

Heterophilic Interactions of DM-GRASP: GRASP-NgCAM Interactions Involved in Neurite Extension

Angela P. DeBernardo and Susannah Chang

Department of Neuroscience, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Abstract. DM-GRASP is an immunoglobulin superfamily cell adhesion molecule that is expressed in both the developing nervous and immune system. Specific populations of neurons respond to DM-GRASP by extending neurites. Neurite extension on DM-GRASP substrates appears to require homophilic interactions between DM-GRASP molecules. We were interested in determining whether DM-GRASP interacts heterophilically with other ligands as well. We have found that eleven proteins from embryonic chick brain membranes consistently bind to and elute from a DM-GRASP-Sepharose affinity column. One of these proteins is DM-GRASP itself, consistent with its known homophilic binding. Another protein, at 130 kD, is immunoreactive with monoclonal antibodies to NgCAM. Other neural cell adhesion molecules were not detected

in the eluate. The DM-GRASP-Sepharose eluate also contains a potent neurite stimulating activity, which cannot be accounted for by either DM-GRASP or NgCAM. To investigate the interaction of DM-GRASP and NgCAM, antibodies against DM-GRASP were added to neuronal cultures extending neurites on an NgCAM substrate. The presence of antibodies to DM-GRASP decreased neurite extension on NgCAM. Antibodies to DM-GRASP did not affect neurite extension on laminin, suggesting that the antibody is not toxic or generally inhibiting motility. We present two possible models for the DM-GRASP-NgCAM association and a hypothesis for neural cell adhesion function that features the dimerization of cell adhesion molecules.

THE development of specific connections between neurons and their targets is a critical step in the making of a mature and functional nervous system. Axons actively explore the environment as they extend to their target areas. Guidance cues within the environment may be localized on cell surfaces, in extracellular matrix, or as diffusible factors (for recent reviews see Goodman and Shatz, 1993; Hynes and Lander, 1992). The recognition of these molecules is thought to be mediated through receptors on the surface of the growth cone that, when activated, lead to changes in the actin cytoskeleton (Lin and Forscher, 1993; Fan and Raper, 1995). A number of cell adhesion molecules have been isolated and shown to be expressed on axons (for reviews see Rathjen, 1991; Goodman and Shatz, 1993). They fall into three broad classes of molecules: cadherins, integrins, and members of the immunoglobulin superfamily. Many of these molecules have been shown to be involved in neurite extension, either as a substrate that supports extension, as in the case of L1 or

N-cadherin (Lagenauer and Lemmon, 1987; Chang et al., 1987; Bixby and Zhang, 1990), or as receptors for neurite promoting molecules, as in the case of integrins (Tamkun et al., 1986; Reichardt et al., 1989).

The manner in which Ig superfamily molecules promote neurite extension is unclear. Classically, immunoglobulin superfamily molecules are thought to function by binding homophilically, as demonstrated by monitoring the aggregation of protein-coated beads or transfected cell lines. Recently, heterophilic interactions between Ig superfamily molecules have been identified as well (for review see Brummendorf, 1994). For example, NgCAM, which is expressed on many growing axons, mediates neurite extension *in vitro* through homophilic interactions between NgCAM on the neuronal cell surface and purified NgCAM on the substrate (Lemmon et al., 1989; Felsenfeld et al., 1994). However, recent studies show that cell surface NgCAM participates in heterophilic interactions with both axonin-1 (Kuhn et al., 1991) and F11 (Brummendorf et al., 1993). Furthermore, heterophilic interactions with an unidentified partner may be a significant aspect of NgCAM-dependent neuronal migration in the adult songbird forebrain (Barami et al., 1994). Thus, cell surface NgCAM can act as a receptor for a number of different ligands other than itself. Other homophilic cell adhesion molecules that have now been shown to bind heterophilically include axo-

Address all correspondence to A.P. DeBernardo, Department of Neuroscience, School of Medicine, University of Pennsylvania, 36th Street Service Drive, Stemler Hall Rm. 215, Philadelphia, PA 19104. Tel.: (215) 898-2180; Fax: (215) 573-9050.

nin-1/TAG-1 binding to NgCAM as its cellular receptor (Felsenfeld et al., 1994; Kuhn et al., 1991) and F11/F3 binding to NrCAM as its cellular receptor (Morales et al., 1993).

DM-GRASP, or simply GRASP, is a member of a subfamily within the immunoglobulin superfamily that is distinguished by extracellular domains consisting of two V-type followed by three C2-type Ig domains (Burns et al., 1991). GRASP is also identified in chickens by the monoclonal antibodies SC1 and BEN (Tanaka et al., 1991; Pourquie et al., 1992). The protein is expressed in a temporally dynamic and spatially restricted pattern within the developing nervous system. For example, in the spinal cord it is expressed by sensory projections in the dorsal funiculus but not by any axonal tracts in the lateral or ventral funiculi. It is also expressed by motorneurons in the spinal cord and hindbrain. Levels of GRASP expression on neurons are highest during the period of active axonal extension, and disappears from axons after they have reached their targets. Monoclonal antibodies to GRASP have been shown to decrease the extension of sympathetic neurites growing on sympathetic axons (Burns et al., 1991) and purified GRASP supports neurite extension selectively from a subset of neuronal cell types *in vitro* (DeBernardo and Chang, 1995).

Like many other members of the immunoglobulin superfamily, GRASP has been shown in bead aggregation and cell binding assays to bind homophilically (el-Deeb et al., 1992; Tanaka et al., 1991). Homophilic GRASP-GRASP interactions between the growing neuron and a GRASP substrate appear to be an essential component of GRASP's activity in stimulating neurite extension (DeBernardo and Chang, 1995). Recently, the human homologue of GRASP, ALCAM, was isolated from leukocytes as a CD6-binding protein (Bowen et al., 1995). Interestingly, other Ig superfamily proteins that belong to the same structural subfamily as GRASP have been reported to act heterophilically as well as homophilically. Gicerin, a novel 82-kD chick protein that belongs to the GRASP subfamily, was identified as the cellular receptor for NOF, a large (700 kD) extracellular matrix protein that promotes neurite extension and cell migration (Taira et al., 1994). IrreC-rst, a *Drosophila* protein that plays a role in patterning axonal projections within the adult optic lobes and also in regulating cell death during earlier steps in ommatidial development, is also part of the GRASP subfamily and may act heterophilically as well as homophilically (Ramos et al., 1993; Boschert et al., 1990; Schneider et al., 1995). These findings suggest that heterophilic interactions between GRASP and other molecules may also be important to GRASP function.

