The Neural Cell Adhesion Molecule (NCAM) as a Regulator of Cell-Cell Interactions

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The neural cell adhesion molecule (NCAM) can influence a number of diverse intercellular events, including junctional communication, the association of axons with pathways and targets, and signals that alter levels of neurotransmitter enzymes. These pleiotropic effects appear to reflect the ability of NCAM to regulate membrane-membrane contact required to initiate specific interactions between other molecules. Such regulation can occur through changes in either NCAM expression or the molecule's content of polysialic acid (PSA). When NCAM with a low PSA content is expressed, adhesion is increased and contact-dependent events are triggered. In contrast, the large excluded volume of NCAM PSA can inhibit cell-cell interactions through hindrance of overall membrane apposition.

CELL ADHESION MOLECULES (CAM's) ARE LIGANDS THAT can participate in cell-cell recognition during the formation of tissue structures. In the nervous system, a number of membrane glycoproteins have been identified as CAM's (*1*). Several of these CAM's have been associated with a specific recognition function, such as selective bundling of axons, the choice of synaptic target, or segregation of cells into different tissues. In contrast, the function of the neural cell adhesion molecule (NCAM), one of the most abundant adhesion molecules, appears to affect a wide variety of different cellular events. To understand the biological role of NCAM, it is necessary to explain how and why so many different types of interactions appear to be influenced by its function.

There are two ways in which NCAM is believed to affect cell-cell adhesion: (i) developmental changes in the number of homophilic bonds between NCAM polypeptides on apposing membranes, and (ii) the modulation of adhesion according to the molecule's content of polysialic acid (PSA), an unusual oligosaccharide present primarily, if not exclusively, on NCAM (*2–4*). The interpretation of NCAM-associated phenomena previously has emphasized adhesive preferences that directly reflect these variations in NCAM binding (*5*). However, as illustrated below, both NCAM expression and its PSA content can also serve as indirect and permissive regulatory elements in other cell interactions. Experimental evidence and a model are presented suggesting that this situation reflects changes in the overall state of membrane-membrane contact, rather than direct alteration of a specific ligation event.

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NCAM binding affects the function of another cell-cell adhesion mechanism. While adhesion mediated by NCAM is calcium-independent, a different class of CAM that requires calcium often exists on the same cell surface (*1, 6–8*). To investigate a possible functional interdependence between these two adhesion mechanisms, we prepared membrane vesicles such that both of these CAM's were present. As expected, adhesion between the membranes included both a calcium-dependent and a calcium-independent component. Although antibody to NCAM (anti-NCAM) does not affect aggregation of cells with only the calcium-dependent mechanism (*8*), under these conditions both types of adhesion were blocked by this antibody (Table 1). The converse was not true in that adhesion was only partially blocked by removal of calcium, a

