

The NC1 Domain of Type IV Collagen Promotes Axonal Growth in Sympathetic Neurons through Interaction with the $\alpha_1\beta_1$ Integrin

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Abstract. We have examined the effects of collagen IV on the morphological development of embryonic rat sympathetic neurons in vitro. In short-term (≤ 24 h) culture, collagen IV accelerated process outgrowth, causing increases in the number of neurites and total neuritic length. Analysis of proteolytic fragments of collagen IV indicated that the NC1 domain was nearly as active as the intact molecule in stimulating process outgrowth; in contrast, the 7S domain and triple helix-rich fragments of collagen IV were inactive. Moreover, anti-NC1 antiserum inhibited neuritic outgrowth on collagen IV by 79%. In long-term (up to 28 d) cultures, neurons chronically exposed to collagen IV maintained a single axon but failed to form dendrites. Thus, the NC1 domain of collagen IV can alter neuronal development by selectively stimulating axonal growth.

Comparison of collagen IV's effects to those of laminin revealed that these molecules exert quantitatively different effects on the rate of initial axon growth and the number of axons extended by sympathetic neurons. Moreover, neuritic outgrowth on collagen IV, but not laminin, was blocked by cycloheximide. We also observed differences in the receptors mediating the neurite-promoting activity of these proteins. Two different antisera that recognize β_1 integrins each blocked neuritic outgrowth on both collagen IV and laminin; however, an mAb (3A3) specific for the $\alpha_1\beta_1$ integrin inhibited collagen IV but not laminin-induced process growth in cultures of both sympathetic and dorsal root neurons. These data suggest that immunologically distinct integrins mediate the response of peripheral neurons to collagen IV and laminin.

EXTRACELLULAR matrix (ECM)¹ molecules influence numerous aspects of neural development, including cellular migration, process outgrowth, synaptogenesis, and myelination (reviewed in Sanes, 1989). The most potent and effective protein within neural ECM appears to be laminin, which has been shown to be involved in nerve regeneration in situ (Sandrock and Matthew, 1987). Tissue culture studies reveal that laminin can increase the number of processes extended by neurons in vitro and/or the rate at which they grow (reviewed by Reichardt et al., 1989; Sanes, 1989; Sephel et al., 1989), and can also participate in the guidance of growth cones (Hammarback et al., 1988). Additionally, laminin may play a role in neuronal morphogenesis since its actions have recently been shown to be process-specific. That is, laminin has been shown to selectively modulate axonal but not dendritic growth in sympathetic (Lein and Higgins, 1989) and hippocampal neurons in vitro (Lein, P. J., G. A. Banker, and D. Higgins, manuscript in preparation).

In addition to laminin, the peripheral nervous system contains other ECM components such as collagen IV, nidogen/entactin, fibronectin, and heparan sulfate proteoglycan. That these other molecules play significant roles in neuronal development and regeneration is suggested by demonstrations that laminin alone is not sufficient to explain either migration or process outgrowth and guidance phenomena in situ (Reichardt et al., 1989; Sanes, 1989). One ECM protein which may be an important modulatory factor in the development and regeneration of the peripheral nervous system is collagen IV. Type IV collagen, which is synthesized by glial cells (Carey et al., 1983), is present in both fiber tracts and ganglia of the peripheral nervous system (Bunge et al., 1989). Within the peripheral nervous system, collagen IV serves as a structural component of the endoneurial basement membrane, providing tensile strength as well as a scaffold with which other basement membrane components can associate (Timpl, 1989). However, recent evidence indicates that collagen IV can also directly affect cellular functions. In nonneuronal cells, it regulates attachment, migration, and morphological differentiation (Kleinman et al., 1982a; Aumailley and Timpl, 1986; Chen and Little, 1987;

1. *Abbreviations used in this paper:* ECM, extracellular matrix; PIV, pepsinized collagen IV.

Herbst et al., 1988; Chelberg et al., 1989). Collagen IV also promotes neuritic outgrowth in select neuronal cell types (Carbonetto et al., 1983; Hall et al., 1987; Tomaselli et al., 1987; Politis, 1989; Turner et al., 1987). The domains of the molecule responsible for this latter activity, as well as the identity of the processes affected, have not been previously defined.

