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L1-Mediated Axon Outgrowth Occurs via a Homophilic Binding Mechanism

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Summary

The molecular mechanism by which the L1 cell adhesion molecule mediates neurite outgrowth has been examined. Purified L1 from mouse and L1 from chick brain were attached to nitrocellulose dishes. Both chick and mouse neurons were able to adhere to purified mouse L1 and chick L1. Both molecules promoted neurite extension from chick and mouse neurons. Addition of Fabs specific for chick L1 to the cultures inhibited chick neurite outgrowth on both mouse L1 and chick L1. These findings suggest that L1-like molecules support neurite outgrowth via a "homophilic" binding mechanism.

Introduction

In the experiment reported here we have studied mouse L1 (Rathjen and Schachner, 1984) and its chicken analog, the 8D9 antigen (Lemmon and McLoon, 1986), which will be referred to as chick L1. These molecules are also related to NILE, which is found on PC12 cells (Bock et al., 1985; Sajovic et al., 1986), and Ng-CAM (Friedlander et al., 1986), which is found in chick brain. The mechanism by which L1 and the related chicken molecule, Ng-CAM, act has been investigated using cells (Keilhauer et al., 1985; Grumet et al., 1984b), membrane vesicles (Grumet and Edelman, 1984), and protein-coated Covaspheres (Grumet and Edelman, 1988). Thus far the evidence suggests that the interaction between adjacent neurons is homophilic, while any interaction between neurons and glia must be heterophilic because these glia do not express Ng-CAM (Grumet and Edelman, 1988). These previous studies have used rapid collection-type assays to examine this interaction and have not been able to investigate how these molecules function in a more complicated phenomenon, axon outgrowth. This class of molecule is concentrated on growing axons, it decreases in concentration in most mature axon fascicles (Daniloff et al., 1986; Nieke and Schachner, 1985), and it has been implicated in neurite outgrowth on neurites (Lagenaur and Lemmon, 1987; Chang et al., 1987). We therefore designed an experimental system to investigate the molecular basis by which this type of molecule functions in axon outgrowth.

At present, one of the most common ways of studying cell–cell recognition is to perform cell–cell adhesion or membrane–vesicle adhesion assays. In these experiments, blocking antibodies are used to investigate the role of specific molecules during adhesion. Unfortunately, interpretations of these experiments are complicated by the fact that cells usually have multiple receptors which can mediate adhesion processes. As a result, blocking the function of a given receptor may result in only a negligible effect on cell–cell adhesion. This problem is somewhat obviated in studies of cell–substrate adhesion, in which one can provide a purified cell attachment substrate, such as laminin or fibronectin. A second problem that complicates studies of cell recognition in the nervous system is that some cell adhesion molecules may be concentrated on dendrites or axons rather than cell somata. As a result, cell adhesion assays that use dissociated cells which have had processes sheared off can give false-negative results due to absence of the relevant molecules from the cell soma. Finally, the link between cell adhesion experiments and complicated phenomena like axon outgrowth and guidance or synaptogenesis is tenuous.

In an attempt to overcome these problems, we have used a system of attaching purified proteins to cell culture dishes coated with nitrocellulose (Lagenaur and Lemmon, 1987). Our test system allows us to assess directly whether neurite–substrate interactions can occur via a homophilic or a heterophilic binding mechanism. A homophilic mechanism is one in which a membrane receptor on one cell surface binds to the same type of receptor on an adjacent cell or surface. The binding of N-CAM to N-CAM (Rutishauser et al., 1982) is an example of a homophilic interaction, although the simplicity of this characterization has been questioned (Cole and Glaser, 1986). A heterophilic mechanism is one in which a membrane receptor on one cell binds to a different type of molecule on an adjacent cell surface or substrate, e.g., integrin binding to laminin.

Results

The experiment described below uses a cross-species design suggested by the work of Lund et al. (1985) and Covalt et al. (1987) and depends on the availability of L1 purified from mouse brain and L1 purified from chick brain (8D9 antigen; Lemmon and McLoon, 1986) and a polyclonal antibody specific for chick L1 that does not bind to mouse L1. This antibody is used to block the function of the chick L1. To produce the purified L1 and the polyclonal antibody, it was first necessary for us to generate a new monoclonal antibody (MAb), 74-5H7, that binds to mouse L1 because MAb 8D9 binds only to chick L1. Figure 1 shows a Western blot in which mouse L1 (provided by F. Rathjen) was probed with MAb 74-5H7. This blot demonstrates that MAb 74-5H7 binds to the 190 and 85 kd components of mouse L1.