Using an affinity chromatography approach, we have isolated several putative heterophilic ligands for GRASP. Based on molecular weight and immunoreactivity, one of the proteins that binds to and elutes from the GRASP column is NgCAM. Neurite outgrowth assays suggest that GRASP-NgCAM interactions are functionally involved in extension of sympathetic neurons on immunopurified NgCAM. Of all the heterophilic interactions that have been described for NgCAM, the interaction with GRASP is the first to affect neuronal responsiveness to NgCAM, as measured by neurite outgrowth.

Materials and Methods

Preparation of Recombinant GRASP

A full-length GRASP cDNA clone was subcloned into the pVL1393 baculovirus transfer vector (Pharmingen, San Diego, CA). Sf21 insect cells were cotransfected with the recombinant plasmid and linearized baculovirus DNA (Pharmingen) according to the manufacturer's instructions. Recombinant virus was collected from the supernatant over the cells. After two more rounds of infection, recombinant virus was routinely used at a 10^{-3} dilution to infect High Five insect cells (Invitrogen, San Diego, CA) for production of recombinant GRASP protein.

High five insect cells were maintained in Ex-Cell 400 medium. For infection, the medium was replaced with diluted virus for 1 h, and the infected cells were then incubated in Ex-Cell 400 for another 5 d. The cells were harvested, washed, and extracted with 1% Triton X-100 according to established procedures (DeBernardo and Chang, 1995). Recombinant GRASP was purified using an immunoaffinity column prepared with the monoclonal antibody DM1. Approximately 100 μ g of purified protein could be obtained from a T-150 cm² flask of confluent cells.

Column Preparation and Protein Purification

DM1 (binds to GRASP; Burns et al., 1991) and F4C (binds to NgCAM) monoclonal antibodies were purified from ascites fluid over a protein A-Sepharose column. 3–5 mg purified antibody was coupled to 1 ml swollen CNBr-activated Sepharose according to the manufacturer's instructions (Pharmacia LKB Biotechnology, Piscataway, NJ). Using the monoclonal antibody affinity columns, native GRASP and NgCAM were purified from E15 chick brains as previously described (DeBernardo and Chang, 1995).

GRASP-Sepharose columns were prepared by coupling 0.5–1 mg of purified recombinant GRASP to 1 ml swollen CNBr-activated Sepharose according to the manufacturer's instructions. All solutions contained 0.1% Triton X-100 and the temperature was maintained at 4°C.

Isolation of GRASP-binding Proteins

A membrane preparation from fresh E15 chick brains was prepared as previously described (Burns et al., 1991). Membranes were extracted with 1% Triton X-100/PBS, 2 mM EDTA at a ratio of 1 ml crude membranes to 20 ml extraction buffer. The solution was homogenized with six strokes of a Dounce homogenizer and clarified by centrifugation at 100,000 g for 60 min.

GRASP-Sepharose columns were preequilibrated with 30–40 column volumes of 0.1% Triton X-100/PBS. The clarified extract from 75–100 chick brains was passed over the GRASP-Sepharose column at 15–20 ml/h at 4°C. After the extracted proteins were loaded, the column was washed with 40–50 column volumes of preequilibration buffer and eluted with 100 mM diethylamine, 0.1% Triton X-100, pH 10.2. 500 μ l fractions were immediately neutralized with 10 μ l 1 M HEPES, pH 3.0. Fractions were dialyzed against two changes of PBS for 48 h immediately after purification and stored at 4°C. All procedures were carried out at 4°C.

To eliminate known proteins from the eluate, two precolumns were added to the purification scheme. The clarified extract from 75–100 chick brains was passed over a DM1 antibody column followed by an F4C antibody column before passing through the GRASP-Sepharose column (see schematic in Fig. 3). The extract was loaded at 15–20 ml/h at 4°C. After the extracted proteins were loaded, the columns were separated and individually washed with 40–50 column volumes of preequilibration buffer. All columns were eluted with 100 mM diethylamine, 0.1% Triton X-100, pH 10.2. 500 μ l fractions were immediately neutralized with 10 μ l 1 M HEPES, pH 3.0. Fractions were dialyzed against two changes of PBS for 48 h immediately after purification and stored at 4°C. All procedures were carried out at 4°C.

Protein concentration was calculated with the Bradford protein assay (BioRad Labs, Hercules, CA) according to the manufacturer's instructions.

Electrophoresis and Western Blotting

Electrophoresis on 7.5% polyacrylamide gels and transfer of proteins to nitrocellulose was performed according to standard protocols. High range molecular mass markers were purchased from BioRad Labs. Gels were

silver stained according to Anson (1985). Immunoblots were blocked with 3% BSA, 2.2% polyvinylpyrrolidone (30/60/300), and 0.01% Tween 20 in PBS before overnight incubation at 4°C with either monoclonal antibody supernatant containing 0.01% Tween 20 (in the case of GRASP, G4, F3/F11, neurofascin, NrCAM/BRAVO, and axonin) or antibody supernatant diluted 1:5 in blocker solution (in the case of NCAM and chicken B_1 integrin). After washing in PBS, immunoblots were incubated at room temperature for 2 h with the appropriate alkaline phosphatase-conjugated secondary antibody (Jackson Laboratories, West Grove, PA). The enzyme label was developed with 0.35 mg/ml nitroblue tetrazolium and 45 μ g/ml 5-bromo-4-chloro-indolyl phosphate in 100 mM CO_3 buffer (pH 10.2).

Cell Culture

10-mm-round glass coverslips (EM Corp) were cleaned and silanized as previously described (DeBernardo and Chang, 1995). Substrate proteins, in 0.1% Triton X-100, were adsorbed directly to the surface of the coverslips for 1 h at 37°C. The coverslips were then blocked with 500 μ g/ml BSA for 1 h at 25°C. Coverslips were then washed extensively in Hanks buffered saline solution (HBSS) and placed in 48-well plates (Costar Corp., Cambridge, MA). For explants, E7 dorsal root ganglia and E6 retinas were aseptically dissected from chick embryos. The neural tissue was cut into small pieces and dropped in the center of each coverslip. Explant cultures were grown in a defined F12 media supplemented with 2 mM L-glutamine, 6 g/L-glucose, 100 U/ml penicillin and 100 ng/ml streptomycin, 10 ng/ml NGF (Collaborative Research, Inc., Waltham, MA), and N2 hormones as described by Bottenstein et al. (1980). For dissociated cultures, sympathetic ganglia were aseptically dissected from E9 chick embryos in HBSS. The collected tissue was incubated in 1 ml of 0.05% trypsin solution containing 0.02% EDTA at 25°C for 20 min. The trypsin solution was then replaced with 1 ml of serum-containing medium, and the tissue was triturated through a fire polished Pasteur pipette until a single cell solution was obtained. Dissociated cells were pelleted by centrifugation at 1,000 rpm for 10 min. The pellet was resuspended in F12 media supplemented with 5% FCS, 5% chick serum, 2 mM L-glutamine, 6 g/L-glucose, 100 U/ml penicillin and 100 ng/ml streptomycin, 10 ng/ml NGF, and N2 hormones as described by Bottenstein et al. (1980). Cells were plated at a density of 1,000 cells per coverslip.