To further elucidate the role of collagen IV in the peripheral nervous system, the present study was initiated to (a) define the domain(s) of the collagen IV molecule which affect neuritic outgrowth; (b) characterize collagen IV's effects on axonal and dendritic development in sympathetic neuron *in vitro*; and (c) compare these effects to those induced by the well-characterized neurite-promoting factor, laminin. In addition, we sought to identify the receptor for collagen IV in peripheral neurons. Previous *in vitro* studies indicate that the neurite-promoting activities of collagen IV and laminin are mediated primarily by receptors of the β_1 integrin family (Hall et al., 1987; Tomaselli et al., 1987, 1988). The recent development of antisera specific for individual α integrin subunits has led to the identification of the $\alpha_3\beta_1$ integrin as a receptor that mediates process outgrowth on laminin in human neuroblastoma cells (Clegg et al., 1989); and the $\alpha_1\beta_1$ integrin as a dual collagen IV/laminin receptor in PC12 cells (Turner et al., 1989; Tawil et al., 1990; Tomaselli et al., 1990). In this study, we demonstrate that an mAb (3A3) specific for the $\alpha_1\beta_1$ integrin inhibits collagen IV but not laminin-induced process outgrowth in primary cultures of sympathetic and dorsal root sensory neurons. These data suggest that, for neurons of the peripheral nervous system, the $\alpha_1\beta_1$ integrin serves primarily as a collagen IV receptor and a different integrin mediates responses to laminin. Since mAb 3A3 has also been shown to retard axonal regeneration in the sciatic nerve *in situ* (Toyota et al., 1990), our observations suggest a regulatory role for collagen IV in the growth of axons of peripheral neurons.

Materials and Methods

ECM Proteins and Enzymatic Digests

Collagen IV and laminin were purified from the murine Engelbreth-Holm-Swarm tumor as previously described (Kleinman et al., 1982b). Laminin was radiolabeled by the Iodogen method (Haas and Bright, 1985), using Na^{125}I (28 $\mu\text{Ci}/\mu\text{l}$) from Amersham Corp. (Arlington Heights, IL) and Iodogen from Pierce Chemical Co. (Rockford, IL). Type IV collagen and collagen IV-derived peptides were radioiodinated as described by Markwell (1982) using Na^{125}I (10 $\mu\text{Ci}/\mu\text{l}$) from ICN K & K Laboratories Inc. (Plainview, NY) and IODO-BEADS from Pierce Chemical Co. Human plasma fibronectin was purchased from Bethesda Research Laboratories (Gaithersburg, MD); human serum fibrinogen was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Rat tail tendon type I collagen was purified according to the procedure of Bornstein (1958). Previously described methods were used to prepare the NC1 (Timpl et al., 1985) and 7S domains (Timpl et al., 1981) of native type IV collagen. Pepsinized type IV collagen depleted of the 7S domain was prepared as described by Timpl et al. (1983). The purity of all collagen IV peptides was assessed by SDS-PAGE and by diode array multi-wavelength analysis spectrophotometry downstream from a C18 μbond pac reverse phase column.

Antibodies

Goat antimouse laminin antibody (polyclonal, monospecific), rabbit antimouse type IV collagen antibody (affinity purified), and anti-7S domain of type IV collagen (also affinity purified) were prepared as previously described (Terranova et al., 1980; Liotta et al., 1981; Foidart et al., 1980).

Specificity was determined by ELISA and dot blot assays. Antilaminin antibodies did not cross-react with native type IV collagen, fibronectin, or heparan sulfate proteoglycan. Anticollagen IV antibody did not recognize the native 900-kD laminin molecule or heparan sulfate proteoglycan. Anti-7S antibody did not cross-react with the NC1 domain of type IV collagen, with laminin, or with heparan sulfate proteoglycan. Rabbit antimouse polyclonal antiserum specific for the NC1 domain of type IV collagen (Tsilibary and Charonis, 1986) was generously donated by Dr. Effie Tsilibary (University of Minnesota). A goat antiserum (anti-gpl40, here referred to as anti-ECMR) which was raised against purified adhesion-related, 140 kD, integral membrane glycoproteins from BHK cells (Knudsen et al., 1981) was the generous gift of Dr. Karen Knudsen (Lankenau Medical Research Center, Philadelphia, PA). The IgG fraction of antiserum recognizing the rat β_1 integrin subunit (Gullberg et al., 1989) was kindly provided by Dr. Kristofer Rubin (University of Uppsala). The mAb designated 3A3 which specifically recognizes the $\alpha_1\beta_1$ integrin (Ignatius et al., 1990; Tawil et al., 1990) was prepared as previously described (Turner et al., 1989).

