter in the presence or absence of endo-N. These data probably represent an underestimate of the defasciculation caused by endo-N, since in control dishes the density of growth cones in very thick fiber bundles

was too high to allow resolution among them, and therefore the entire group was tallied only as a single growth cone. In contrast, extension of neurites on the substrate always permitted clear resolution. Despite this underestimate of the "on" category in controls, there was a clear decrease in the 100% "on" category and a corresponding increase in the 0% category after endo-N treatment (Fig. 7). The possibility that the removal of PSA might simply have selected a population of neurites capable of greater interaction with the substrate was ruled out by adding endo-N to cultures with pre-existing growth and observing that the "on/off" ratio was reduced to the same values shown in Fig. 7.

#### **Decreased Fasciculation Caused by Removal of PSA Is Reversed by Anti-LN But Not Anti-NCAM**

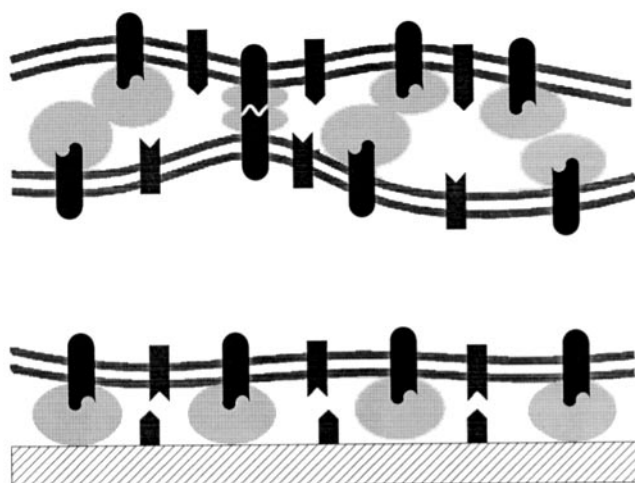
To evaluate the contribution of different adhesion systems to the defasciculation caused by endo-N, NCAM-mediated cell-cell adhesion, and LN-mediated cell-substrate adhesion were independently blocked in cultures by addition of specific antibodies known to inhibit either NCAM or LN function *in vitro*. Anti-NCAM alone produced only a slight reduction in fasciculation (Fig. 6, *A* vs. *C*), indicating that in this system, fasciculation largely reflects the function of molecules other than NCAM. Furthermore, in the presence of anti-NCAM, the defasciculating effect of endo-N (Fig. 6, *A* vs. *B*), was still apparent (Fig. 6, *C* vs. *D*), and just as extreme (Fig. 6, *D* vs. *B*). Thus the major modulation of fasciculation by endo-N in this system appeared to be independent of adhesion directly mediated by NCAM. Parallel cultures were exposed to different dilutions of antibodies to LN, and screened for effects on fasciculation. With high concentrations of anti-LN, all outgrowth of neurites was blocked (not shown), demonstrating that LN is the major ligand in growth cone-substrate interaction. At lower concentrations the anti-LN had no apparent effects in the presence of PSA (Fig. 6, *A* vs. *E*), but reversed the effects of endo-N, producing cultures with thick fascicles (Fig. 6, *F*) indistinguishable from controls (Fig. 6, *E* and *A*). Together, these results suggest that for cultured SpC neurons the decrease in fasciculation produced by endo-N is more dependent on LN function than on NCAM function.

#### **Discussion**

The present studies provide new evidence that PSA on NCAM can be a potent regulator of cell interactions involving membrane contact (Rutishauser et al., 1988). A proposed basis of this regulation, the apparent ability of PSA's unusual physical/chemical properties to reduce the efficiency of overall membrane-membrane contact, makes two striking experimental predictions: that ligands other than NCAM can be affected, and that its influence could include contact with any ligand-bearing surface. A simple schematic of these mechanisms is illustrated in Fig. 8.

