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Domain-Specific Activation of Neuronal Migration and Neurite Outgrowth-Promoting Activities of Laminin

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Summary

The ECM glycoprotein laminin has profound and varied actions on neurons *in vitro*. Little is known about how laminin's multiple domains and receptor-binding sites interact in determining its overall effects. Here, it is shown that laminin's ability to promote migration of olfactory epithelium neuronal cells maps to distal long arm domain E8 and is mediated by $\alpha_6\beta_1$ integrin. Surprisingly, treatment of laminin with antibodies against its short arms (domains E1' or P1') uncovered a new neuronal migration-promoting activity, mediated by a β_1 integrin other than $\alpha_6\beta_1$. Laminin treated with anti-short arm antibodies also promoted β_1 integrin-dependent neurite outgrowth from late embryonic retinal neurons, which are normally unresponsive to laminin. These "antibody-induced" migration and neurite outgrowth activities mapped to laminin's distal long arm, far from the site(s) of antibody binding. Evidence is presented that the induced activities are not actually cryptic in laminin, but are suppressed by an activity that is located in laminin's P1' domain and that may be lacking in the laminin homolog merosin.

Introduction

The extracellular matrix (ECM) has profound effects on cells: it can influence cell shape, proliferation, motility, differentiation, growth factor responsiveness, and gene expression. The ECM glycoproteins that elicit these responses, including laminin, fibronectin, thrombospondin, tenascin, and collagens, are large, multidomain molecules, the individual domains of which appear to have distinct biological activities (see reviews by Martin and Timpl, 1987; Erickson and Bourdon, 1989; Beck et al., 1990; Hynes, 1990; Lander and

Calof, 1993). Little is known, however, about how these component activities are integrated into the overall biological effects of ECM molecules.

The neuronal response to laminin provides unique opportunities for studying how ECM molecules work. Laminin has diverse and striking effects on many types of neurons and neuronal precursors, including promotion of cell attachment, neurite outgrowth, migration, differentiation, and survival (Lander, 1989). In addition, some of laminin's activities have already been mapped to distinct molecular domains (reviewed by Sephel et al., 1989; Beck et al., 1990; Mecham, 1991; Reichardt and Tomaselli, 1991; Lander and Calof, 1993). In a recent study of the migration of neuronal cells (olfactory receptor neurons and neuronal precursor cells) derived from cultured embryonic mouse olfactory epithelium (OE), it was found that laminin stimulated and guided neuronal cell migration, but was an extremely poor substratum for cell adhesion (indeed, laminin was anti-adhesive [Calof and Lander, 1991]). The results raised the possibility that the effects of laminin on certain neurons might be mediated through some form of signaling, rather than through promotion of cell-substratum adhesion.

To understand better how such signaling might occur, a study was undertaken to map the site(s) on laminin responsible for promotion of OE neuronal migration and to identify the cellular receptors involved. As described below, the experiments at first yielded a simple picture, in which a single laminin domain (E8, at the distal end of laminin's long arm) and a single integrin receptor ($\alpha_6\beta_1$) accounted for all of laminin's neuronal migration-promoting activity. However, control experiments led to the unexpected discovery of an additional, β_1 -dependent, migration-promoting activity, which also mapped to E8 but was not mediated by $\alpha_6\beta_1$. The additional activity was not detected by OE neuronal cells unless substratum-bound laminin was treated with antibodies that bind domain P1', part of laminin's short arms. Since E8 and P1' are not adjacent domains (indeed, they are quite distant), it appeared that something other than local conformational activation of a cryptic site might be responsible for the effects of anti-short arm antibody binding on laminin function. Experiments undertaken to address this issue led to the conclusions that the change induced in laminin by antibody binding leads not only to increased neuron migration, but also to neurite outgrowth by neurons that normally show no response to laminin; that the short arms of laminin are likely to contain a "suppressor" activity that selectively interferes with one of the integrin-dependent functions of E8; and that the laminin isoform merosin behaves as though it lacks that suppressor activity. These results suggest that functional interactions between the domains of ECM molecules can play crucial roles in dictating a molecule's biological activity.

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Figure 1. Migration of OE Neuronal Cells on Laminin and Laminin Fragments

Solutions (85 $\mu\text{g}/\text{ml}$ applied concentration) of laminin or laminin fragments were adsorbed to acid-cleaned glass coverslips overnight at 4°C. The coverslips were washed and OE explants were plated onto them in complete LCM. Eighteen hours later, cultures were fixed and migratory distances of OE neuronal cells were measured as described. Values on the abscissa indicate the distances that individual cells migrated, and values on the ordinate are a measure of the number of cells that migrated greater than or equal to any given distance. (A) Effect of laminin and laminin fragments on migration. (B) Effect of treating a laminin substratum (applied at 50 $\mu\text{g}/\text{ml}$) with antibodies to fragment E8 (10 $\mu\text{g}/\text{ml}$), prior to plating OE explants. (C) A schematic view of the laminin molecule and the domains represented by several of its fragments.

Results

The Neuronal Migration-Promoting Activity of Untreated Laminin Maps to the E8 Domain and Is Mediated by $\alpha_6\beta_1$ Integrin

To identify the region(s) of the laminin molecule responsible for stimulating neuronal migration, four proteolytic fragments of laminin—the elastase fragments E1', E3, E4, and E8—and one recombinant fragment—the G domain (the C-terminal globule of the A chain), expressed in insect cells (Yurchenco et al., 1993)—were adsorbed to glass coverslips, and explants of embryonic mouse OE were cultured upon them for ~18 hr. Migration of neuronal cells was quantified by determining, for each of several explants, the total number of cells that were present on the substratum surrounding the explant and the distance from each such cell to the nearest edge of the explant. In Figure 1, these data are presented in the form of a running histogram, in which migration distances are shown on the abscissa, and the ordinate indicates the number of cells that migrated greater than or equal to any given distance. To correct for variations in the size of explants analyzed, the data have been normalized to total explant area.

The results in Figure 1A show that, on a laminin substratum, 3600 neuronal cells migrated per mm² of explant, moving a median distance of 75 μm . Only one laminin fragment promoted cell migration: E8, which represents the distal end of laminin's long arm (Figure 1C). In the experiment shown, E8 stimulated about 2300 cells per mm² of explant to migrate a median distance of 74 μm ; however, the exact number of cells migrating on E8 substrata varied among experiments and in some cases exceeded that on laminin

substrata, especially when plastic, rather than glass, substrata were used. In addition to stimulating cell migration, E8 also promoted neurite outgrowth by OE neurons (data not shown), as was observed previously for intact laminin (Calof and Lander, 1991). In contrast to E8, substrata treated with E1', E3, E4, or G supported minimal cell migration and neurite outgrowth, equivalent to that on coverslips treated with bovine serum albumin (BSA) or with fibronectin (see Calof and Lander, 1991).