Cell Surface Labeling and Immunoprecipitation

Membrane proteins of sympathetic neurons (~ 20 dissociated ganglia) in culture at high density for 1 wk were iodinated enzymatically with lactoperoxidase and Na^{125}I (1 $\mu\text{Ci}/\text{ml}$) as described by Gullberg et al. (1990). Labeled cells were rinsed extensively with DME then extracted with 1 ml of PBS containing 1% Triton X-100, 1 mM CaCl_2 , 1 mM MgCl_2 , 5 $\mu\text{g}/\text{ml}$ leupeptin, 0.5 mM phenylmethanesulfonylfluoride, and 2 $\mu\text{g}/\text{ml}$ pepstatin A. Immunoprecipitates of these cell extracts were obtained using purified mAb 3A3 coupled to Affi-Gel 10 beads (Bio-Rad Laboratories, Cambridge, MA) as described by Turner et al. (1989). Protein bound to 3A3-coupled beads was eluted in nonreducing SDS sample buffer (Laemmli, 1970) and analyzed by SDS-PAGE (5% acrylamide).

Tissue Culture

Using previously described methods, suspensions of dissociated superior cervical ganglion (20–21 d; Lein and Higgins, 1989) or lumbar dorsal root ganglion (15–16 d; Bunge et al., 1983) neurons were obtained from Holtzmann Co. (Madison, WI) rat fetuses, plated at densities ranging from 3–8 cells/ mm^2 onto glass coverslips (Bellco Glass, Inc., Vineland, NJ) precoated with poly-D-lysine (100 $\mu\text{g}/\text{ml}$; Sigma Chemical Co., St. Louis, MO), and maintained in a serum-free medium (Lein and Higgins, 1989). This medium contains a maximally effective concentration (100 ng/ml) of β -nerve growth factor that allows the long-term survival (≥ 2 mo) and growth of sympathetic neurons in culture (Bruckenstein and Higgins, 1988a). Cytosine- β -D-arabinofuranoside (1 μM) was added to the medium of all cultures on the second and third days and this exposure was usually sufficient to render them virtually free of nonneuronal cells for 30–40 d (Tropea et al., 1988).

Cultures were exposed to ECM proteins either by binding these proteins to a polylysine-coated substrate before the addition of cells (precoating), or by adding these factors directly to the culture medium. To precoat, polylysine-coated coverslips were soaked in 1 ml of a solution containing the factor at concentrations indicated in the text for at least 4 h at 4°C. Collagens I and IV, collagen IV-related peptides, fibronectin, and fibrinogen were diluted in sterile H_2O . Laminin was diluted in DME (Gibco Laboratories, Grand Island, NY). Before the addition of cells, coverslips were rinsed twice with DME. The amount of collagen IV, collagen IV-derived proteolytic fragments, or laminin that bound to the substrate under these conditions was quantitated using radiolabeled proteins.

Immunocytochemistry

Cultures were immunostained with antibodies previously shown to selectively react with either dendritic or axonal antigens, both *in situ* and *in vitro*. An mAb to the nonphosphorylated forms of the M and H neurofilament subunits (SMI32; Sternberger-Meyer Immunochemicals, Jarrettsville, MD) was used as a dendritic marker (Sternberger and Sternberger, 1983; Bruckenstein and Higgins, 1988a). Axonal probes included antibodies specific for synaptophysin (SY 38; Boehringer-Mannheim Biochemicals [Indianapolis, IN]; Weidenmann and Franke, 1985), and phosphorylated forms of either the H (NE14; Boehringer-Mannheim; Shaw et al., 1986) or the M and H (SMI31; Sternberger-Meyer Immunochemicals; Sternberger and Sternberger, 1983) neurofilament subunits. All antigens were localized by indirect immunofluorescence as described in Lein and Higgins (1989).