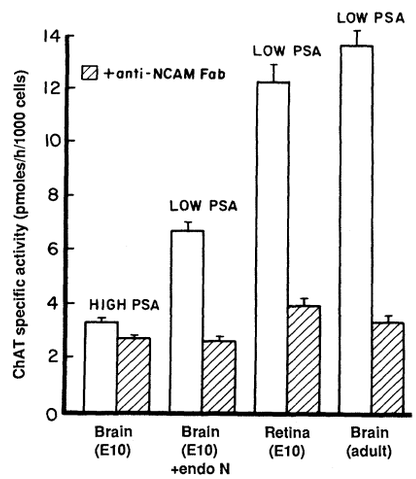

Fig. 1. Membrane-mediated increase in ChAT activity in chick sympathetic neurons: role of NCAM. The increase in ChAT activity by addition of neural membranes to cells in culture (open bars) required both NCAM function, as indicated by inhibition with anti-NCAM Fab (hatched bars), and a low content of PSA. Selective removal of PSA from brain membranes was carried out with endo N (*4*). E10 retina and adult brain represent natural sources of the low PSA form of NCAM (*13, 14*). E10 sympathetic ganglia were dissociated and the cells (2000 per square centimeter) were cultured on a laminin substrate (*12*). Five hours later, crude membranes, prepared freshly from the tissues indicated, were added at a concentration of 30 μg of protein per milliliter. The relative NCAM PSA content of the membrane fractions was estimated from the mobility of NCAM in immunoblots. Membrane vesicles were first incubated with 50 μg of anti-NCAM Fab fragment per microliter of original pellet volume for 30 minutes on ice before they were added to the cells. Eighteen hours after the addition of membranes to cells, ChAT-specific activity (picomoles per hour per 1000 cells) was determined (*37*). Activity measured by this assay was blocked by the specific ChAT inhibitor naphthylvinylpyridine (*12*). The number of cells per dish was determined as in (*12*). Data are the means ± SEM obtained from five dishes in two independent experiments. Values obtained from dishes that were coated with membranes but contained no cells were subtracted. These values ranged between 2 to 5 picomoles per hour per dish, as compared with total activities for membrane-treated cells of 60 to 260. Other control conditions were (in picomoles per hour per 1000 cells): cells without added membranes, 4.74 ± 0.33; cells plus endo N–treated E10 brain membranes and nonimmune Fab, 6.72 ± 0.40; cells plus E10 retinal membranes plus nonimmune Fab, 12.75 ± 0.63; and cells plus adult chicken brain membranes plus nonimmune Fab, 13.9 ± 0.55.

treatment that does not affect adhesion mediated by NCAM alone (Table 1). If one assumes that short-term aggregation assays directly reflect the binding properties of CAM's, these data suggest that NCAM function can regulate the action of another class of CAM's when present on the same membrane.

Both NCAM binding and PSA content can influence cell contact-dependent events. Membrane-membrane contact regulates the levels of neurotransmitter biosynthetic enzymes in vitro for a variety of neural crest-derived cells (*9–11*). With both rat and chick sympathetic neurons, cell-cell contact results in an increase in choline acetyltransferase (ChAT) activity (*11, 12*). In our studies, chick sympathetic neurons were exposed, in the presence or absence of anti-NCAM Fab, to membranes containing NCAM in either its high or low PSA form. Under these conditions, membrane contact-mediated increases in ChAT were found to require both NCAM binding function, as indicated by inhibition with anti-NCAM Fab, and the presence of the molecule in its adhesion-promoting, low PSA form (Fig. 1). The effects of antibody were specific, in that antibodies to another abundant adhesion molecule present on these cells, the L1/G4 glycoprotein, did not alter ChAT levels (*12*). The low PSA requirement was met either by specific enzymatic removal of PSA from NCAM on embryonic brain membranes by endoneuraminidase N (endo N) (*4*) (Fig. 1; compare E10 brain membranes, which are ineffective, with the same membranes treated with endo N) or by the use of membranes that naturally express the low PSA form of NCAM (*13, 14*) (Fig. 1; E10 retina and adult brain). Thus, NCAM appears to serve as a permissive regulatory factor in this system in two different ways: its binding function holds membranes together and its PSA content independently regulates the ability of cells to transmit the relevant biochemical signal. This conclusion is also supported by recent evidence that the ability of anti-NCAM Fab to block increased ChAT levels can be reversed by addition of a plant lectin, but again only when the PSA content of the endogenous NCAM is low (*12*).

NCAM PSA content alone can regulate the function of other cell surface ligands. Additional evidence for an influence of NCAM PSA on cell interactions involving molecules other than NCAM has been obtained from studies of the bundling patterns of neurites. We previously demonstrated that neurite outgrowth from dorsal root ganglia onto a collagen substrate displays increased fasciculation after treatment with endo N, suggesting that the removal of PSA can result in augmented fiber-fiber adhesion (*4*). However, subsequent analysis reveals that the effects of endo N can also be exactly the opposite, depending on the type of neuron and substrate used. For example, embryonic chick (E7) spinal cord neurites, whose NCAM is very heavily sialylated, grow as large fascicles on laminin