Mouse L1 was immunoaffinity-purified from mouse

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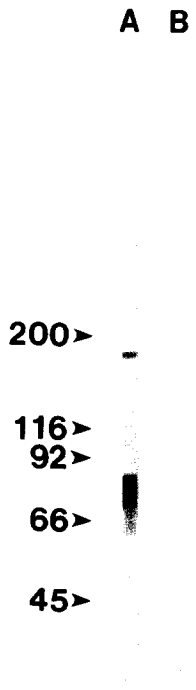

Figure 1. Biochemical Characterization of MAb 74-5H7 Antigen

Western blot of mouse L1 using MAb 74-5H7 (A) and no primary antibody (B). MAb 74-5H7 recognized the 190 and 85 kd components of mouse L1. Molecular weight standards in kilodaltons are indicated at left.

brain using MAb 74-5H7. Some of the mouse L1 was reserved for cell culture experiments. Mouse L1 (100 μg) was linked onto 1 ml of Affigel beads and used to preadsorb the previously produced rabbit anti-chick L1 (RaCL1) Fabs. These preadsorbed Fabs showed no cross-reactivity with mouse membrane proteins using a dot-blot assay (data not shown).

We have previously shown that chick L1 is a potent substrate for neurite outgrowth for both chick and mouse neurons (Lagenaur and Lemmon, 1987) and that this activity is independent of the means of attaching the chick L1 to the dish (Farr et al., submitted). If L1 acts via a homophilic binding mechanism, then RaCL1 Fabs should prevent binding of chick neurite outgrowth on either chick L1 or mouse L1 because the antibody would cover the only type of cell surface receptor for the substrate. If L1 acts via a heterophilic mechanism, then, in the presence of RaCL1 Fabs, chick neurites would not grow on chick L1 but would still be capable of growing on mouse L1. In this case, the function of the chick L1 on the substrate and cell surface would be compromised but the heterophilic binding partner and the mouse L1 would be unaffected. If mouse neurons are used instead of chick neurons, then the RaCL1 Fabs would be expected to prevent neurite outgrowth on chick L1 but not on mouse L1, regardless of whether this molecule acts via a homophilic or heterophilic mechanism. This is an essential control because it demonstrates the species specificity of the Fabs, i.e., they do not bind to mouse L1. Another control is to show that RaCL1 Fabs do not affect the ability of chick neurites to grow on laminin, thereby demonstrating that the Fabs do not generally inhibit neurite growth but are specific for interactions dependent on chick L1 function.

The following experiment was therefore performed. Four dishes were prepared, each having separate spots of chick L1, mouse L1, and laminin. Chick neural cells were plated in two dishes, and mouse neural cells were plated in the other two. One dish containing mouse cells and one containing chick cells had Fabs added at the time of plating. The next day, the following results were observed. In the two control dishes to which no Fabs were added, all three substrates, chick L1, mouse L1, and laminin, produced substantial neurite outgrowth from both chick and mouse neurons (Figures 3A–3C; 3G–3I). In the Fab-treated dish with chick neurons, there were no neurons with neurites on either the chick L1 or the mouse L1 (Figures 3D and 3E), but there were neurons with neurites on laminin (Figure 3F). The number of cells attached to these two molecules was reduced to background levels. In the Fab-treated dish with mouse neurons, there were no neurons with neurites on chick L1 (Figure 3J) and the number of attached neurons was reduced, but neurons on mouse L1 and laminin (Figures 3K and 3L) did have neurites. The mean values of the neurite lengths are given in Table 1, and the distribution of neurite lengths are shown in Figure 4.

Discussion

The mechanisms responsible for cell–cell adhesion mediated by individual adhesion molecules are under

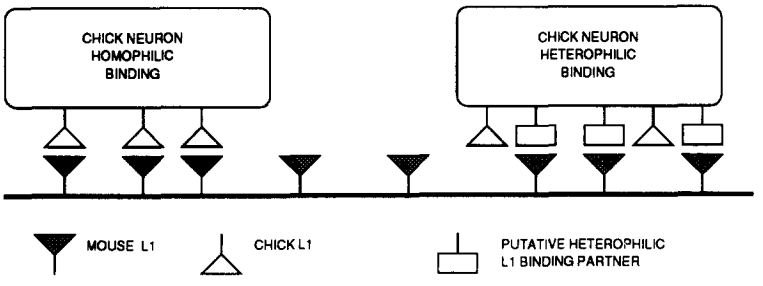

Figure 2. Models of L1 Binding Mechanisms

Illustration of possible binding mechanisms of chick neurons attaching to purified mouse L1 linked to a tissue culture dish. If the chick neurons bind via a homophilic mechanism (shown on left), then inactivation of the chick L1 would block binding. However, if the mouse L1 bound to a heterophilic binding partner (shown on right), then inactivation of chick L1 would not inhibit binding.