Figure 2. Anti- α_6 Antibodies Block Migration on Laminin

OE explants were cultured on laminin-treated glass coverslips (laminin applied at 60 $\mu\text{g}/\text{ml}$) in the presence of monoclonal rat anti- α_6 (GoH3), or nonimmune IgG (10 $\mu\text{g}/\text{ml}$). GoH3, at 2 or 10 $\mu\text{g}/\text{ml}$, decreased cell migration to background levels.

Figure 3. Inhibitory and Stimulatory Effects of Domain-Specific Anti-Laminin Antibodies on Neuronal Migration

(A–E) Glass coverslips were coated with laminin (50 μ g/ml applied concentration) overnight at 4°C, washed 3 times in CMF–HBSS, and either held in complete LCM or treated with domain-specific antibodies (diluted to 10 μ g/ml in complete LCM) for 6 hr at 4°C. Coverslips were washed 3 times in complete LCM, and OE explants were plated on them in 1–1.5 ml of complete LCM per coverslip and placed into culture. After 18 hr, cultures were fixed by underlay, as described in Experimental Procedures, and photographed in the culture plate without rinsing, to minimize disturbance of weakly adherent cells. Photographs of typical explants were taken using a Zeiss Axiovert microscope equipped with a 10 $\times$ phase-contrast objective. (F) Migration of OE neuronal cells was quantified for the coverslips shown in (A)–(E) and plotted as in Figure 1. Essentially identical results were obtained when anti-fragment antibodies were applied at 40 μ g/ml.

Figure 1B shows that treatment of a laminin substratum with an affinity-purified antibody to the E8 domain completely inhibited neuronal migration. Thus, in addition to being sufficient to promote neuronal migration, the E8 region is also necessary for this activity in the intact laminin molecule. Consistent with this, the migration-promoting effects of laminin on OE neuronal cells could easily be destroyed by heat (90°C for 10 min; data not shown), a characteristic of activities that map to E8 (Goodman et al., 1991).

Among the cellular receptors that could mediate the responses of OE neuronal cells to laminin, $\alpha_6\beta_1$ integrin is known to recognize the E8 domain of laminin (Sonnenberg et al., 1990), is highly expressed in the developing nervous system (Bronner-Fraser et al., 1992), and mediates neurite outgrowth on laminin by some classes of neurons (Cohen and Johnson, 1991), but not all (Tomaselli et al., 1990, 1993). The data in Figure 2 show that the GoH3 monoclonal antibody, which binds to and blocks the function of α_6 -containing integrins (Sonnenberg et al., 1988), completely inhibits the ability of laminin to promote OE neuronal migration. Since an antiserum to the β_1 integrin subunit also blocks neuronal migration (see below), the data imply that the migratory response of OE neuronal cells to laminin is mediated by $\alpha_6\beta_1$ integrin.

Antibodies to Short Arm Domains E1' and P1' Enhance Laminin's Ability to Promote Cell Migration

As a control for the experiment shown in Figure 1B, in which a laminin substratum was treated with anti-E8 antibodies, laminin substrata were treated with antibodies to other fragments, namely E1', E3, and P1'. These antibodies, like the anti-E8 antibodies, had been affinity purified and cross-absorbed against nonoverlapping fragments (see Experimental Procedures). Laminin substrata were treated with individual anti-fragment antibodies (at 10 μ g/ml), unbound antibodies were removed by extensive washing, and OE explants were plated onto the substrata and cultured for 18 hr. The results are shown in Figure 3. Clearly, no antibody besides anti-E8 could completely eliminate neuronal migration, although anti-E3 antibodies caused a small,

but reproducible decrease (Figures 3C and 3F), which did not become more profound at higher antibody concentrations (40 μ g/ml; data not shown). Since E3 and E8 are adjacent fragments, the partial effect of anti-E3 antibodies might result from steric hindrance of sites in E8. Alternatively, sites in E3 may contribute partially to the migration-promoting effects of laminin.

Unexpectedly, treatment of laminin substrata with either anti-P1' or anti-E1' antibodies dramatically *increased* neuronal migration (Figures 3D and 3E). The most noticeable change was a more than doubling of the number of cells that migrated from explants on anti-P1'- and anti-E1'-treated laminin substrata (Figure 3F). Since P1' is effectively a subfragment of E1' (Figure 1C), these data suggest that the binding of antibodies to a site or sites within the P1' region of substratum-bound laminin actually enhances laminin's ability to promote cell migration.

Several experiments were undertaken to control for ways in which these results might arise by artifact. Most important to consider was the possibility that the anti-P1' antibodies might contain molecules that themselves promote migration in some manner. To test this, anti-P1' antibodies were applied to BSA or fibronectin substrata; these substrata did not support OE neuronal cell migration. In addition, anti-P1' antibodies were applied to E8 substrata; cell migration on these substrata was not enhanced over the level seen with E8 alone (data not shown). These results argue that anti-P1' antibodies are not simply contaminated with migration-promoting molecules that deposit on the substratum.

A second possibility is that anti-P1' antibodies, when bound to substratum-adsorbed laminin, might somehow interact with cells and cause them to migrate. To test this, anti-P1' antibodies were applied to E1' substrata. Since E1' contains all the epitopes recognized by anti-P1' (in fact, anti-P1' was isolated from anti-E1' [see Experimental Procedures]), the substrata thus generated should contain the same complement of substratum-bound antibodies as laminin substrata treated with anti-P1' antibodies. Nonetheless, as shown in Table 1, E1' substrata treated with anti-P1'

Table 1. Absorption of Anti-P1' Antibodies with E1' and E8

Substratum Applied	Antibody Treatment	Migration (Cells/mm ²)
Laminin	None	1964
Laminin	Anti-P1' (10 μ g/ml)	6178
Laminin	Anti-P1' Fab fragments (25 μ g/ml)	5366
E1'	None	199
E1'	Anti-P1' (10 μ g/ml)	241
Laminin	Anti-P1' (10 μ g/ml) + E1' (40 μ g/ml)	1927
Laminin	Anti-P1' (10 μ g/ml) + E8 (40 μ g/ml)	6503

Glass coverslips were treated with laminin or laminin fragment E1' (50 μ g/ml), washed, blocked, and treated with the reagents shown above. The coverslips were washed, and OE explants were plated onto them in complete LCM. Eighteen hours later, cultures were fixed, and migratory distances of OE neuronal cells measured as described in Experimental Procedures. Shown above is the number of cells that migrated >20 μ m, per mm² of explant, in each condition.