Both predictions were tested by quantifying F11 cell attachment to different tissue culture substrates. For example F11 cells were shown to attach specifically to an LN-containing substrate, through integrin-type receptors or possibly an LN-heparin affinity (Edgar et al., 1984). Although NCAM itself was shown not to be directly involved in mediating F11 cell attachment to this substrate, removal of PSA from NCAM increased the rate of attachment. These data are con-



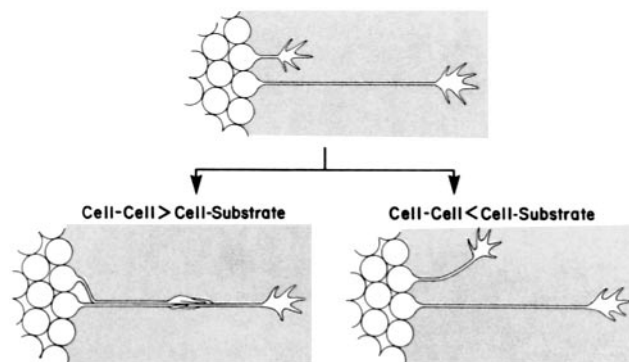


**Figure 8.** Proposed mechanisms for inhibition of cell interactions by PSA. (Top) Two cells, each containing NCAM with PSA (ellipses) as well as other receptor pairs, interact poorly because of the interference of the PSA with membrane-membrane contact. Bottom: The presence of PSA also affects attachment of cells to a tissue culture substrate (hatched surface).

sistent with the idea that the function of the LN receptor(s) on these cells is “masked” or hindered in some way by NCAM PSA. When PSA is removed, the overall efficiency of the interaction is increased. The fact that similar increases in attachment also occur with TC plastic and polyornithine argue against the possibility that a more receptor-specific regulation is involved, such as the apparent modulation of integrin function by sialic acid in gangliosides (Mugnai et al., 1988).

The present studies of F11 cells also support the prediction that PSA can regulate the function of cell-cell adhesion molecules other than NCAM. In particular, we have assessed the ability of L1 to promote F11 cell aggregation in the presence or absence of PSA. With untreated F11 cells (having sialylated NCAM), anti-NCAM Fab fragments blocked most of the aggregation, with L1 mediating the remainder. However, removal of PSA from NCAM, a procedure which did not alter the amount or electrophoretic mobility of L1, reversed this picture, such that anti-L1 blocked about three-quarters of the aggregation. Thus, removal of PSA from NCAM augmented the role of L1, allowing it to function better and even to dominate over NCAM.

These data therefore represent a situation in which one adhesion molecule can alter the function of another. Interactions between L1 and NCAM function also have been reported by Kadmon et al. (1990a,b). However, their findings have been interpreted via a mechanism whereby L1 is involved in two types of binding, one in which L1 binds to itself to form cell-cell bonds, and another in which L1 is modulated by a *cis* interaction with NCAM so as to enhance its *trans* adhesion properties. Although these studies did not investigate the role of PSA in such a *cis* interaction directly, the putative NCAM-L1 interaction depends on high mannose glycan chains whose synthesis is blocked by castanospermine (Kadmon et al., 1990b). In any case, such a mechanism cannot easily account for the diversity of cell interactions that appear to be regulated by PSA, and the fact that the presence of PSA inhibits rather than promotes cell interactions.



**Figure 9.** Cell adhesion during in vitro neurite fasciculation. The top of this schematic depicts an explant with cellular components (circles) which has extended one long neurite and growth cone upon prepared substrate (stipples), and a second neurite just beginning to extend. Enhanced cell-to-cell adhesion (lower left), allows the second neurite to extend along the previous neurite, producing a bundle of fibers and overall a fasciculated pattern of outgrowth. Enhanced cell-to-substrate interaction (lower right) permits the second neurite to follow a new path over the open substrate, producing distinct unbundled fibers and overall a defasciculated pattern of outgrowth.