Data Analysis and Statistics

Cultures were fixed in 4% paraformaldehyde containing 10% sucrose in PBS. Coverslips were transferred out of 48-well plates into Petriperm (Bachofen GMBH) petri dishes, and viewed on a Zeiss inverted microscope with a 40 \times neofluor lens. The distance between the center of the cell body and the tip of the longest neurite was measured with the aid of a microcomputer (Apricot) equipped with a mouse and a measurement program written by J.A. Raper (University of Pennsylvania, Philadelphia, PA). Neurites were counted only if they (a) were longer than one cell body in length (b) did not collide with other neurons and (c) did not extend from a clump of cells. The measurement recorded was a straight line between the cell body and the tip of the neurite, and thus tend to underestimate the actual length of curved neurites. 100–150 neurites were counted across three coverslips in each experimental condition in each experiment. The non-parametric Wilcoxon rank test was used to determine whether antibody treated and control cultures were statistically different. We considered a one-tailed P value of <0.05 to be significant.

Results

Purification of GRASP-binding Proteins Using a GRASP-Sepharose Affinity Column

Triton X-100 extracts of E15 chick brain membrane proteins were loaded onto a GRASP-Sepharose affinity column, after which the column was washed extensively and then eluted at pH 10.5. The elution profile from six independent columns made from six separate purifications of recombinant GRASP consistently contained 11 major bands. The pattern on silver-stained gels is shown in Fig. 1 A, lane 1 and consists of a faint band at 210 kD, more

prominent bands at 130, 115, and 100 kD, a triplet of bands running between the 97- and 66-kD marker bands, a faint band at 66 kD followed by a stronger band at 60 kD, a second faint band at 48 kD, and a final strong band at 45 kD.

Western Blot Analysis of GRASP-binding Proteins

The possibility that known neural cell surface molecules were present in the GRASP-binding fractions was analyzed on immunoblots. The 100-kD band was shown on Western blots to be immunoreactive with GRASP monoclonal antibodies (Fig. 1 A, lane 2). This is consistent with previous reports that GRASP binds homophilically (el-Deeb et al., 1992; Tanaka et al., 1991). Monoclonal antibodies to NgCAM consistently identified a band at 130 kD (Fig. 1 A, lane 3) in immunoblots of proteins eluted from four separate GRASP columns. In matched experiments, immunoblots with antibodies to other neural immunoglobulin superfamily proteins including NCAM, axonin, neurofascin, F11/F3, and NrCAM/BRAVO were consistently negative as were immunoblots with antibodies against integrin B_1 (SP2/OAG14 obtained from the Developmental Hybridoma Bank) (selected examples shown in Fig. 1 A, lanes 4–7). The 200-kD band visible in lanes 2, 6, and 7 was seen consistently in immunoblots incubated with hybridoma supernatants and is probably nonspecific. Immunoblots were also performed with antibodies to cytoskeletal-associated proteins such as talin, vinculin, and α -actinin and were negative (data not shown).

To deplete the known proteins GRASP and NgCAM from the brain extract, monoclonal antibody columns that bind GRASP and NgCAM were added as precolumns in front of the GRASP-Sepharose column (Fig. 1 B). The addition of the monoclonal antibody columns reduced the total amount of protein purified through the GRASP affinity column, but did not alter the banding pattern significantly for bands above 70 kD except for the elimination of the 100-kD band (compare Fig. 1 C, lanes 1–2). With the addition of the two monoclonal antibody precolumns, GRASP protein was effectively removed from the eluate, as shown by Western blot analysis (Fig. 1 C, lane 3). However, the presence of a G4 monoclonal antibody precolumn was not sufficient to remove the 130-kD NgCAM immunoreactive band from the eluate (Fig. 1 C, lane 4). The three column purification strategy was used on all subsequent purifications.

Neurite Extension on GRASP-binding Proteins

A potential heterophilic interaction that may be hypothesized for GRASP is that it participates in a ligand-receptor complex. If GRASP acts as a receptor for a neurite outgrowth promoting molecule, then the proteins eluting off the GRASP-Sepharose column might contain the ligand. Therefore, GRASP-binding proteins purified by the procedure outlined in Fig. 1 B were tested for neurite stimulating activity. GRASP-binding proteins support neurite extension from both dorsal root ganglion (Fig. 2 C) and retinal ganglion explants (Fig. 2 D) when applied to coverslips at concentrations of 0.5–1 μ g/ml total protein content. In contrast, immunopurified GRASP applied to coverslips at a concentration of 40 μ g/ml supports neurite