Figure 4. Migration on Anti-P1'-Treated Laminin Is Only Partially Mediated by α_6 Integrins

Glass coverslips were coated with laminin (50 μ g/ml applied concentration) for 4 hr at 4°C, washed 3 times in CMF-HBSS, and treated with anti-P1' IgG (10 μ g/ml) overnight at 4°C. Coverslips were then washed into complete LCM, and OE explants were plated onto them in either complete LCM (No antibody) or complete LCM containing 10 μ g/ml anti- α_6 (GoH3) for 18 hr. After fixation, migration was measured and plotted as in Figure 1. The effect of GoH3 is an approximately 50% decrease in cell migration. This result contrasts markedly with the complete blockade of migration that is observed when GoH3 is added to explants plated on an untreated laminin substratum (i.e., the experiment shown in Figure 2; data from a repetition of that experiment, but carried out in parallel with the one shown above, were essentially identical to those shown in Figure 2).

antibodies did not promote cell migration. In addition, Table 1 shows that anti-P1' antibodies did not need to be divalent in order to enhance neuronal migration on laminin, as monovalent Fab fragments were also effective. Finally, Table 1 confirms the fragment specificity of the anti-P1' antibodies, by showing that their ability to enhance cell migration on laminin can be neutralized by preabsorbing them with E1', but not by preabsorbing them with E8. Taken together, the results indicate that increased cell migration on anti-P1'-treated laminin is due to a change induced in laminin by the binding of antibodies specifically to laminin's P1' domain.

Enhanced Neuronal Migration on Anti-P1'-Treated Laminin Is Not Mediated by $\alpha_6\beta_1$ Integrin

Since previous experiments had found the effects of laminin on OE neuronal migration to be mediated through integrin $\alpha_6\beta_1$ (Figure 2), it was of interest to determine whether the enhancement of neuronal migration by anti-P1' treatment of laminin involved enhancement of $\alpha_6\beta_1$ -mediated activity. As shown in Figure 4, this appears not to be the case. Whereas the GoH3 antibody effectively blocked neuronal migration on untreated laminin, it only partially inhibited migration on anti-P1'-treated laminin, even when used

Table 2. Effect of Anti- β_1 Integrin Antibodies on Neuronal Migration

Treatment of Laminin Substratum	Antiserum Added to Culture Medium	Migration (Cells/mm ²)
None	Control	724
Anti-P1'	Control	2988
None	Anti- β_1 integrin	84
Anti-P1'	Anti- β_1 integrin	69

Tissue culture wells were treated with laminin (40 μ g/ml), washed, blocked, and either left untreated, or treated with anti-P1' (10 μ g/ml), as described in Table 1. OE explants were plated in LCM containing rabbit anti- β_1 integrin anti-serum (1:300), or control, nonimmune rabbit serum (1:300), and cultured for 18 hr. Cell migration was measured as described in Experimental Procedures and is presented as in Table 1.

at a concentration 5 times that required for complete blockade of migration on laminin. Interestingly, the level of cell migration that remained on anti-P1'-treated laminin when cells were cultured in the presence of GoH3 was approximately equal to the difference between the level of migration on anti-P1'-treated and untreated laminin. This was the case regardless of whether the level of migration was measured in terms of the numbers of cells that migrated (which declined by 45% in the presence of GoH3), or in terms of the median distance that cells migrated (which declined by 48% in the presence of GoH3). Taken together, these results suggest that the migration activity induced in laminin by anti-P1' antibodies and the $\alpha_6\beta_1$ -mediated migration activity that exists in untreated laminin are independent and additive.

In contrast to the results with the GoH3 monoclonal antibody, a polyclonal antibody directed against the β_1 chain common to many integrins reduced neuronal migration to background levels on *both* untreated and anti-P1'-treated laminin substrata (Table 2). Taken together, these data indicate that there is an additional type of β_1 integrin receptor, other than $\alpha_6\beta_1$, that participates in mediating the neuronal migratory response to anti-P1'-treated, *but not untreated*, laminin.

Functionally, Anti-P1'-Treated Laminin Resembles the Laminin Homolog Merosin

Merosin, a homolog of laminin that shares two of three polypeptide chains (Ehrig et al., 1990), was previously shown to promote OE neuronal cell migration (Calof and Lander, 1991). Interestingly, merosin was found consistently to promote a significantly greater level of cell migration than laminin. This is illustrated in Figure 5, which also compares the effects of the GoH3 antibody on cell migration on laminin and merosin substrata. As shown, cell migration on merosin substrata was only partially inhibited by GoH3, even at antibody concentrations 5 times the level that inhibited all cell migration on laminin (see Figure 2). Nevertheless, all the migration-promoting activity of

Figure 5. Neuronal Migration Promoted by Merosin Resembles That Promoted by Anti-P1'-Treated Laminin

In an experiment conducted in parallel with the one shown in Figure 2, OE explants were plated on coverslips treated with merosin (60 μ g/ml) and cultured in the presence of GoH3 or control IgG. By comparing these data with Figure 2, it can be seen that merosin promotes considerably more neuronal migration than laminin and that migration on merosin is only partly inhibitable by GoH3. In both these regards, the effects of merosin resemble those of anti-P1'-treated laminin (see Figures 3 and 4).

merosin could be blocked by a polyclonal anti- β_1 anti-serum (data not shown). Thus, like anti-P1'-treated laminin, merosin promotes a higher level of cell migration than untreated laminin, and merosin engages

not only $\alpha_6\beta_1$ integrins, but also another β_1 integrin receptor that untreated laminin does not engage.

Anti-P1'-Treated Laminin Promotes Neurite Outgrowth by Late Embryonic Retinal Neurons

Although merosin and laminin usually have similar effects on cells in vitro, a few reports exist of quantitative or qualitative differences (Cohen and Johnson, 1991; Brown and Goodman, 1991; Tomaselli et al., 1993). The data described above raise the intriguing possibility that anti-P1' antibodies activate in laminin functions that are normally present constitutively in merosin. To explore this possibility further, experiments were undertaken involving retinal neurons, the cells that have been reported to show the most dramatic differences in response to merosin versus laminin. Specifically, Cohen and Johnson (1991) showed that vertebrate retinal neurons, which normally lose the ability to adhere to or extend neurites upon laminin as they mature (Cohen et al., 1986; Hall et al., 1987), retain the ability to respond to merosin. For example, by embryonic day 9 in the chicken or day 15 in the rat, retinal ganglion neurons show little response to laminin, yet they readily extend neurites on merosin. Moreover, the late embryonic retinal response to merosin was β_1 dependent, but α_6 independent (Cohen and Johnson, 1991).

If merosin's non- α_6 -dependent neurite outgrowth activity for late embryonic retinal neurons is related or identical to merosin's non- α_6 -dependent neuronal migration activity for OE neuronal cells and, furthermore, if the latter is functionally equivalent to the

Figure 6. Effect of Domain-Specific Anti-Laminin Antibodies on Retinal Neurite Outgrowth

Dissociated embryonic day 18 rat retinal neurons were cultured on laminin substrata (laminin applied at 40 μ g/ml) that had been treated with no antibody (A), anti-P1' (10 μ g/ml) (B), anti-E1' (10 μ g/ml) (C), anti-E3 (10 μ g/ml) (D), or anti-E8 (10 μ g/ml) (E). Profuse neurite outgrowth is seen only in (B) and (C). Bar, 100 μ m. (F) shows that the activating effect of anti-P1' antibodies is dose dependent, being half-maximal at $\sim$ 2 μ g/ml. Similar results were obtained with E16 mouse and E12 chicken retinal neurons (data not shown).