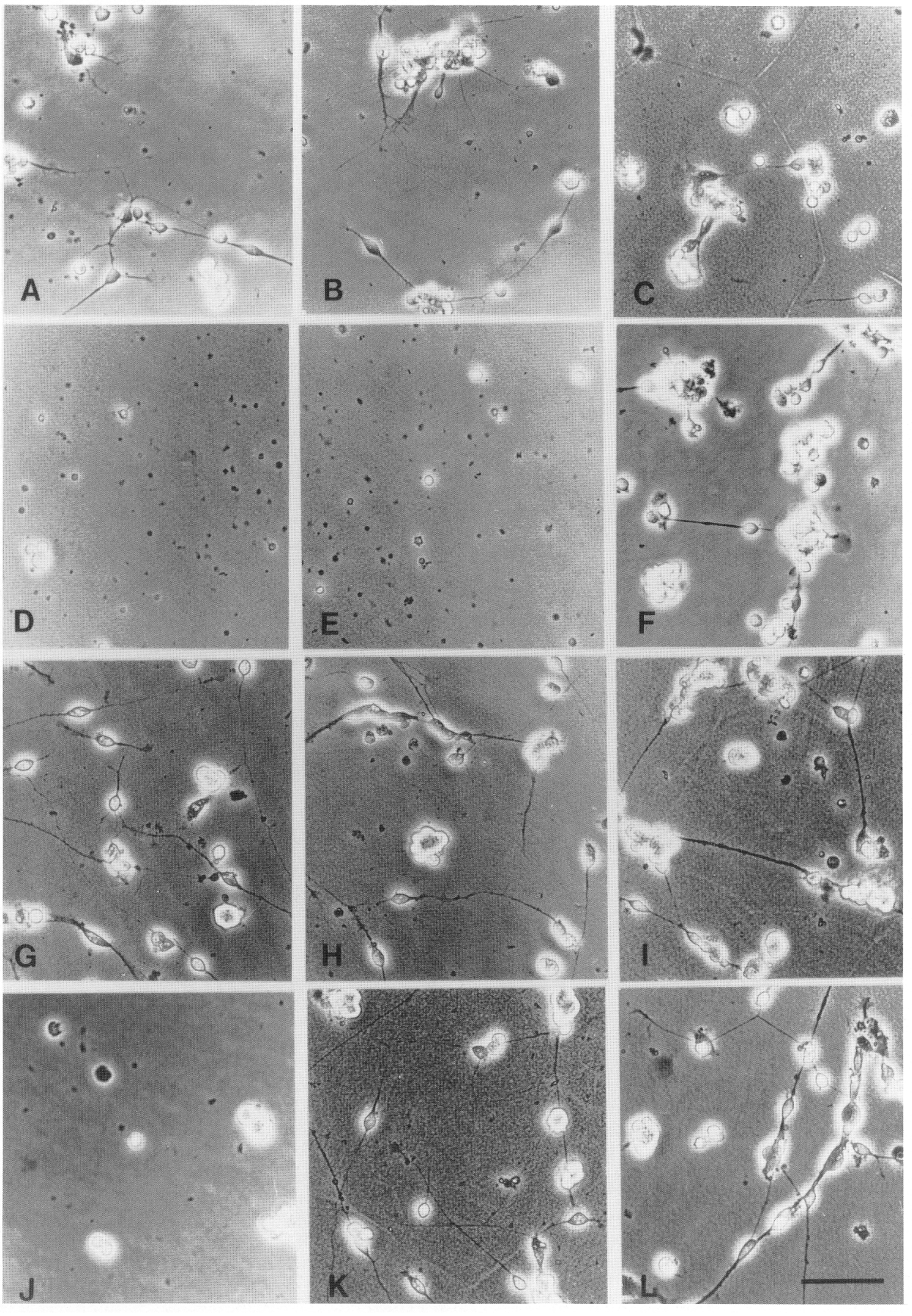

Figure 3. Effects of RaCL1 on Neurons in Culture

Phase photographs of chick neurons (A–F) and mouse neurons (G–L) plated on chick L1 (A, D, G, and J), mouse L1 (B, E, H, and K), or laminin (C, F, I, and L) in the presence (D, E, and F; J, K, and L) or absence (A, B, and C; G, H, and I) of rabbit anti-chick L1 Fabs.