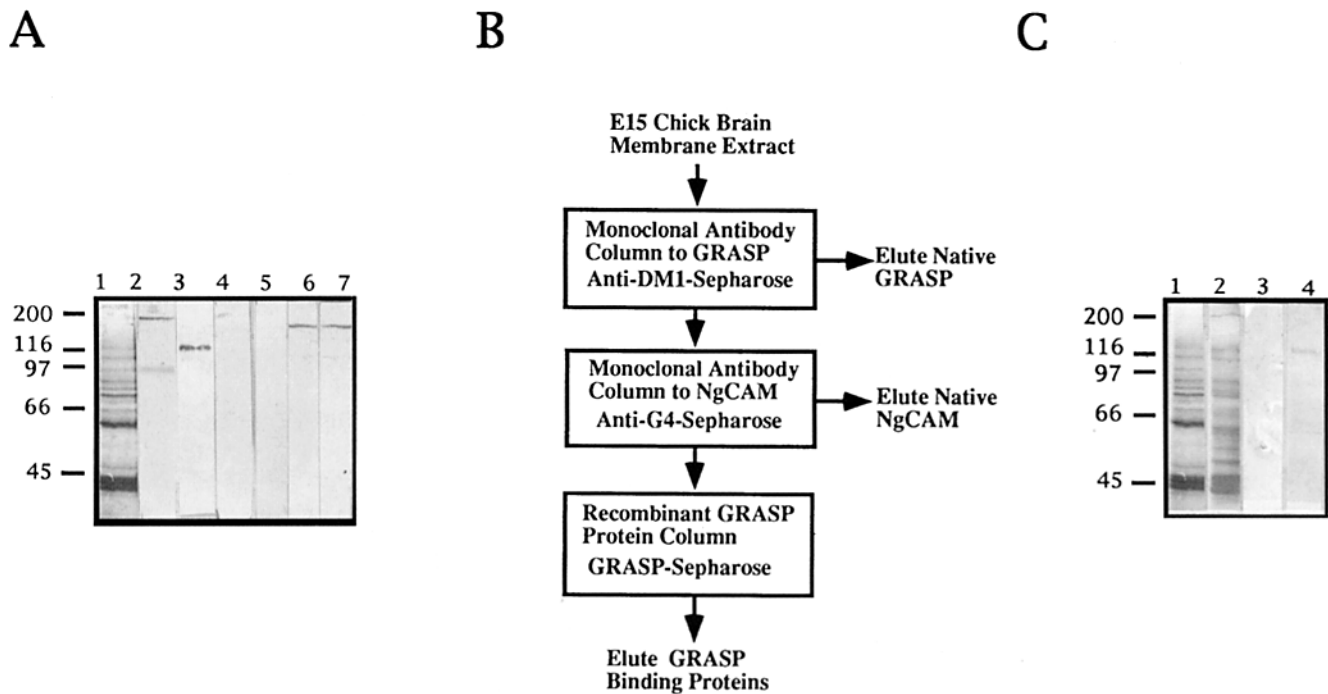


Figure 1. (A) Total protein from the fourth 500 μ l fraction eluted from a GRASP-Sepharose column was run on a 7.5% polyacrylamide gel under nonreducing conditions and transferred to nitrocellulose. This fraction contained all bands visible across the elution. A duplicate gel was silver stained (lane 1). The resulting immunoblot was cut into strips and incubated with primary and secondary antibodies as described in Materials and Methods. Immunoblots were analyzed using (lane 2) anti-GRASP, (lane 3) anti-NgCAM, (lane 4) anti-axonin, (lane 5) anti-NCAM, (lane 6) anti-F11, and (lane 7) anti-neurofascin. The 200-kD band visible in lanes 2, 6, and 7 was seen inconsistently in immunoblots incubated with hybridoma supernatants and is thought to be nonspecific. (B) Schematic of purification scheme. (C) Comparison of banding pattern and immunoblot analysis after the addition of precolumns. (Lane 1) Silver stain gel of a single fraction eluted from a GRASP-Sepharose column loaded with E 15 brain membrane extract. This fraction contained all bands visible at any point in the elution. (Lane 2) Silver-stained gel of a single fraction eluted from a GRASP-Sepharose column loaded with E15 brain membrane extract depleted of GRASP and NgCAM by the addition of precolumns. This fraction contains all bands visible across the elution. (Lane 3) Anti-GRASP immunoblot of the fraction shown in lane 2. (Lane 4) Anti-NgCAM immunoblot of the fraction shown in lane 2.

extension only from dorsal root ganglion explants (Fig. 2 A), but not from retinal ganglion explants (Fig. 2 B).

GRASP was eliminated from the GRASP-Sepharose eluate by the addition of a DM1 monoclonal antibody pre-column to the purification scheme. Further incubation of GRASP-binding proteins with function blocking antibodies to GRASP did not affect their ability to stimulate neurite extension (Fig. 3). Hence, it is highly unlikely that GRASP is responsible for the neurite stimulating activity present in GRASP-binding proteins. NgCAM could not be completely eliminated from the GRASP-Sepharose eluate by the addition of a G4 monoclonal antibody pre-column. Purified NgCAM supports neurite extension from both retinal explants (see Lemmon et al., 1992) and dorsal root ganglion explants (Fig. 4 A). Function blocking Fab fragments of polyclonal antibodies against NgCAM completely neutralized the ability of purified NgCAM to stimulate neurite outgrowth from dorsal root ganglion explants (Fig. 4, A and B). In matched coverslips, preincubation of GRASP-binding proteins with polyclonal Fab fragments against NgCAM did not eliminate their neurite promoting activity (Fig. 4, C and D). Thus, neither GRASP nor NgCAM accounts for all the neurite stimulating activity that binds to the GRASP-Sepharose column.

Effects of Anti-GRASP on NgCAM Stimulated Neurite Extension

Potential functional interactions between GRASP and NgCAM were investigated further through antibody perturbation experiments. The monoclonal antibody DM2 has been previously shown to decrease neurite extension of sympathetic neurons growing on a substrate of sympathetic axons by \sim 20% (Burns et al., 1991). On Western blots, DM2 identifies immunopurified GRASP as a single band at 100 kD; it also identifies a single 100-kD band on Western blots of E14 chick brain membrane preparations (Burns et al., 1991). This strongly suggests that DM2 binds specifically to GRASP. Consistent with this, preincubation of GRASP-coated coverslips with DM2 eliminates their neurite outgrowth promoting activity (Burns et al., 1991; DeBernardo and Chang, 1995).

The monoclonal antibody DM2 was added to cultures of sympathetic neurons extending neurites on a purified NgCAM substrate. If GRASP and NgCAM interact and this interaction is important for NgCAM stimulated neurite extension, blocking GRASP function on the neurons would be expected to result in a change in neurite extension on NgCAM substrates. The average neurite length of neurites extending on NgCAM in the presence of the