Figure 7. Characteristics of Antibody-Induced Retinal Neurite Outgrowth-Promoting Activity

Substrata were treated with laminin (40 µg/ml), merosin (40 µg/ml) or laminin fragment E1' (20 µg/ml), blocked, and then further treated with antibodies as indicated. Dissociated embryonic day 18 rat (A-D) or day 16 mouse (E) retinal neurons were plated onto the substrata and cultured for 18-19 hr. Neurite outgrowth from duplicate cultures was scored as described in Experimental Procedures. (A) Treatment of laminin substrata with anti-P1' or anti-E1' antibodies rendered them strongly neurite promoting,

migration activity induced in laminin by anti-P1' antibodies, then late embryonic retinal neurons would be predicted to respond to anti-P1'-treated laminin as they do to merosin and not as they do to (untreated) laminin.

Figures 6 and 7 show that this prediction is in fact borne out: E18 rat retinal neurons extended few neurites on untreated laminin (Figure 6A), as expected for their developmental stage (Cohen and Johnson, 1991).

to nearly the same degree as a merosin substratum (all antibodies were applied at 10 µg/ml). (B) The ability of anti-P1' antibodies (10 µg/ml) to activate laminin could be neutralized by fragment E1' (50 µg/ml), but not fragment E8 (50 µg/ml), confirming the specificity of the antibody preparation. (C) Treatment of uncoated, or E1'-coated substrata with anti-P1' antibodies (10 µg/ml) did not result in the appearance of neurite outgrowth activity; this confirms that the antibodies themselves are not the source of neurite outgrowth-promoting molecules. (D) The neurite outgrowth-promoting activity of laminin that was treated with anti-P1' antibodies (10 µg/ml) could be completely blocked by adding to the culture medium a polyclonal antibody directed against the mammalian β_1 integrin subunit (1:400 dilution; kindly provided by Clayton Buck). Nonimmune rabbit serum, at the same dilution, was without effect. (E) The neurite outgrowth-promoting activity of laminin that was treated with anti-P1' antibodies (10 µg/ml) could not be blocked by adding the GoH3 antibody (5 µg/ml) to the culture medium. Since the GoH3 antibody does not recognize rat $\alpha_6\beta_1$, E16 mouse retinal neurons were used in this experiment. The results in (D) and (E) indicate that the neurite outgrowth activity induced in laminin by anti-short arm antibodies is mediated by a β_1 integrin other than $\alpha_6\beta_1$.

Figure 8. The Neurite Outgrowth- and Migration-Promoting Activities Unmasked by Anti-P1' Antibodies Are Blocked by Anti-E8 Antibodies

(A) Retinal neurite outgrowth. Plastic substrata were treated with laminin (40 µg/ml), washed, and blocked with albumin as described. Anti-fragment antibodies were prepared at 10 µg/ml in culture medium and applied for 4 hr at 4°C. Substrata were washed, and E18 rat retinal neurons were prepared, plated, and cultured as in Figure 6. Neurite outgrowth is given as the percentage of cells or cell aggregates that bore neurites >2 cell diameters in length. Data are the means $\pm$ range from 2 independent determinations in which ≥ 100 neurons were counted. The data indicate that anti-E8 and anti-E3 antibodies block the neurite outgrowth activity induced in laminin by anti-P1' antibodies. Additional experiments indicated that anti-E8 and anti-E3 were half-maximally inhibitory at ~ 0.3 µg/ml and ~ 2 µg/ml, respectively (data not shown). (B) OE neuronal migration. Glass coverslips were coated with laminin (50 µg/ml applied concentration) for 4 hr at 4°C, washed, blocked, and treated with domain-specific antibodies overnight at 4°C, as follows: anti-P1', 10 µg/ml in complete LCM; anti-E8, 40 µg/ml in complete LCM; anti-P1' + anti-E8, 10 and 40 µg/ml, respectively. Coverslips were washed into complete LCM, OE explants were plated onto them, and cultures were incubated for 18 hr. After fixation, migration was measured and plotted as in Figure 1. The data indicate that anti-E8 antibodies block the additional neuronal migration-promoting activity induced in laminin by anti-P1' antibodies.

Table 3. Further Evidence that Laminin's Distal Long Arm Is Required for the Neurite Outgrowth-Promoting Activity of Anti-P1'-Treatment Laminin

Substratum Applied	Treatment	% with Neurites
(A) Laminin	—	1.5 ± 0.0
Heat-treated laminin	—	0.8 ± 0.8
Laminin	Anti-P1' (10 µg/ml)	94 ± 4.6
Heat-treated laminin	Anti-P1' (10 µg/ml)	1 ± 0.6
(B) Laminin	—	6 ± 1.5
Laminin	Anti-laminin (1:50)	1 ± 1.4
Laminin	Anti-laminin (1:50) + E8 (50 µg/ml) + E3 (25 µg/ml)	76 ± 2.6
Laminin	Anti-laminin (1:3200)	8 ± 0.7
Laminin	Anti-laminin (1:3200) + E8 (50 µg/ml) + E3 (25 µg/ml)	9 ± 0.2

Substrata were treated with 40 µg/ml laminin and further treated with antibodies as indicated. The substrata were then washed and E18 rat retinal neurons prepared, plated, and cultured as in Experimental Procedures. Neurite outgrowth is presented as the percentage of cells or cell aggregates that bore neurites >2 cell diameters in length. Data are the means ± range from 2 independent determinations in which ≥100 neurons were counted. (A) When laminin was briefly heated under conditions that specifically denature its long arm, but not its short arms (70°C × 10 min [Goodman et al., 1991]), before being adsorbed to coverslips, anti-P1' antibodies were not able to induce the appearance of neurite outgrowth activity. (B) When isolated E3 and E8 fragments were mixed with a crude, commercial anti-laminin antiserum (see Experimental Procedures), that antiserum acquired a dose-dependent ability to induce neurite outgrowth activity in laminin.

In contrast, on laminin substrata that had been treated with anti-P1' or anti-E1' antibodies (Figures 6B and 6C), but not with anti-E3 or anti-E8 antibodies (Figures 6D and 6E), substantial neurite outgrowth, involving >90% of plated cells or cell clusters, occurred (Figure 6F). Neurite-bearing cells on anti-P1'-treated or anti-E1'-treated laminin substrata included retinal ganglion cells, as judged by Thy-1 immunocytochemistry (data not shown; see Cohen and Johnson, 1991).

Figure 7 confirms that the degree of neurite outgrowth on anti-P1'- and anti-E1'-treated laminin is comparable to that seen on merosin substrata (Figure 7A), although some differences in cell morphology were noticed (cells were somewhat more spread on merosin; data not shown). Figure 7 also shows that the effects of anti-P1' antibodies on laminin can be neutralized by the E1' fragment but not the E8 fragment (Figure 7B); that application of anti-P1' antibodies to BSA or E1' substrata is insufficient to generate substrata that promote neurite outgrowth (Figure 7C); that β_1 integrins are required for the neurite outgrowth response to anti-P1'-treated laminin (Figure 7D); and that α_6 integrins are not required for this response (Figure 7E).