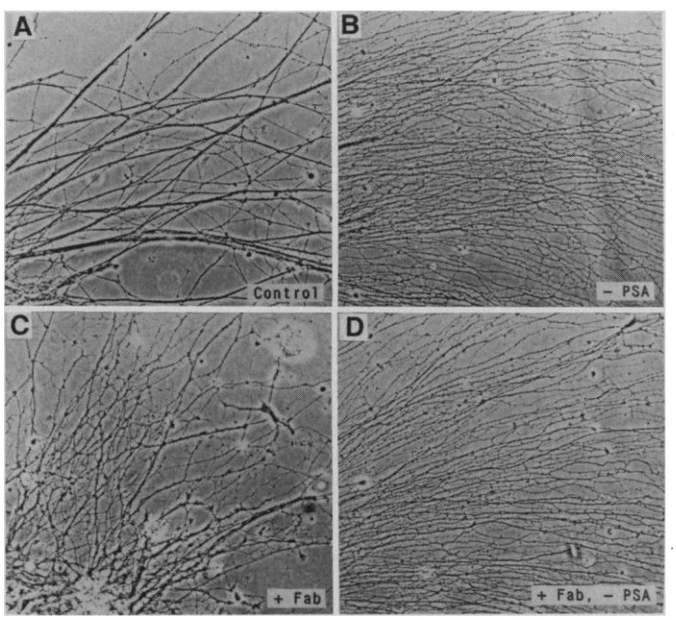

Fig. 2. Axon bundling of chick embryonic spinal cord on a laminin substratum. (**A**) Control with large fascicles. (**B**) Treatment with endo N to remove NCAM PSA caused a large decrease in fasciculation. (**C**) The presence of anti-NCAM Fab caused a partial reduction in the diameter of axon bundles. (**D**) The effect of endo N was not altered by the presence of anti-NCAM Fab. Spinal cords from embryonic day 7 chicks were dissected free of meninges and dorsal root ganglia, and were cut into pieces (1 to 2 mm^2). Such explants were cultured on a laminin substratum in F14 medium containing 10 percent horse serum and muscle extract at 20 μl/ml (*38*). Anti-NCAM Fab fragments (0.5 mg/ml) or purified endo N (30 unit/ml) (*4*) were added to the medium prior to plating. To facilitate adhesion of explants to the dish, 1 ml of medium was removed from each dish after plating, and was added back 4 hours later. Explants were fixed in 0.5 percent glutaraldehyde 40 to 48 hours after the initial plating.