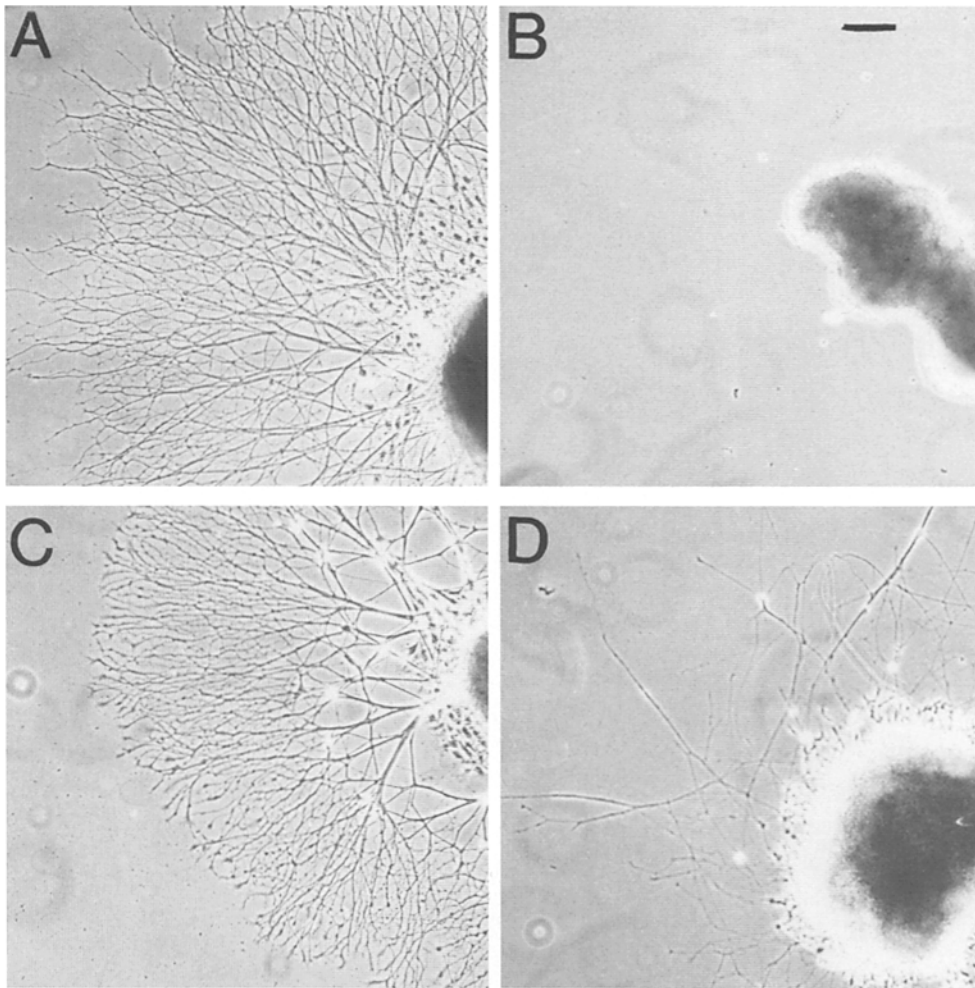


Figure 2. Neurite outgrowth from dorsal root ganglion explant cultures on either (A) immunopurified GRASP or (C) GRASP-binding proteins is compared to retinal outgrowth on either (B) GRASP or (D) GRASP-binding proteins. Cultures were grown for 36 h in serum-free media. Bar, 100 μm .

monoclonal antibody DM2 was significantly shorter than the average length of neurites extending on NgCAM in the absence of the antibody. Data from a representative experiment is shown in Fig. 5 A. Neurite lengths in the presence of anti-DM2 were reduced by more than 20% as compared to neurite lengths in matched control cultures. The lengths of neurites extending on laminin in the presence or absence of DM2 did not differ significantly (Fig. 5 C), suggesting that the effect seen on an NgCAM substrate is not due to general toxicity of the antibody or to other nonspecific effects. The results from individual experiments are summarized in Table I.

A second monoclonal antibody, DM1, was tested for its ability to perturb outgrowth on NgCAM. DM1 binds to GRASP on immunoblots but does not decrease extension of sympathetic axons growing on a substrate of sympathetic axons (Burns et al., 1991). Interestingly, addition of DM1 antibodies did not significantly alter neurite lengths on NgCAM (Fig. 5 B) or on laminin (Fig. 5 D). The results from individual experiments are summarized in Table I.

Discussion

Many cell adhesion molecules have been shown to bind homophilically, as is the case for cadherins (Takeichi, 1990), and for most neural immunoglobulin superfamily

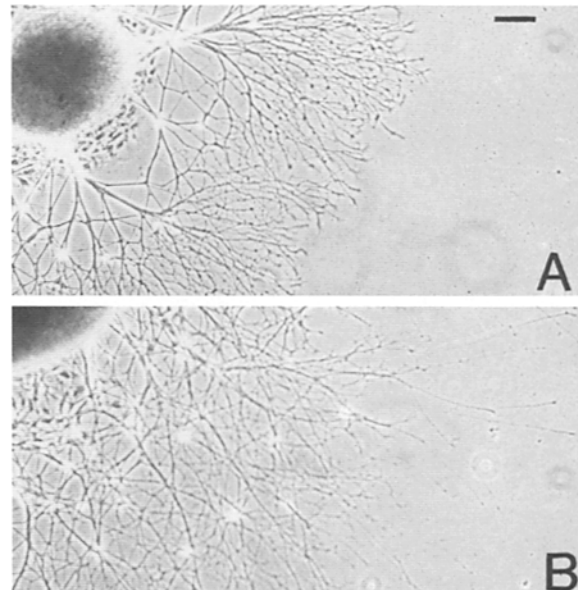


Figure 3. Neurite outgrowth from dorsal root ganglion explant cultures on coverslips (A) coated with GRASP-binding proteins or (B) coated with GRASP-binding proteins and subsequently blocked with anti-DM2 before the addition of explant. Cultures were grown for 18 h in serum-free media. Bar, 100 μm .