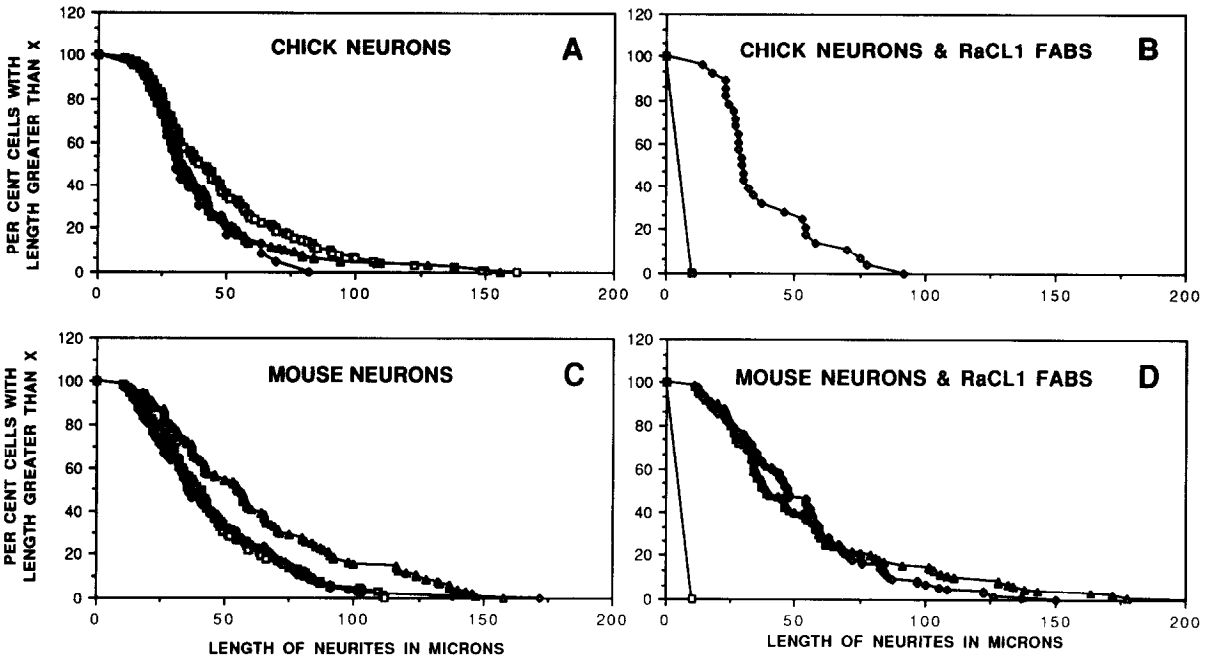

Figure 4. Effects of RaCL1 on Neurite Length

Distribution of neurite lengths of chick neurons (A and B) or mouse neurons (C and D) in the presence (B and D) or absence (A and C) of rabbit anti-chick L1 Fabs. Different substrates were chick L1 (squares), mouse L1 (triangles), and laminin (diamonds). Distribution was plotted as percentage of neurons with neurites (y axis) longer than a given length (x axis).

intense investigation. In the case of the molecules variously identified as NILE, L1, Ng-CAM, 8D9 antigen, or G4, evidence for heterophilic as well as homophilic binding has been presented by several laboratories. Additionally, L1-like proteins have been associated with both neuron–neuron and neuron–glia adhesion. Several studies have shown that L1 and related molecules play an important role in fasciculation between axons (Stallcup and Beasley, 1985; Rathjen et al., 1987; Friedlander et al., 1986). In the original description of Ng-CAM, het-

Table 1. Neurite Growth on Different Substrates

Substrate	% Single Neurons with Neurites	Neurite Length[a]	Total Neurons per mm^2
Chick Neurons			
Laminin	6	37 ± 4	329
Chick L1	48	49 ± 3	204
Mouse L1	49	41 ± 3	246
Uncoated	0	0	113
Chick Neurons + RaCl1 Fabs			
Laminin	8	38 ± 4	380
Chick L1	0	0	68
Mouse L1	0	0	101
Uncoated	0	0	81
Mouse Neurons			
Laminin	60	44 ± 3	339
Chick L1	60	44 ± 3	497
Mouse L1	65	61 ± 4	315
Uncoated	0	0	177
Mouse Neurons + RaCl1 Fabs			
Laminin	41	51 ± 3	422
Chick L1	0	0	85
Mouse L1	64	55 ± 4	317
Uncoated	0	0	90