With the F11 cell studies as an interpretive basis, we then turned our attention to the phenomenon of neurite fasciculation. Changes in the degree of fasciculation often reflect a shift in the balance between two competing forces (Fig. 9): cell-cell and cell-substrate interactions (Rutishauser et al., 1978; Landmesser et al., 1988, 1990). Enhancement of cell-to-cell adhesion produces thicker fascicles, while dominance of cell-to-substrate adhesion results in defasciculation.

In dissecting the different parameters that contribute to the pattern of fasciculation produced by SpC axons on an LN substrate, we observed by time-lapse cinematography that endo-N treatment enhances the relative “attractiveness” of the LN substrate for individual SpC growth cones, but without affecting the extent of their growth. Given the enhanced attachment of endo-N-treated F11 cells to LN, we propose that this behavior, as well as the overall pattern of defasciculation caused by endo-N, is due to an increase in cell-substrate interaction. However, if PSA serves as a regulator of overall membrane contact, it should also affect neurite-neurite interactions. Thus the additional conclusion must be drawn that in this case any increase in cell-cell adhesion is overshadowed by the increase in cell-LN interaction. That endo-N’s effects on fasciculation were reversed by anti-LN, but not by anti-NCAM, is consistent with this interpretation.

Alternatively, one could argue that NCAM contributes to neurite outgrowth patterns via a more complex cell-cell interaction that is enhanced by the presence of PSA (Doherty et al., 1990a). Although such a possibility cannot be ruled out, it is not supported by the extensive evidence that PSA inhibits both NCAM-dependent and -independent interactions between purified surface membranes as well as live cells (Hoffman and Edelman, 1983; Rutishauser et al., 1985, 1988; and the present studies).

Although treatment with endo-N causes SpC axon defasciculation on an LN substrate, in other experimental situations the removal of PSA from axon fascicles appears to shift the balance of interactions in the opposite direction. During

the innervation of chick limb muscle by SpC motor axons, the L1 adhesion molecule on the neurites again appears to be largely responsible for fasciculation. However, in vivo it is the low sialic acid form of NCAM on the underlying muscle cells, which serves as a major substrate (Landmesser et al., 1988). In support of the hypothesis that PSA can serve as a regulator of fasciculation, there are developmental changes in the pattern of innervation that are not correlated with levels of NCAM or L1 expression but rather with the amount of PSA on the ingrowing motor axons (Landmesser et al., 1990). However, in contrast to the present in vitro studies using LN, removal of PSA from NCAM by endo-N during the initial stages of motor axon growth results in a dramatic decrease in the extent of nerve branching and an increase in nerve fasciculation. Thus, in this situation axon-axon interactions appear to be enhanced relative to those between the axons and substrate (Fig. 9). Nevertheless, in agreement with the overall mechanism of regulation proposed for PSA, the effect of endo-N can be reversed in this system by co-administration of antibodies to L1, but not by antibodies to NCAM. That is, PSA again appears to be able to modulate other adhesion mechanisms independently of NCAM's own binding functions.

In summary, the PSA moiety associated primarily with NCAM on the neuronal surface appears to regulate the function of a variety of cell surface receptors (see also Rutishauser et al., 1988), involved in both cell-cell and cell-substrate interactions. This regulation appears to play a key role in determining the pattern of neurite outgrowth both in vitro (this study) and in vivo (Landmesser et al., 1990). Whether the effect of PSA removal is increased bundling, decreased bundling and/or increased branching depends on the type of neuron, the growth substrate and the relative abundance of other CAMS or cell-substrate adhesion molecules. The physiological importance of fasciculated versus defasciculated growth has been established in many neural systems, including retinotectal projections (Thanos et al., 1984) and motor axon growth in the chick hindlimb (Dahm and Landmesser, 1988; Landmesser et al., 1988, 1990). In these examples, a transition from fasciculated to defasciculated growth is necessary to produce the normal pattern of innervation. Since the PSA content of NCAM is developmentally regulated in both systems (Schlosshauer et al., 1984; Landmesser et al., 1990) it is likely that PSA is a significant factor in this aspect of their development.

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