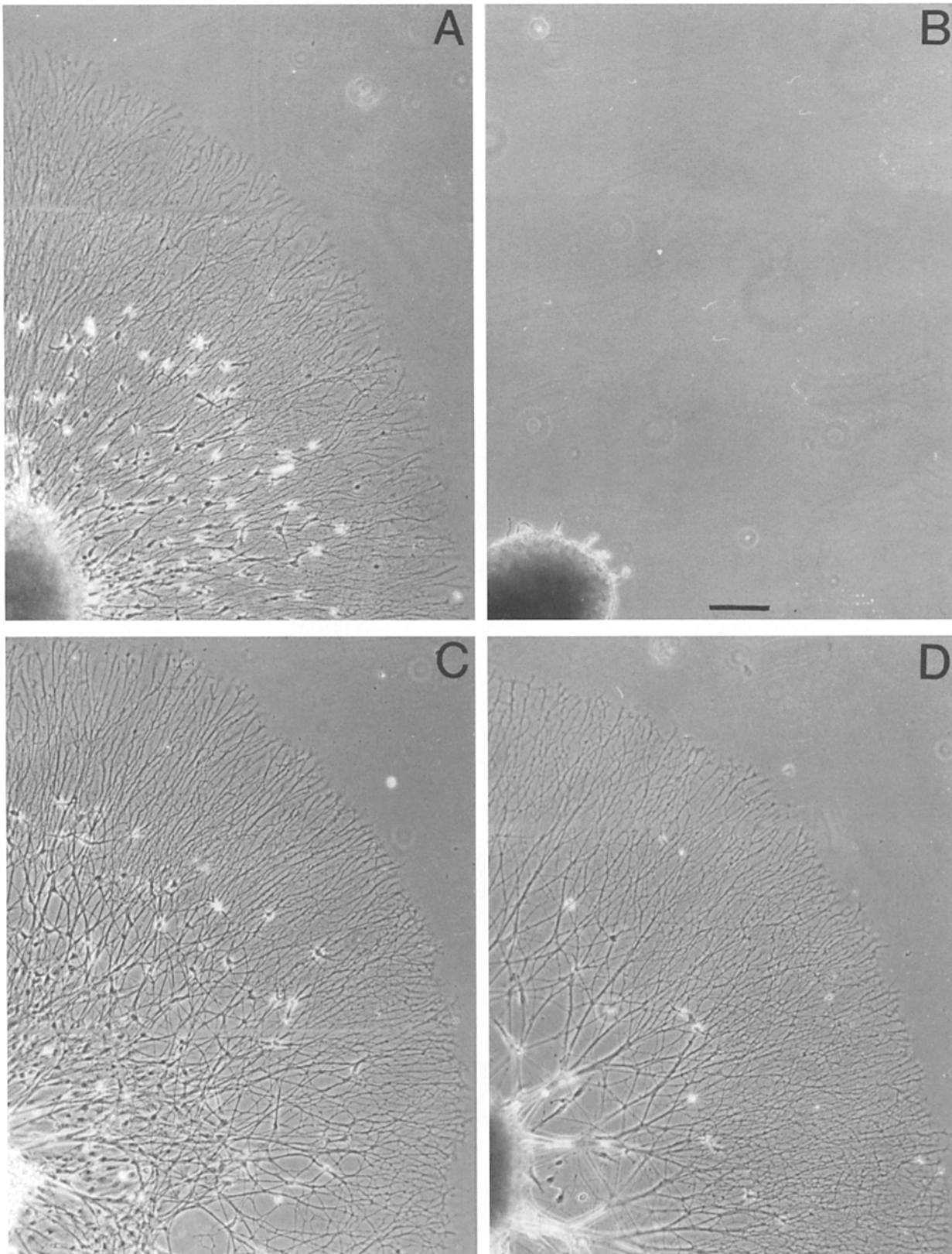


Figure 4. Neurite outgrowth from dorsal root ganglion explant cultures on (A) NgCAM-coated coverslips and (B) NgCAM-coated coverslips preincubated with function blocking polyclonal Fab fragments to NgCAM. Neurite outgrowth from dorsal root ganglion explant cultures on (C) GRASP-binding proteins coated coverslips and (D) GRASP-binding proteins coated coverslips preincubated with function blocking polyclonal Fab fragments to NgCAM. Cultures were grown for 26 h in serum-free media. Bar, 100 μm .

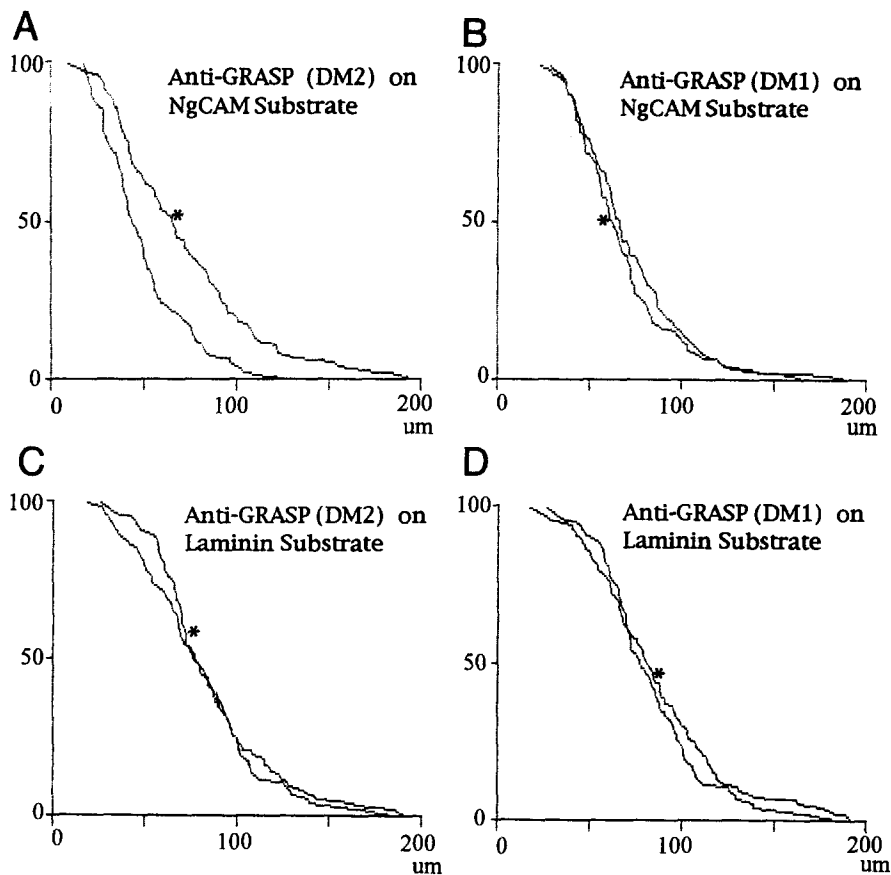


Figure 5. Distribution of neurite lengths in the presence or absence of antibody. Percentage of neurons with neurites (vertical axes) longer than a given length (horizontal axes). The distribution of lengths on an NgCAM substrate in the presence and absence (*) of 25 $\mu\text{g/ml}$ (A) anti-DM2, an antibody against GRASP that decreases the extension of sympathetic neurites on axonal substrates or (B) anti-DM1, an antibody against GRASP that has no effect on the extension of sympathetic neurites on axonal substrates. These data were taken from experiments number 6 and number 8, respectively, presented in Table I. The distribution of lengths on a laminin substrate in the presence or absence (*) of 25 $\mu\text{g/ml}$ (C), anti-DM2, or (D) anti-DM1. These data were taken from experiments number 4 and number 5, respectively, presented in Table I. Cultures were grown for 24 h in serum containing media.

proteins (Hoffman et al., 1984; Rathjen, 1991). However, heterophilic interactions mediated by proteins of the immunoglobulin superfamily have been identified, first in interactions between proteins in the immune system (for review see Springer, 1990) and more recently in interactions between proteins in the nervous system (for review see Brummendorf, 1994). Heterophilic interactions open the possibility that cell adhesion molecules can act as receptors or ligands for proteins other than themselves, or that their binding properties can be modulated. As such, heterophilic interactions may contribute in important ways to the physiological function of cell surface proteins.