These results indicate that the binding of antibodies to the P1' domain of laminin induces β_1 -dependent neuronal migration and neurite outgrowth activity that are α_6 -independent and may be related or identical to an activity that is normally present in merosin. Because late embryonic retinal neurons apparently lack functional receptors required to interact with untreated laminin, the effects of anti-P1' antibodies on laminin were particularly dramatic when studied using these cells. This situation contrasts with that seen with OE neuronal cells, for which anti-P1' treatment of laminin caused only a 2- to 3-fold increase in cell migration. For this reason, retinal neurons were used for much of the subsequent analysis (see below) of the mechanism of antibody "activation" of laminin.

Investigation of the Mechanism of Antibody Enhancement of Laminin Activity

The results described above imply that there is a functional site on laminin, the presence of which is not detected by neurons unless anti-P1' antibodies are bound to the laminin molecule. One possibility is that there is a cryptic functional site within or near the P1' domain, and this site becomes conformationally exposed when antibodies bind nearby. Interestingly, the data do not support this explanation. As shown in Figure 8, the neuronal migration and neurite outgrowth activities induced in laminin by anti-P1' antibodies are blocked by the application of anti-E8 antibodies. To a somewhat lesser degree, anti-E3 antibodies can also block anti-P1'-induced activity.

Thus, the activity induced in laminin by anti-short arm antibodies is an activity that apparently requires the function of the distal long arm of the laminin molecule. Consistent with this view, when laminin was briefly heated to denature its long arm (70°C, 10 min; see above), anti-P1' antibodies were no longer able to induce retinal neurite outgrowth activity (Table 3A). Further confirmation that only antibodies to laminin's distal long arm inhibit the activity induced by anti-P1' antibodies is provided in Table 3B, which shows that a commercial anti-laminin antiserum, if applied to a laminin substratum, does not induce retinal neurite outgrowth activity, but if the same antiserum is first mixed with fragments E8 and E3 (to neutralize just those antibodies directed against these domains), the antiserum acquires the ability to induce retinal neurite outgrowth activity in laminin (i.e., the serum behaves like anti-P1' antibodies). This result is additionally significant because it shows that the ability to activate laminin is not limited to the particular batch of affinity-purified anti-P1' antibodies used exclusively up to this point.

How might antibodies to the P1' domain of laminin (the short arms) activate a function that involves the

E8 and/or E3 domains (the distal long arm)? Perhaps anti-P1' antibodies cause a conformational change that exposes a cryptic functional site in the distal end of the long arm. Although such a mechanism is theoretically possible, features of laminin structure suggest that it is unlikely. Electron microscopic and hydrodynamic data indicate that the laminin molecule adopts a semi-rigid cross shape, with insufficient flexibility to permit the short arms and the distal long arm to interact physically within a single laminin molecule (Engel et al., 1981). Binding studies further argue that short and long arm domains on separate laminin molecules do not interact directly with each other, even at concentrations much higher than those used in the present study (Yurchenco and Cheng, 1993). Consequently, any conformational change induced by the binding of anti-P1' antibodies to laminin would have to propagate through at least 40 nm of polypeptide (the proximal long arm) to influence any site in E8, or at least 70 nm (the entire long arm) to affect any site in E3 (Engel et al., 1981; Paulsson et al., 1985; Beck et al., 1990). Since these are unusually long distances over which to expect the propagation of protein conformational changes, it is prudent to consider two alternative explanations for how anti-P1' antibodies might activate laminin:

One possibility is that the activity induced in laminin by anti-P1' antibodies was already present in the distal long arm even *before* antibody addition, but it was antagonized by a second, "suppressor" activity residing elsewhere, most likely in or near the P1' region. According to this model, anti-P1' antibodies block the suppressor activity, and "uncover" the preexisting migration and neurite outgrowth activity.

Alternatively, the activity uncovered by anti-P1' antibodies may be a "synthetic" one, composed of two partial activities: one in the distal long arm that exists even before antibody addition and one in the short arms, which is created by local conformational changes in response to binding of anti-short arm antibodies. According to this model, neither the activity in the distal long arm nor the cryptic activity in the short arms is sufficient, on its own, to promote detectable cell migration or neurite outgrowth, but together they are sufficient.

These two models can be distinguished experimentally because they make opposite predictions about the consequences of destroying or removing the short arms of laminin. According to the first model, destroying or removing laminin's short arms should eliminate the suppressor activity and thereby have the same effect on laminin as anti-P1' antibodies. In contrast, according to the second model, removal or destruction of laminin's short arms should *preclude* the appearance of the activity that is induced in laminin by anti-P1' antibodies.

As shown in Figure 9, data obtained using retinal neurons clearly support the first (suppressor) model. Fragment C8-9 (which is derived exclusively from laminin's long arm and includes the E8 domain), as well

Figure 9. Long Arm Fragments Containing the E8 Region Strongly Promote Retinal Neurite Outgrowth

Plastic substrata were treated with laminin (40 µg/ml) or laminin fragments E8, E3, or C8-9 (50 µg/ml), then washed and blocked with albumin as described. One set of laminin-treated wells was further treated overnight with anti-P1' antibodies (10 µg/ml). E18 rat retinal neurons were then prepared, plated, and cultured as in Figure 6. Neurite outgrowth is given as the percentage of cells or cell aggregates that bore neurites >2 cell diameters in length. Data are the means ± range from 2 independent determinations in which ≥100 neurons were counted. Both fragments E8 and C8-9 promoted neurite outgrowth equivalent to that seen on anti-P1'-treated laminin.

as E8 itself, was found to be just as effective as anti-P1'-treated laminin at promoting late embryonic retinal neurite outgrowth. Thus, the data support the view that the biological activity revealed in laminin by the binding of anti-P1' antibodies is a function that already exists in the isolated E8 domain. The E3 domain appears not to be required for this activity, since E3 sequences are not present in E8. Also, E3 alone does not possess such activity (Figure 9).

Although these data strongly support the model that E8 alone possesses the activity induced in laminin by anti-P1' antibodies, they lead to the expectation that E8 ought also to promote OE neuronal cell migration to the same high level as anti-P1'-treated laminin. Yet, in Figure 1, E8 promoted OE neuronal migration somewhat less well than untreated laminin (as mentioned earlier, cell migration on E8 varied substantially among experiments and sometimes did exceed that on untreated laminin). The explanation for these results may be trivial: E8 is considerably less stable than intact laminin, and preparations of it lose activity upon storage, or when exposed to air during the rinsing of substrata (Deutzmann et al., 1990; and unpublished data). Since it is impossible to know in a given experiment what fraction of adsorbed E8 is active, measurements of the amount of neuronal migration promoted by E8 substrata may not provide a fair test of whether E8 possesses all the activities of anti-P1'-treated laminin.