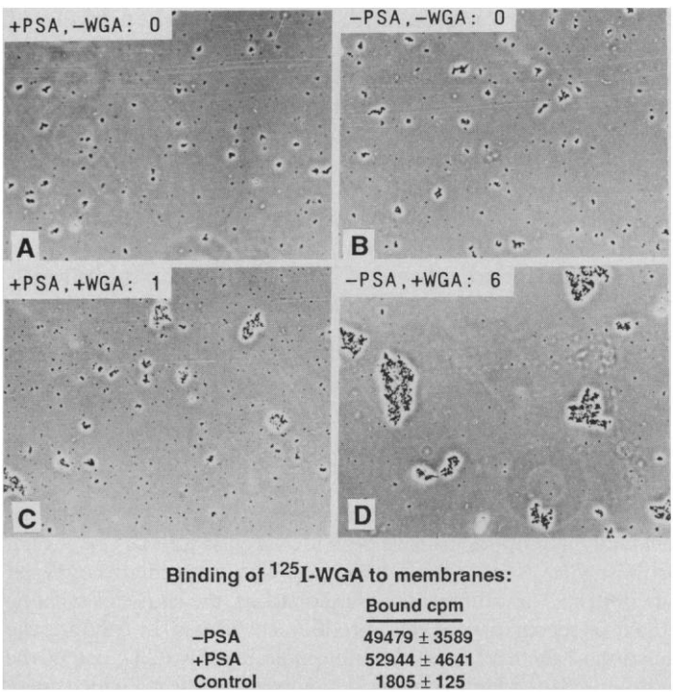

Fig. 3. Wheat germ agglutinin (WGA)–mediated agglutination of membranes: role of NCAM PSA in the absence of NCAM-mediated adhesion. NCAM binding function was blocked by anti-NCAM Fab, and the extent of aggregation of brain vesicles was assessed after 5 minutes. The relative rate of decrease in particle number for each set of conditions is indicated in the upper left-hand corner of each panel. (**A**) No aggregation was observed in the absence of WGA, (**B**) even after the membranes were treated with endo N to remove NCAM PSA. (**C** and **D**) When WGA was present, agglutination was enhanced by the removal of NCAM PSA. Bottom: removal of NCAM PSA had no effect on the number of ^{125}I-labeled WGA binding sites. Membrane vesicles were prepared from E10 chick brain, and some were treated with endo N for 30 minutes at 4°C (*4*). All vesicles were treated with 50 μg of anti-NCAM Fab fragment per microliter of original pellet volume for 10 minutes at 4°C. Aggregation was then carried out at room temperature with rotation at 70 rpm in the presence or absence of WGA at 400 μg/ml. For binding studies, WGA was iodinated with chloramine T, yielding a specific activity of 190,000 cpm/μg. A 1:20 suspension of brain vesicles (150 μl) was incubated with unlabeled WGA (400 μg/ml) and ^{125}I-labeled WGA (1.25 μg/ml; about 119,000 cpm total) in a total volume of 500 μl. The binding reaction was carried out at 4°C for 10 minutes with continuous rotation. The vesicles were then washed three times with phosphate-buffered saline (PBS) containing 10 percent horse serum and then twice with PBS. Bound radioactivity was quantified in the final pellet. Control binding was measured with heat-denatured ligand (100°C, 10 minutes). Data are means ± SEM of nine values obtained in two independent experiments.

(Fig. 2A), and removal of PSA from these axons actually reduces fasciculation (Fig. 2B). How can these seemingly disparate results be explained? In fact, the size of bundles reflects not only neurite-neurite binding mediated by several adhesion molecules including NCAM (*4, 15–17*), but also an opposing force exerted by the individual growth cones as they adhere to and migrate along the substrate (*18*). The simplest explanation of the neurite patterns obtained is therefore that with ganglion-collagen cultures membrane-membrane adhesion is enhanced relative to growth cone-substrate adhesion whereas with spinal cord–laminin the opposite is the case. Consistent with this interpretation is the fact that in the spinal cord studies, the effect of endo N occurred even in the absence of NCAM-mediated adhesion (Fig. 2D), was even greater than that produced by anti-NCAM Fab fragments (Fig. 2C), and could be reversed by an antibody to laminin. In the ganglion-collagen studies, there was no effect of endo N in the presence of anti-NCAM (*4*).

A second illustration of this phenomenon is the effect of PSA on an artificial cell-cell interaction, wheat germ agglutinin (WGA)–mediated agglutination of embryonic chick (E10) brain membrane vesicles. As with spinal cord neurons, the NCAM on these membranes is heavily sialylated (*3, 4, 13*). In these experiments, the ability of WGA to agglutinate membranes in the absence of NCAM binding function (that is, in the continuous presence of anti-NCAM Fab) was examined as a function of NCAM PSA content. The amount of PSA did not affect the extent of aggregate formation in the absence of WGA (Fig. 3, A and B). However, removal of NCAM PSA greatly facilitated the ability of WGA to promote aggregation (sixfold increase; compare Fig. 3D with 3C). This effect did not reflect a change in the number of lectin receptors on membranes (Fig. 3, bottom), but rather an enhanced ability of the lectin to function when PSA was removed from the endogenous NCAM.

Membrane and molecular parameters in NCAM-mediated adhesion. It is known that NCAM mediates cell-cell binding, and that the rate of adhesion is enhanced by the removal of PSA from the molecule (*3, 4*), but how are these effects manifested in terms of membrane-membrane contacts? Electron microscopic examination of aggregates formed by embryonic chick (E7) brain cells, which express NCAM with a high PSA content, showed intermittent regions of close membrane-membrane apposition between neighboring cells (Fig. 4B). Specific removal of the NCAM PSA by endo N resulted in a fivefold increase in the area of these closely apposed regions (Fig. 4C; see lower panel for quantitation and legend for details of the morphometric criteria used). As a control for nonspe-