GRASP belongs to a recently identified subfamily within the Ig superfamily. The members of this subfamily are transmembrane proteins that share a common domain structure in their extracellular region, consisting of two V-type followed by three C2-type Ig loops. The proteins are all heavily glycosylated, with more than 20% of their molecular mass attributed to this posttranslational modification. The family includes GRASP, gicerin, IrreC-rst, MUC-18, and B-CAM. Aside from structural similarities, the members of this family share several features. All members have been suggested to play a role in cell motility, either in tumor metastasis, as is the case for MUC-18 and B-CAM (Shih et al., 1994; Campbell et al., 1994), or in neurite extension, as is the case for GRASP, gicerin, and IrreC-rst (DeBernardo and Chang, 1995; Taira et al., 1994; Schneider et al., 1995). GRASP, gicerin, and IrreC-rst have been shown to interact homophilically in bead or cell aggregation experiments. All members, except for the re-

Table I. Effects of Anti-GRASP Monoclonal Antibodies on Average Neurite Lengths on Immunopurified NgCAM

	N_t/N_c	L_t/L_c	L_t/L_c
		μm	%
NgCAM substrate			
1 [‡]	126/131	33/83	40*
2 [‡]	112/114	46/77	60*
3	70/70	61/92	66*
4	70/70	50/68	73*
5	100/100	60/72	83*
6	100/100	49/72	68*
7 [§]	100/100	68/72	94
8 [§]	100/100	67/72	93
Laminin substrate			
1 [‡]	101/102	105/109	96
2	70/70	91/93	97
3	70/70	68/67	101
4	100/100	82/83	98
5 [§]	100/100	86/83	103

Cultures containing antibody (25 $\mu\text{g/ml}$ except for experiment 1 in which 10 $\mu\text{g/ml}$ was used) or the equivalent volume of carrier solution (F12 media) were grown for ~ 18 h at 37°C, fixed, and neurite lengths were measured on NgCAM or laminin-coated coverslips. All experiments were run with monoclonal antibody DM2 unless otherwise noted. Column one indicates the experiment number. N_t is the number of antibody-treated neurites measured. N_c is the number of untreated control neurites measured. L_t is the average neurite length in the antibody-treated cultures. L_c is the average neurite length in the untreated control cultures.

*Indicates that the result was significant within a 95% confidence interval.

[‡]Indicates that the experiment was run in serum-free media.

[§]Indicates that experiment tested the effect of monoclonal antibody DM1.

cently discovered B-CAM, are suspected or known to have heterophilic binding partners. This is well established for gicerin, which was originally isolated as the cellular receptor for a 700-kD neurite outgrowth factor related to laminin (Taira et al., 1994). IrreC-rst is a *Drosophila* protein isolated through a mutant screen for axonal pathfinding errors. The explanation for mutant phenotypes seen can only partially be explained by homophilic binding (Schneider et al., 1995). Finally, MUC-18 is expressed in 80% of late stage and metastatic melanoma and is thought to function through a heterophilic interaction (Lehmann et al., 1987; Shih et al., 1994).

Using an affinity chromatography approach, we have begun to characterize heterophilic binding partners for GRASP. We biased our purification towards surface molecules by using a crude membrane extract as our starting material for purification. We reasoned that the proteins isolated from this preparation could act as cell surface ligands, if GRASP functions as a receptor, or as receptor molecules, if GRASP itself is a cell surface ligand. However, since full-length recombinant GRASP protein was coupled to the column in random orientations, it is possible that proteins could also interact with intracellular portions of the GRASP molecule. These interactions may contribute to attachment to the cytoskeleton or to signaling. GRASP-Sepharose affinity columns consistently purify a mixture of eleven protein bands visible on silver stained SDS-PAGE from a detergent extract of E15 chick brain membrane proteins. If these proteins are all specific and physiological ligands for GRASP, this would suggest that GRASP participates in a large number of protein interactions. However, it is important to note that single proteins often purify as multiple bands; thus, eleven bands on a silver-stained gel do not necessarily represent eleven individual proteins.

Several lines of evidence suggest that the proteins that bind to the GRASP-Sepharose column do so specifically. The pattern of bands eluting from the column is highly reproducible, occurring over multiple preparations of GRASP protein and multiple preparations of GRASP-Sepharose columns. Monoclonal antibodies coupled to Sepharose and used in the same purification scheme do not bind proteins other than the antigen of interest, suggesting that the Sepharose matrix itself is not responsible for the proteins interacting with the GRASP column. These findings would suggest that the proteins binding to the GRASP-Sepharose column do so reproducibly and specifically; still, the question remains whether specific interactions detected under these experimental conditions can or do occur in a physiological context. Since it is possible that functionally irrelevant binding occurs on the column, we have made an effort to confirm the functional relevance of any protein-protein interactions detected on the GRASP affinity column.

We identified two of the proteins that are in the GRASP-Sepharose eluate. Not surprisingly, GRASP itself purifies on the GRASP-Sepharose column. This serves as a good positive control, since homophilic binding between GRASP molecules is well established. GRASP does not elute during prewashes of the column with elution buffer before loading, eliminating the possibility that GRASP is simply leaching off the Sepharose matrix (DeBernardo, A.P.,

and S. Chang, unpublished observations). The GRASP-Sepharose column also consistently binds to NgCAM. A second, independent assay relying on antibody perturbations suggests that these two cell surface molecules interact with each other. GRASP-binding proteins also contain a potent neurite stimulating activity for both dorsal root sensory neurons and retinal neurons. Neither GRASP nor NgCAM account for all of this neurite stimulating activity, suggesting that GRASP, like the related protein gicerin, binds a potent neurite outgrowth promoting molecule.

It is interesting to note that although GRASP-binding proteins support outgrowth from retinal neurons, purified GRASP itself does not. We have previously demonstrated that GRASP expression on the surface of a cell correlates with its ability to extend on purified GRASP substrates (DeBernardo and Chang, 1995). GRASP expression in neural retina is minimal by immunofluorescence, and, accordingly, retinal explants do not extend processes on purified GRASP substrates. However, since retinal explants extend neurites on GRASP-binding proteins, it may be that, although GRASP is present in the retina at levels too low to support neurite extension on GRASP, those levels are sufficient to respond to GRASP-binding proteins. It is also possible that GRASP interacts with an unidentified molecule that can be bound by multiple cell surface receptors.

The association of NgCAM with GRASP on the column was intriguing. NgCAM has been shown to interact with a number of molecules. It interacts homophilically in bead and cell binding assays (Grumet and Edelman, 1988; Keilhauer et al., 1985; Grumet et al., 1984). NgCAM has also been shown to interact with axonin-1, a GPI-linked Ig superfamily molecule. Neurite extension on an axonin-1 substrate, which is robust, is substantially reduced by addition of antibodies against NgCAM to the culture media (Kuhn et al., 1991; Felsenfeld et al., 1994). NgCAM has also been reported to bind to F11 (Brummendorf et al., 1993), and antibody perturbations seem to indicate the potential for interactions between L1 and NCAM in the mouse (Kadmon et al., 1990*a,b*). Thus, NgCAM participates in an array of heterophilic interactions.