Fortunately, a fair test can be devised by other means: as shown above (Figures 2 and 4), OE neuronal migration on a laminin substratum is entirely depen-

dent on $\alpha_6\beta_1$ integrin, whereas on anti-P1'-treated laminin, migration is only partly dependent on this integrin. If E8 behaves like anti-P1'-treated laminin, then it too should support neuronal migration that is only partly dependent on $\alpha_6\beta_1$. Table 4 shows that this is in fact the case. Whereas the GoH3 (anti- α_6) antibody nearly abolished migration on a laminin substratum, considerable cell migration remained on an E8 substratum in the presence of GoH3 at a concentration well above that needed for complete blockade of α_6 integrin function (see Figure 2).

Discussion

In the present study, the ability of laminin to promote migration of OE neuronal cells was mapped to the E8 region of the molecule (the distal long arm) and found to be mediated by $\alpha_6\beta_1$ integrin. Only fragment E8 promoted neuronal migration (Figure 1A), antibodies to E8 blocked the migration-promoting effects of intact laminin (Figure 1B), and antibodies to either the α_6 or β_1 integrin subunits completely blocked migration on laminin substrata (Figure 2; Table 2). In view of the fact that laminin promotes OE neuronal migration without measurably promoting cell-substratum adhesion (Calof and Lander, 1991), it is interesting that $\alpha_6\beta_1$ was found to be involved, since on other types of cells, this integrin clearly mediates cell-laminin adhesion (e.g., Sonnenberg et al., 1990; Cooper et al., 1991). These data suggest that $\alpha_6\beta_1$ can signal cells in ways other than simply promoting cell-substratum binding.

Unexpectedly, it was found that treatment of laminin substrata with affinity-purified anti-short arm antibodies (anti-P1' or anti-E1) increased laminin's migration-promoting activity by 2- to 3-fold (Figure 3). The additional activity could not be blocked by anti- α_6 antibodies (Figure 4), but could be blocked by anti- β_1 antibodies. Thus, anti-short arm antibodies uncovered "latent" β_1 -dependent migration activity in laminin. In principle, this latent activity could represent either an enhancement of migration by OE neuronal cells in general or, alternatively, the specific recruitment of a large population of migrating cells that were otherwise incapable of migrating on laminin. Although not tested here, the latter possibility is argued against by previous results (Calof and Lander, 1991) suggesting that few, if any, OE neuronal cells are not capable of migrating in response to (untreated) laminin.

Anti-short arm antibodies also uncovered latent neurite outgrowth activity in laminin, a result that was dramatically illustrated with late embryonic retinal neurons (Figure 6), cells that ordinarily exhibit little to no neurite outgrowth on laminin. This activity was also β_1 dependent and α_6 independent (Figure 7). Further examination of both the migration and neurite outgrowth activities uncovered in laminin by anti-short arm antibodies indicated that they required the distal long arm (Figure 8; Table 3). Ultimately, evidence was obtained that the distal long arm itself possessed

Table 4. Isolated Fragment E8 Possesses the Non- α_6 -Mediated Neuronal Migration Activity That Untreated Laminin Does Not

Substratum	Antibody Added to Culture Medium	Migration (Cells/mm ²)
Laminin	None	2810
E8	None	2903
Laminin	GoH3	167
E8	GoH3	956

Tissue culture plastic substrata were treated with laminin or E8 at 50 μ g/ml, washed, and blocked. OE explants were plated in LCM, or LCM containing monoclonal anti- α_6 GoH3 (at 10 μ g/ml), and cultured for 18 hr. Cell migration was measured as described in Experimental Procedures and is presented as in Table 1. Whereas GoH3 prevented virtually all cell migration on a laminin substratum (see Figure 2), it only partially reduced (by about two-thirds) the migration on an E8 substratum.

such activities when separated from the rest of the laminin molecule (Figure 9; Table 4).

What Is the Source of Antibody-Induced Neuronal Migration and Neurite Outgrowth Activity?

To explain how anti-short arm antibodies induce a potent biological activity that maps to laminin's distal long arm, three hypotheses were considered. The one most consistent with the data proposes that a suppressor site exists in or near the short arms of laminin which, when functional, blocks cell response to a β_1 -binding site (different from the $\alpha_6\beta_1$ -binding site) in the E8 region. According to this view, anti-short arm antibodies block the suppressor site and thereby unmask the function of the E8 site. This explanation is consistent with the expectation that polyclonal antibodies act by blocking the functions of the protein domains they bind to, and it correctly predicts that isolated E8-containing fragments should be functional on their own.

An alternative hypothesis is that anti-short arm antibodies induce a conformational change in laminin's distal long arm, exposing a cryptic integrin-binding site. In view of the structure and relative inflexibility of the laminin molecule, it was argued (see Results) that this hypothesis is less likely, since it requires a conformational change to propagate through at least 40 nm of long arm structure, an unusually long distance. In addition, the data indicate that laminin can be activated not just by antibodies specific for laminin's short arms, but also by crude anti-laminin antibodies absorbed only with E8 and E3 (Table 3), by proteolytic cleavage between laminin's short arms and long arm (see Figure 9, C8-9), or by proteolytic cleavage in the middle of the long arm (see E8). Thus, if the activation of laminin reflects a conformational change in E8, then it must be the case that any of several different manipulations (including antibody binding and proteolysis) can each produce that change. At present the idea that laminin's long arm can be conformationally activated from a consider-

able distance and by multiple means seems less parsimonious, but cannot be ruled out.

The third hypothesis that was considered proposes that the activity induced in laminin by anti-short arm antibodies is synthetic, consisting of the combination of an activity (or activities) in the distal long arm with a second, cryptic activity that maps to the short arms and that is conformationally activated by anti-short arm antibodies. The facts that the isolated C8-9 and E8 fragments (which contain no short arm sequences) promote retinal neurite outgrowth (Figure 9) and that E8 promotes α_6 -independent neuronal migration (Table 4) argue strongly against this hypothesis.

If the first of these three hypotheses is correct, and laminin's short arms are the site of a suppressor activity, what might be the physical basis for such an activity? At one extreme, one might imagine that laminin's short arms interact with cell surface receptors to send a signal which down-regulates the function of an E8-binding integrin receptor (intracellular signals are known to regulate integrin function [reviewed by Hynes, 1992]). At the other extreme, laminin's short arms may send no specific signal of their own, but may physically hinder the access of the E8 region to some integrin receptors. For example, laminin's short arms might compete for the same non- α_6 integrin that E8 recognizes (provided that short arm binding to that integrin does not itself trigger cell migration or neurite outgrowth). Alternatively, laminin's short arms may simply bind tightly to some other cell surface component(s), such that—owing to the relative inflexibility of the laminin molecule—parts of the E8 region are simply physically unable to contact cell surface receptors at the same time.