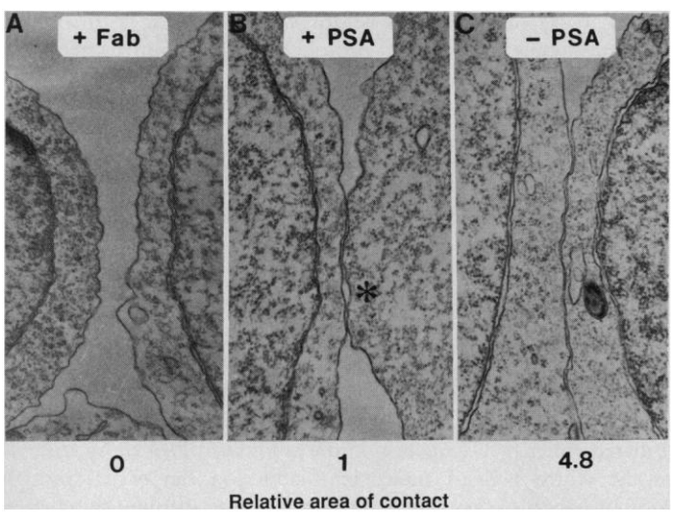

Fig. 4. Apposition of cell membranes in the absence of NCAM binding and after removal of PSA from NCAM. (**A**) In the presence of anti-NCAM Fab fragments (0.5 mg/ml), cells were rarely in contact, and their membranes were never closely apposed (within 50 nm). (**B**) With NCAM having a high content of PSA, plasma membranes came into contact, but short areas of close apposition were frequently interrupted by areas of no contact (asterisk). (**C**) After enzymatic removal of NCAM PSA with endo N (*4*), large regions of the plasma membrane were in close, continuous apposition. In addition, the cells were often deformed by the extensive attachment between their membranes. The numbers below indicate the percentage of cell membrane in close apposition, which was evaluated after digitization by dividing the apposition length by the cell circumference for each cell in a bond. The mean cell circumference did not differ with any treatment. The percentage of membrane contact for chick brain cells was 3.53 ± 0.03 (mean ± SEM, $n = 72$) and 7.63 ± 0.05 (mean ± SEM, $n = 75$) after the enzymatic removal of NCAM PSA, yielding a 2.2-fold increase in apposed membrane per cell (modified t test, $P < 0.0005$, 117 df). Since the area of contact is proportional to the square of the apposed membrane in sections, these values corresponded to a 4.8-fold increase in bond area after the enzymatic removal of PSA from NCAM. Embryonic (day 7) chick brain cells were prepared by light trypsinization in the absence of calcium (*8*). They were allowed to aggregate at 37°C for 30 minutes before being gently pelleted (1000*g* for 2 minutes), onto agar and prepared for electron microscopy (*39*) in 0.08*M* sodium cacodylate buffer, *p*H 7.4.

Table 1. Effect of NCAM on calcium-dependent adhesion.

Functioning adhesion mechanism*	Rate of adhesion†			
	Calcium	EDTA	Anti-NCAM	Control Fab‡
NCAM plus calcium-dependent	56 ± 3	30 ± 3	8 ± 2	63 ± 5
Calcium-dependent alone	28 ± 2	1 ± 1	26 ± 2	ND
NCAM alone	31 ± 2	34 ± 3	9 ± 1	32 ± 3

*NCAM plus calcium-dependent: embryonic brain vesicles were incubated in the presence of 0.01*M* calcium (no trypsinization). Calcium-dependent alone: embryonic brain cells were isolated by treatment with 0.1 percent trypsin in the presence of 0.01*M* calcium, which preserves calcium-dependent adhesion but destroys NCAM. NCAM alone: embryonic brain cells were isolated by treatment with 0.005 percent trypsin in the presence of 0.001*M* EDTA, which preserves NCAM but destroys calcium-dependent adhesion (see *7, 8*). †Percent decrease in particle number (*4*). Data are means ± SEM of six determinations obtained from two independent experiments. Data from different assays were normalized with respect to the rate of aggregation of cells or membranes alone. ‡Control is a polyclonal Fab against intact neural cells; this antibody was then absorbed with NCAM. The amount of the absorbed Fab that bound to membranes was similar to that with anti-NCAM Fab, indicating that the inhibition obtained with the latter does not reflect a nonspecific steric effect.