Functional interactions between GRASP and NgCAM were tested by adding monoclonal antibodies against GRASP to cultured sympathetic neurons growing on NgCAM. When neurite lengths were measured, it was clear that antibodies to GRASP resulted in shorter neurites on NgCAM. These data suggest that GRASP and NgCAM interact during neurite extension. Of all the heterophilic interactions that have been described for NgCAM, the interaction with GRASP is unique in that it is the first to affect neuronal responsiveness to NgCAM.

Two simple models of how GRASP-NgCAM interactions might occur are presented in Fig. 6. The most straightforward possibility is that GRASP and NgCAM molecules bind across cell membranes directly to the other molecule. This heterophilic "trans"-binding model is illustrated in Fig. 6 A, and suggests that GRASP or NgCAM can serve as an alternative cellular receptors for each other. For example, blocking the function of GRASP on neurons would reduce the cell's ability to respond to NgCAM, since only NgCAM-NgCAM binding can now take place. The result would be observed as shorter neurites. If this model is cor-

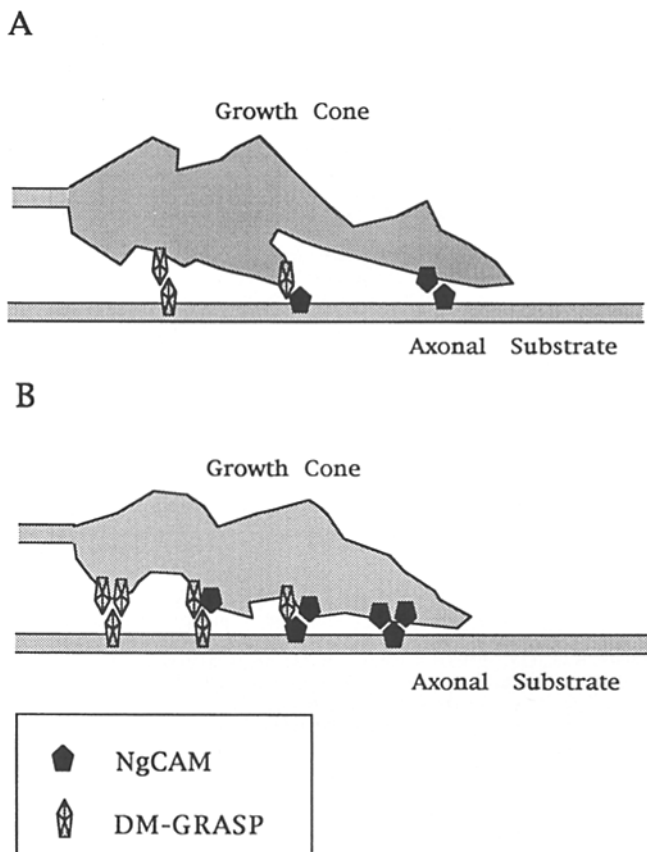


Figure 6. Two possible models of NgCAM-GRASP interaction. (A) GRASP serves as an alternate cellular receptor for NgCAM substrates. (B) GRASP and NgCAM interact on the surface of the cell to form a dimeric receptor complex. See Discussion for details.

rect, it is interesting to note that NgCAM by itself is not sufficient to confer responsiveness to GRASP, since neurons which express high levels of NgCAM but very little GRASP, such as retinal ganglion neurons, are unable to extend neurites on a purified GRASP substrate (DeBernardo and Chang, 1995).

A second possibility is that GRASP-NgCAM interactions could occur within the plane of the membrane in a "cis" configuration to form dimeric receptors. This heterophilic *cis*-binding model is illustrated in Fig. 6 B. In this model, homodimers of NgCAM-NgCAM or GRASP-GRASP, or heterodimers of NgCAM-GRASP, form within the plane of the membrane and associate with GRASP or NgCAM in the substrate. The presence of antibody would disrupt the function of heterodimers, but homodimers would continue to be functional. Thus, a partial reduction in neurite extension would be obtained. Both models are consistent with our results.

The heterophilic *cis*-binding model can be extended to suggest the more general hypothesis that the active receptor form of neural cell adhesion molecules is a dimer or multireceptor complex. Although completely hypothetical for GRASP and other neural IgSF proteins, dimerization is a common motif for other members of the Ig superfamily (Williams and Barclay, 1988). Many Ig superfamily re-

ceptor molecules of the immune system exist as dimers. Examples are the immunoglobulins and the T-cell receptor, which are held together by disulfide linkages, and the MHC class I and class II molecules. The type of dimerization that we propose would be a transient interaction; the neural cell adhesion molecules exist independently on the surface of the cell as well as in association with other neural Ig molecules. Examples of other Ig superfamily molecules known to participate in this type of *cis* interaction include the PDGF receptor, which forms either a homodimer or heterodimer upon binding of PDGF (Bishayee et al., 1989; Eriksson et al., 1992), and the transient heterodimeric interactions between CD8 or CD4 with the T-cell receptor complex. CD8 and CD4 serve as coreceptors for class I and class II MHC molecules, respectively (Bierer et al., 1989). They are present independently on the surface of the cell, but are brought together with the T-cell receptor complex by corecognition of ligand on the opposing cell surface. The main physiological importance of this interaction is in signaling. When either coreceptor is blocked, as much as 100-fold increases of ligand are needed to induce responsiveness (Janeway et al., 1988; Dembic et al., 1987). Synergism in the signaling response initiated by the *cis* interaction of the T-cell receptor with CD8 has been directly demonstrated (Salter et al., 1990).

The recent crystal structure of the N-cadherin amino terminal domain strongly suggests that this domain exists as a dimer, held together by hydrophobic interactions and salt bridges (Shapiro et al., 1995). The single domain in solution also behaves as a dimer rather than a monomer, as measured by techniques such as gel filtration chromatography. The existence of N-cadherin dimers supports our hypothesis that dimers, either homodimers or heterodimers, of cell adhesion molecules are the functionally operational unit within the cell membrane.

While the existence of cell surface dimers of neural cell adhesion molecules remains to be proven, this idea offers increased flexibility for how these proteins function. If cell surface adhesion molecules form dimeric receptors which are interchangeable and fluid within the plane of the membrane, and if various dimeric receptors have different binding affinities and cellular actions, it is possible that growth cones could respond quickly to environmental cues simply by rearranging the composition of dimers on its surface without having to alter expression of any given protein. This is especially attractive considering the distance that the growth cone is away from the cell body and from major sites of protein synthesis.

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