[a] Given in micrometers; mean ± SEM.

erophilic binding was assumed, since Ng-CAM was detected on neuronal cells and mediated binding to astroglia, which lack Ng-CAM (Grumet et al., 1984a). In contrast, studies of L1 have provided evidence for the involvement of L1 in the adhesion of neurons to neurons or neurons to Schwann cells (Bixby et al., 1988; Seilheimer and Schachner, 1988) but not to astroglia (Keilhauer et al., 1985). Similar studies by Grumet and Edelman (1988) have provided conflicting results, supporting a role for chick Ng-CAM and mouse L1 in both neuron–neuron and neuron–astroglia adhesion. Our studies with substrates coated with purified chick L1 revealed attachment of only neuronal cells (Lagenaur and Lemmon, 1987). Using Ng-CAM derivatized Covaspheres, Grumet and Edelman (1988) have shown that binding of Covaspheres to both neurons and glia could be mediated by Ng-CAM. These workers have further demonstrated that binding of Ng-CAM-derivatized Covaspheres to neurons can be inhibited by pretreatment of neurons with anti-Ng-CAM Fabs, supporting the conclusion that the Ng-CAM-mediated binding between neurons is homophilic. Pretreatment of glia with Fabs did not block the binding of Covaspheres to glia. Clearly, the conclusions that can be drawn about the nature of NILE/L1/Ng-CAM/8D9-mediated adhesion are highly dependent on the type of assay used to test adhesion; it is possible that differences in parameters such as relative shear forces occurring during washing after cell or vesicle attachment may produce differing results. Since these studies are all based on short-term assays of cell–cell or cell–vesicle adhesion, they do not address the role of NILE/L1/Ng-CAM/8D9 in supporting neurite outgrowth. Recent sequencing of mouse L1 by Moos et al. (1988) has identified two Arg-Gly-Asp (RGD) sequences. Such sequences have been implicated in adhesion occurring between fibronectin and laminin and their respective receptors. Whether these sequences are important in the function of L1-like proteins, by perhaps providing a heterophilic binding site for an integrin-type receptor remains to be seen.

The studies presented here strongly suggest that adhesive interaction between neuronal processes mediated by the L1 family of molecules is homophilic. Our results show that monovalent antibody binding to one of two putative binding partners (the cell-bound chick L1) prevents chick neurons from attaching and extending neurites on mouse L1 immobilized on the surface of a petri plate. Since chick L1 can support attachment of mouse neurons and since mouse L1 can support the attachment of chick neurons, it must be inferred that the binding sites on these two molecules mediating the homophilic binding have been conserved and therefore should be similar in structure. If this is the case, it is perhaps surprising that it is possible to produce polyclonal antibodies against chick L1 that inhibit binding and are species-specific. Inhibition may be due to antibody binding to species-specific sites near the chick L1 binding site that interferes with the binding to mouse L1 via steric hindrance.

Since the RaCL1 Fabs interfered with both cell attachment and neurite outgrowth, it is not possible to determine whether these two activities reside at the same binding site. It is important to note that the few mouse or chick neurons that succeeded in attaching to plates coated with chick L1 in the presence of RaCL1 Fabs showed little neurite outgrowth. This suggests that the failure of neurite outgrowth was not due only to the inability of cells to attach. While neurite outgrowth probably requires cell attachment, it is not clear that all molecules mediating cell attachment are able to mediate neurite outgrowth. Tomaselli et al. (1986) have reported that with chick brain monolayers, neurite outgrowth observed on highly adhesive poly-L-lysine requires laminin (which can be produced by astrocytes). We have also observed that highly purified mouse cerebellar granule cells have great difficulty in extending neurites on poly-L-lysine without other substrate molecules, although they adhere very well to the substrate (authors' unpublished data). It should be possible to distinguish differences between cell binding sites and neurite outgrowth promoting sites (if indeed such sites exist) by using species-specific blocking antibodies raised against defined regions of the chick L1 or mouse L1 molecule.