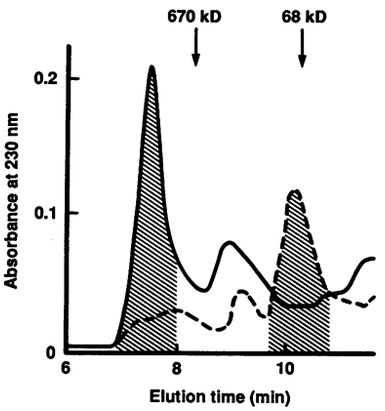

Fig. 5. HPLC gel filtration chromatography of NCAM peptide fragments containing large amounts of PSA. Proteolytic fragments of NCAM were separated, and elution fractions were tested for PSA immunoreactivity (hatched areas). With the PSA moiety intact (solid line), these fragments had an apparent excluded volume greater than that of thyroglobulin (670 kD). After treatment of the fragments with endo N (dotted line), the remaining PSA eluted with bovine serum albumin (68 kD). The column was a DuPont Bio-series GF-450, equilibrated and eluted with 0.2*M* sodium phosphate, *p*H 7.3. The PSA-rich fragment was prepared by digesting a 40 percent suspension of embryonic day 14 chick brain vesicles (*13*) with V-8 protease (final concentration 50 μg/ml) for 1 hour at 37°C. After centrifugation to remove vesicles, the supernatant was passed over a monoclonal 5e–anti-NCAM coupled Sepharose column to remove the cell binding domains (*20*). The presence of PSA in 1-minute eluate fractions was determined from slot immunoblots with polyclonal immunoglobulin M against PSA (*4*). The endo N digestions were conducted with purified enzyme (30 unit/ml) at 4°C for 30 minutes (*4*).

cifically apposed membranes, anti-NCAM Fab fragments were added which completely block aggregation of these cells. This antibody prevented the formation of areas of close contact (Fig. 4A). Therefore, as might be expected for an abundant adhesion ligand with a uniform distribution on the cell surface (*19*), there were gross physical changes in the overall degree of adhesion-induced membrane apposition as a result of either enhanced or decreased NCAM function.

The observation that alterations in PSA content affect other cell-cell interactions even when NCAM's binding function is blocked is a striking phenomenon. This carbohydrate comprises up to one-third of the molecule's mass (*13*), and causes a very large decrease in the molecule's electrophoretic mobility in SDS–polyacrylamide gel electrophoresis (*3, 4, 13, 14*). To examine the physical properties of the PSA moiety independent of the molecule's binding and cytoplasmic domains, NCAM-bearing membranes were digested with V-8 protease to release extracellular protein regions, and proteolytic fragments containing the binding region of the molecule [as defined by immunoreactivity with monoclonal antibody 5e (*20*)] were removed from the mixture. The remaining peptides were subjected to high-performance liquid chromatography (HPLC) gel filtration chromatography, and elution fractions were tested for the presence of PSA immunoreactivity (Fig. 5; see legend for experimental details). The peptide fragment containing PSA (Fig. 5) had an apparent excluded volume greater than that of thyroglobulin (molecular size, 670 kD). After treatment with endo N, the residual glycopeptide had an apparent size of less than 100 kD (Fig. 5). Thus, the excluded volume of PSA was equivalent to that of a globular protein several times the estimated size of the intact NCAM polypeptide. On this basis, we suggest that the steric properties of the PSA moiety are not only likely to affect NCAM-NCAM binding but, in view of the abundance of NCAM in the membrane, could produce a thin screen of carbohydrate around the cell. In this respect, it is intriguing to note that the other reported occurrences of PSA are the protective outer coat of certain bacteria (*21*) and the perivitelline zone of trout eggs (*22*), both large structures that surround cells.