In principle, some of the above possibilities could be tested by culturing cells on substrata containing mixtures of short arm and long arm fragments of laminin, in an attempt to reconstitute the suppression that apparently occurs in the intact molecule. In fact, when the E1' fragment and the E8 fragment were mixed and applied to substrata, retinal neurite outgrowth activity was indeed not detected, but neither was it detected when such mixed substrata were further treated with anti-short arm antibodies (unpublished data). In other words, the E1' fragment inhibited the function of E8, but apparently by some mechanism other than the one operating when both are present in the same molecule. In other studies, we have found that the E1' fragment is strongly anti-adhesive, a property that seems to reflect the ability of substratum-bound E1' to prevent physical access of cells to codeposited substratum-bound molecules (L. C. Plantefaber, R. M. Kindt, A. L. C., P. D. Y., and A. D. L., unpublished data). This property of E1' cannot be blocked by antibodies (unpublished data) and could potentially explain the inhibition seen when E1' was mixed with E8. In any case, the data indicate that other approaches will be needed to determine whether antibody-inhibitable suppression of E8 function by E1' can in fact be reconstituted using separated fragments.

If laminin's short arms do contain a region that suppresses—by whatever means—a long arm function, it will be important to map that region in greater detail. The fact that, in the present study, the neuronal activities of merosin more closely resembled those of anti-P1'-treated laminin than untreated laminin (Figures 5 and 7) suggests that the proposed suppressor activity in laminin may be lacking in merosin. This suggests that the activity may reside in the short arm derived from the A chain, the one chain not shared by merosin.

"Cryptic" Activities in ECM Proteins

Many studies have documented the existence in ECM molecules of biological activities that have been called cryptic, because they are not observed in the intact, isolated molecule. For example, type I collagen and laminin both exhibit cryptic cell attachment activities, as revealed by proteolysis (Gullberg et al., 1992; Goodman et al., 1991). A proteolytic fragment of fibronectin exhibits a cryptic anti-proliferative effect on Schwann cells (Muir and Manthorpe, 1992). Vitronectin binds heparin only after partial denaturation (Hayashi et al., 1985). Recombinant fragments of tenascin exhibit cell attachment activity that the parent molecule lacks (Spring et al., 1989). A C-terminal proteolytic fragment of fibronectin strongly promotes neurite outgrowth from neurons of the central nervous system, even though such neurons show no detectable response to intact fibronectin (Rogers et al., 1985). This last example is of particular relevance, as it documents another case—in addition to the present study—in which the ability of an ECM molecule to promote the motile behavior of neurons is activated.

The present study raises the interesting possibility that not all of these activities are cryptic in the biochemical sense (i.e., mediated by protein conformations that are absent or buried in the intact, untreated molecule). Instead, some may reflect the interaction of countervailing functions that reside in distinct molecular domains. Recently, investigators studying the large ECM molecule tenascin appear to have come to similar conclusions. For example, Prieto et al. (1992) presented evidence for interacting adhesive and anti-adhesive activities within individual cytotactin (tenascin) domains. This group also reported that cell attachment to one such domain could be observed only after pretreatment of the cells with trypsin, suggesting that suppression of adhesion could be relieved by the removal of cell surface molecules.

Biological Significance of Latent Migration- and Neurite Outgrowth-Promoting Activity in Laminin

The existence of latent, antibody-induced, migration- and neurite outgrowth-promoting activity of laminin raises interesting questions about the responses of other cells to laminin and about laminin's actions *in vivo*. If the suppressor activity of the short arms of laminin acts through a cell surface receptor, then

some types of cells may lack that receptor, thereby circumventing suppression. Additionally, it is possible that binding proteins exist *in vivo* that interact with laminin's short arms in a manner analogous to anti-short arm antibodies and uncover latent activity *in situ*. Finally, the recent realization that numerous different, but homologous polypeptide chains assort *in vivo* to form an increasing family of laminin isoforms (Ehrig et al., 1990; Sanes et al., 1990; Kallunki et al., 1992; Vailly et al., 1994) creates the potential for considerable diversity in the net biological activities of laminins.

These considerations are especially relevant in the area of retinal development and regeneration. Until recently, it was generally believed that late embryonic and postnatal mammalian retinal ganglion cells were incapable of responding to laminin, owing largely to the developmental loss of α_6 integrins (de Curtis et al., 1991). This view was questioned when Cohen and Johnson (1991) found that merosin promoted neurite outgrowth by late embryonic retinal ganglion cells and is furthered weakened by the present evidence that laminin can easily be activated to promote late retinal neurite outgrowth. Since at least one chain of laminin is expressed postnatally in the retina (and by ganglion cells themselves [Sarthý and Fu, 1990]) and since laminin immunoreactivity can be induced by optic nerve lesion (Zak et al., 1987), it is intriguing to speculate that, with appropriate manipulation, endogenous laminin might be activated to promote outgrowth in retinal axon regeneration.

Experimental Procedures

Materials

ECM Proteins and Fragments

Laminin for testing in cell adhesion, migration, and neurite outgrowth assays was purified from the Engelbreth-Holm-Swarm (EHS) sarcoma according to published methods (Kleinman et al., 1982; Timpl et al., 1982). Proteolytic fragments of laminin were prepared from intact laminin and laminin/entactin that had been isolated from lathyrus EHS tumor according to the EDTA extraction method of Paulsson et al. (1987) and purified as described by Yurchenco et al. (1992). Elastase fragments E1', E3, E4, and E8 were generated at an enzyme:substrate ratio of about 1:250 at 0°C for 1 hr, followed by 25°C for 23 hr, and purified by gel filtration on Sepharose CL-6B (Pharmacia, Piscataway, NJ) and by high pressure liquid DEAE-5PW ion exchange chromatography (15 × 2 cm internal diameter; Toso-Haas, Philadelphia, PA) as previously detailed (Schittny and Yurchenco, 1990) except that only the peak fractions of E1' were pooled after ion exchange for higher purity. Fragment P1' was prepared from a pepsin digest of laminin by Biogel A1.5M gel filtration (Bio-Rad, Richmond, CA) in 1 M CaCl₂ as described (Schittny and Yurchenco, 1990). Recombinant mouse laminin G domain (G), produced as a secreted glycoprotein (fused to the N-terminal signal peptide and first 20 amino acids of rat fibronectin) by sf9 cells infected with recombinant baculovirus, was prepared, purified, and characterized as described (Yurchenco et al., 1993). Protein concentrations of laminin and laminin fragments were measured by amido black binding (Schaffner and Weissman, 1973) or by absorption at 280 nm. Laminin concentrations were also confirmed by amino acid analysis.

Fibronectin (from human plasma) was purchased from the New York Blood Center (New York, NY). Merosin (from human placenta) was obtained from Telios Pharmaceuticals (San Diego,

CA) and represents a laminin isoform in which the A chain is substituted by a homologous chain, known variously as M or A_m. Although the term merosin has sometimes been used to refer to just the M chain itself, in this study it is used only to refer to the complete, holotrimeric, B1- and B2-containing molecule.