While these studies support the concept that L1 molecules on adjacent neurites interact in a homophilic binding mechanism and imply that a heterophilic binding partner for L1 does not exist on neurons, the possibility remains that L1 may be capable of interacting with a heterophilic binding partner on some other type of cell or substrate. M. Grumet and his associates have provided evidence in kinetic binding assays that such a heterophilic binding partner is present on glia. Similarly, we have found evidence that chick retinal glia express a heterophilic binding partner for chick L1 that can help to support neurite outgrowth (Drazba and Lemmon, unpublished data). The identification and characterization of this glial receptor remain important tasks.

Experimental Procedures

Materials and Animals

Nitrocellulose was obtained from Schleicher and Schuell (Type BA 85), Keene, NH. Laminin was from GIBCO, Grand Island, NY. CD-1 mice were raised in our own colony from breeders originally obtained from Charles River Breeding Laboratories, Wilmington, MA.

Monoclonal Antibody Production

A new panel of MAbs to chick L1 were produced by immunizing BALB/c mice with antigen purified using MAb 8D9 (Lemmon and McLoon, 1986). A spleen from a mouse whose serum showed a strong response to the chick L1 was used to fuse with NS-1 cells (De St. Groth and Scheidegger, 1980). Hybridomas were screened using a dot-blot assay (Hawkes et al., 1982) on purified chick L1. Positive hybridomas were then tested on Western blots of chick L1 and mouse neural membrane proteins. Several hybridomas that reacted with proteins of appropriate molecular weight on both the chick L1 and the mouse membrane proteins were cloned several times by limiting dilution. The MAb used in the experiments described in this paper is referred to as 74-5H7.

Antigen Purification

Chick L1 and mouse L1 were purified by immunoaffinity chromatography using methods described elsewhere (Lagenaur and Lemmon, 1987). Briefly, neural membranes from either embryonic day 19 chick brains or postnatal day 6 mouse brains were prepared on

sucrose density gradients, the membranes were extracted with 1% deoxycholate, and the extract was run over MAb affinity columns. The antigens were eluted using 0.1 M diethylamine (pH 11.5) and quickly neutralized with solid Tris-HCl. The antigens were then incubated with Affigel-10 beads coated with rabbit anti-mouse immunoglobulin antibodies to ensure that there were no MAbs from the affinity columns contaminating the purified antigens.

Polyclonal Antibody Production

The rabbit polyclonal antibody to chick L1 used in these experiments has been described previously (Lemmon and McLoon, 1986). Fabs were produced (Mage, 1980) and were observed to produce significant defasciculation of neurites growing from chick retinal explants on laminin substrates (data not shown). These Fabs showed very little cross-reactivity with mouse neurons (e.g., the antibody did not stain live mouse neurons). For the blocking experiments, to ensure that there was no cross-reactivity, the Fabs were preincubated with Affigel-10 beads coated with mouse L1.

Preparation of Nitrocellulose-Coated Petri Plastic

A 5 cm^2 strip of nitrocellulose was dissolved in 12 ml of methanol. Aliquots (0.25 ml) of this solution were rapidly spread over the surface of 35 mm petri plates and allowed to dry under a laminar flow hood. This surface was then spotted with 4 μl aliquots of test samples containing approximately 0.1 mg/ml protein. After 10 min, the droplets were removed by aspiration and the area was washed twice with Hank's calcium- and magnesium-free saline solution. The substrate plates were then blocked with a 10% horse serum in Dulbecco's modified Eagle's medium for 30 min in a 37°C CO_2 incubator. The substrate plates were washed with culture medium (basal Eagle's medium with Earle's salts and 10% horse serum). Two milliliters of freshly dissociated cerebellar cells (1×10^6 cells per ml) from postnatal day 6 mice or 2 ml of tectal cells (5×10^5 cells per ml) from embryonic day 9 chick embryos prepared as previously described (Lagenaur and Lemmon, 1987) was added to the substrate test plates. Cells were allowed to grow for 18 hr. The cultures were then fixed with 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and neurite lengths were measured.

Quantitization of Neurite Length

Culture dishes were examined as previously described (Lagenaur and Lemmon, 1987) using the Bioquant IV Image Analysis system. Neurite length was measured as the distance between the center of the cell soma and the tip of its longest neurite. The neurite had to meet the following requirements: it must emerge from a cell in isolation (not in a clump of cells), it must not contact other cells or neurites, and it must be longer than the diameter of the cell body. In most cases, within an area of approximately 4.0 mm^2, about 100 neurites meeting these requirements were measured. When neurite outgrowth was poor, as many neurites as could be found in the area coated by a given antigen were measured.

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