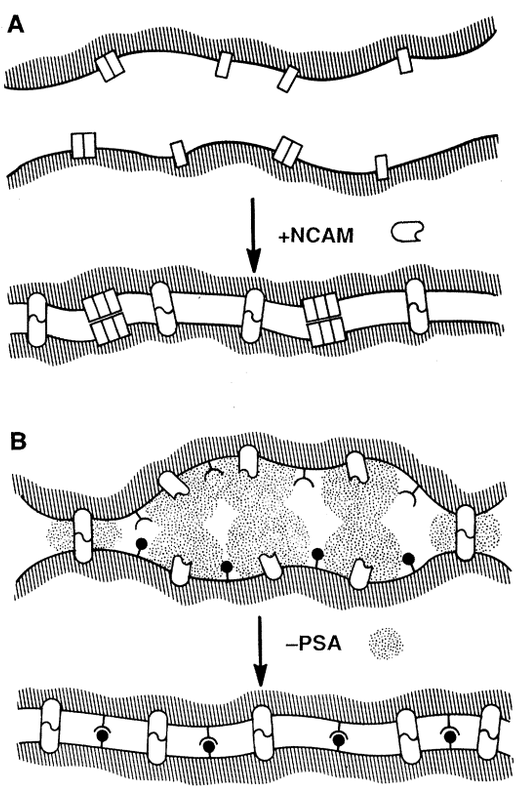

Fig. 6. Two mechanisms for the regulation of cell-cell interactions by NCAM. (**A**) Expression of NCAM on cell surfaces and the resultant formation of NCAM-NCAM bonds enhances the probability of junction formation from interacting subunits (rectangles) by increasing the extent or duration of membrane-membrane contact. (**B**) Initiation of cell interactions via specific ligands (ball and socket) by a reduction in NCAM PSA content (stippled areas). The ability of the ligands to engage is enhanced by reducing the excluded volume of carbohydrate between membranes, which impedes close cell-cell contact. PSA is depicted as also compromising interaction between NCAM's, with adhesion occurring between molecules with relatively low amounts of PSA (see Fig. 4).

Proposed mechanism for NCAM-mediated regulation. Data from the four different experimental systems presented above, together with information about the unusual properties of the molecule itself, have led us to the following conclusion: the presence of NCAM on the cell surface can have either a positive or a negative effect on its overall interaction with other cells or substrates, depending on the molecule's PSA content. To account for these phenomena, two mechanisms are proposed (Fig. 6).

For NCAM with relatively low PSA content, as found predominantly in early development and adult tissues (*14, 23*), the molecule's presence would increase the extent or duration of membrane contact and thereby promote other interactions (Fig. 6A). This mechanism is consistent with the present findings on the relationship between NCAM and calcium-dependent adhesion, which has been linked to the formation of intermediate or adherens-type junctions (*24–26*). We have previously reported two other examples of the ability of NCAM expression to regulate the formation of specialized cell-cell contacts. Although gap junction proteins are unrelated to NCAM, studies of antibody perturbation in vitro suggest that NCAM-mediated adhesion is required for the onset and spatial restriction of extensive junctional communication among neuroepithelial cells, as measured by dye transfer (*27*). A role for NCAM has also been proposed in the innervation of limb muscles by motoneurons (*28–30*). Again, although NCAM itself is not thought to be directly involved in synapse formation (*31*), its developmentally regulated expression on muscle may facilitate the initial interaction with nerves (*28*).

For molecules with high PSA content, the large volume occupied by the carbohydrate would impede membrane-membrane contact so that the function of some ligands, and probably even that of NCAM itself, is hindered (Fig. 6B). In this case, interactions could be initiated either by a reduction in the amount of PSA or by removal of the entire NCAM molecule if adhesion can occur through another ligand. At present it is not possible to distinguish whether the effects of PSA involve changes in the extent, intimacy, or duration of cell-cell contact. Each could, in principle, enhance the efficiency of a particular cell interaction.

The function of multiple cell adhesion mechanisms on a single cell has been explored in several systems (*32–34*). A premise of this work is that combinations of recognition systems can provide additional specificity in cell-cell contacts. Our findings provide evidence for the importance of permissive hierarchies, in which the degree of cell-cell contact required to initiate one interaction is provided by a second molecule. NCAM can function in such a hierarchy in two ways. First, its expression can enhance cell interactions by increasing membrane-membrane adhesion. Such a role for adhesion molecules has been previously proposed in the formation of gap junctions between sponge cells (*35*). A useful feature of this type of permissive regulation is that if a particular cell interaction requires several components, they can be synthesized independently and then functionally activated by expression of a single polypeptide.

Second, NCAM PSA can mediate global regulation of membrane events via its steric or repulsive properties, by producing an oligosaccharide coating around the cell reminiscent of the "glycoca-

lyx" hypothesis proposed by Bennett (*36*). Such alterations of the overall degree of close membrane apposition could allow differential use of several ligand-receptor systems according to their properties. For example, a receptor that is small, relatively immobile, or present in low amounts may require a low NCAM PSA content in order to function, while an abundant and mobile extracellular protein receptor, or a soluble ligand, would be independent of this type of control. Embryonic axons, which have a particularly high PSA content during the formation of tracts and connections may illustrate this type of selection. As an axon grows through a complex environment, it must avoid inappropriate interactions, such as formation of stable junctions, yet remain responsive to guidance and target cues. It should soon be possible to evaluate these ideas experimentally with the use of specific reagents now available for both the localization and degradation of PSA.

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