Antibodies

Purified IgG from the GoH3 hybridoma was purchased from AMAC, Inc. (cat. #0769). Rabbit antiserum against rodent β_1 integrins (Lenny) was obtained from Clayton Buck (Wistar Institute). Antibodies to laminin fragments were prepared by immunizing New Zealand White rabbits (long eared variety) with 0.5–1 mg of protein in complete Freund's adjuvant, with boosting at 2 and 4 weeks. Rabbit polyclonal antibodies specific for laminin fragments E3 and E8 were prepared by affinity purification and characterized as described (Yurchenco et al., 1993). Antibodies specific for P1' were prepared by immunizing with fragment E1', followed by affinity purification of antiserum on immobilized E1', cross-absorption against E4, E8, E3, and entactin (nidogen), and final affinity purification on immobilized P1'. These affinity columns, in which protein was coupled either to Affi-Prep 10 (Bio-Rad) or to CNBr-activated Sepharose CL-4B, were equilibrated in 10 mM sodium phosphate (pH 7.4), 127 mM NaCl and eluted with 0.1 M acetic acid. Acid-eluted fractions from columns coupled with any one laminin fragment were dialyzed into 10 mM sodium phosphate (pH 7.4), 127 mM NaCl and then passed sequentially over columns to which each of the other (nonoverlapping) fragments had been immobilized in the same way. The nonbinding fraction from these cross-absorptions constituted the anti-fragment antibodies that were used in all assays. In some experiments, indicated in the text, a commercial anti-mouse laminin antiserum (cat. #18432, lot 404229, Polysciences, Inc., Warrington, PA) was also tested.

Specificity of anti-fragment antibodies was established by competitive immunoassay. Briefly, polystyrene ELISA plates (Linbro) were coated overnight at 4°C with 1 μ g per well of laminin in 100 μ l. Anti-fragment antibodies (2 μ g/ml) were incubated with competing antigens (laminin fragments, entactin) at concentrations from 0.01 to 100 μ g/ml and applied to laminin-coated plates for 1–2 hr at room temperature. After washing, bound antibody was detected with horseradish peroxidase-conjugated protein A (0.5 μ g/ml) and developed with o-phenylenediamine. In all cases, antibodies isolated as specific for any one fragment could be inhibited by levels of that fragment that were at least 2–3 orders of magnitude lower than the level required for inhibition by any other (nonoverlapping) fragment or entactin.

To prepare Fab fragments of antibodies, 86.9 μ l of 1 mg/ml papain was diluted into 2 ml of 10 mM dithiothreitol, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, then immediately mixed with antibody (1–2 mg/ml in 0.1 M sodium phosphate [pH 7.0], 2 mM EDTA) at an enzyme:substrate ratio of 1:100, and digestion was allowed to proceed for 4 hr at 37°C. The reaction mixture was cooled on ice and adjusted to 1 mM iodoacetamide, tested for degree of digestion by SDS-polyacrylamide gel electrophoresis, dialyzed into 50 mM Tris-HCl (pH 8), and cleared of free Fc and any remaining intact antibody by passage over protein A-agarose.

Other Reagents

Crystallized BSA was purchased from ICN Biomedicals, Inc. Tissue culture media and additives were purchased from GIBCO-BRL or Cellgro-Mediatech (Fisher Scientific Co.). Unless indicated, all other supplies were purchased from Sigma.

Preparation of Cells

Explant cultures of OE, purified from embryonic day 14.5–15.5 mouse embryos (CD-1 random-bred mice; Charles River), were prepared and cultured in serum-free, low calcium medium (complete LCM) as described (Calof and Lander, 1991). Retinal neurons were prepared from retinas of day 18 Sprague-Dawley rat embryos, or day 16 CD-1 mouse embryos, that had been finely minced and incubated at 37°C in trypsin (1 mg/ml) in Leibovitz' L-15 medium with 0.3% glucose. Digestion was terminated with soybean trypsin inhibitor (1.4 mg/ml final concentration) and DNAase (0.1 mg/ml final concentration), the tissue was washed into culture medium (50:50 DME:F12 with 2 mM glutamine, 100

U/ml penicillin, 100 µg/ml streptomycin, 5 mg/ml crystalline BSA, and N2 additives [Bottenstein and Sato, 1979]), and cells were isolated by trituration using a flame-polished Pasteur pipette and collection by centrifugation.

OE explants or dissociated retinal neurons were plated onto culture substrata (glass coverslips or, when indicated, 96 well tissue culture plates [Costar 3596]), treated as described below, and then cultured for 18–19 hr at 37°C in a 5% CO₂, 95% air atmosphere. At the end of this period, cultures were fixed for a minimum of 2 hr at room temperature by underlying the culture medium with a solution of 3.7% formaldehyde, 5% sucrose in phosphate-buffered saline.

Preparation of Substrata

ECM proteins or fragments were diluted in calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS; pH 8.2) and then allowed to adsorb to acid-cleaned glass coverslips (12 mm round, thickness #1, Propper Mfg., Long Island City, NY; see Calof and Chikaraishi, 1989) or 96 well tissue culture plates as indicated, typically either overnight at 4°C or for 3 hr at 37°C. Prior to application of antibodies, substrata were washed 3 times in CMF-HBSS, then blocked with heat-inactivated BSA (10 mg/ml in CMF-HBSS, heat treated for 2 hr at 70°C), and held overnight at 4°C. After blocking, substrata were treated with antibody solutions, diluted in complete culture medium, for a minimum of 4 hr at 4°C. Coverslips were washed 3 times in complete culture medium and placed into 24 well tissue culture trays, and OE explants or retinal cells were plated onto them in a volume of 1–1.5 ml of complete medium per well. Trays (96 well) were washed 3 times in complete medium, and OE explants or retinal cells were plated into them in a volume of 150–200 µl per well.

Measurement of Cell Migration and Neurite Outgrowth

To quantitate cell migration by OE neuronal cells, fixed OE explant cultures were visualized on a Zeiss Axiovert microscope equipped with a 10× objective and phase-contrast optics. The image was magnified further through a 4× (for cell migration measurements) or a 10× (for neurite length measurements) objective placed in front of the video camera (Hamamatsu Photonics, Hamatsu City, Japan) and processed through a series 151 Image Processor (Imaging Technologies, Woburn, MA). Using GBS 151 morphometric software (Version 2, Infrascan, Inc.), measurements of cell migration distances were made as follows: The perimeter of an individual explant (cultured piece of OE) was outlined, and its area (in µm²) was determined. The distance that each cell had migrated was taken as the straightline distance between the center of its cell body and the nearest edge of the explant; this measurement was made for every migratory cell of each explant that was evaluated. All migratory cells were counted for explants totaling a minimum area of 300,000 µm² (usually 4 or more explants). For conditions in which migration was abundant, as with a laminin substratum, this corresponded to measuring the migratory distances of more than 600 total cells for a given condition. Cell migration curves were generated for each condition by plotting migration distances (abscissa) versus the number of cells that migrated equal to or greater than a given distance (ordinate). Cell number (ordinate) values were normalized to the total explant area evaluated in a given condition, to correct for variations in the number and sizes of explants evaluated under different experimental conditions.

To quantitate neurite outgrowth by retinal neurons, fixed cultures were examined as described above and scored for the fraction of cells (or small clusters of cells) bearing neurites >2 cell diameters in length. Large clusters of cells (e.g., >10), which were observed on occasion, were excluded from the analysis.

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