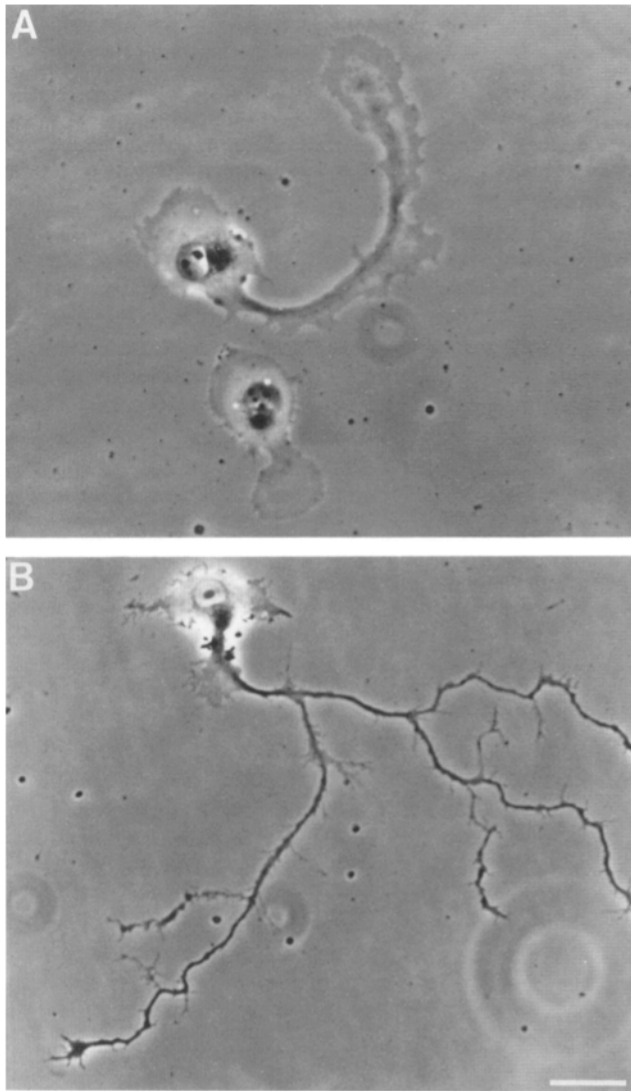


Figure 1. Phase-contrast micrographs of sympathetic neurons after 24 h in vitro. Most neurons plated onto polylysine in the absence of collagen IV generally had not extended neurites by 24 h. Those neurons on polylysine that had initiated neurite outgrowth (A) typically had one short, wide, unbranched process which ended in a prominent growth cone. In contrast, neurons plated onto coverslips precoated with collagen IV at 100 $\mu\text{g/ml}$ (B) typically extended one thin neurite of substantial length which was often highly branched. Under both culture conditions, cytoplasmic veils often surrounded neuronal somata. Bar, 25 μm .

Dye Injections and Morphometry

Intracellular injections of Lucifer yellow (method of Higgins et al., 1988) were used to evaluate the number of axons and number of primary dendrites extended by neurons in long-term cultures (3–28 d). Axons were distinguished from dendrites using standard light microscopic criteria (Bruckenstein and Higgins, 1988a). Previous studies have indicated that the processes of sympathetic neurons in culture identified as axons or dendrites using these light microscopic criteria also express appropriate cytochemical, immunological, and ultrastructural properties (Peng et al., 1986; Bruckenstein and Higgins, 1988a,b; Tropea et al., 1988). To measure total neuritic length in short-term cultures (≤ 24 h), camera lucida drawings of neurons visualized by phase-contrast microscopy were analyzed using a Jandel Scientific digitizing tablet and SigmaScan software (Jandel Scientific, Corte Madera, CA). In all morphometric studies, only isolated neurons, i.e., neurons whose cell bodies were at least 150 μm from the soma of their

nearest neighbor, were analyzed, since earlier studies (Bruckenstein and Higgins, 1988a) demonstrated that density-dependent changes in cellular morphology occur when cell bodies are separated by lesser distances. Only processes whose length was greater than the diameter of the cell body were scored. At least 30 randomly chosen neurons (≥ 10 neurons from three different cultures) were evaluated for each experimental condition. Each experiment was replicated in cultures obtained from at least two different dissections. Unless otherwise specified, all data in the text are from a single representative culture series. Data in the text are expressed as the mean \pm SEM. The percentage of inhibition of neuritic growth was calculated as $100 \times \{1 - [(growth\ on\ ECM\ molecule\ in\ the\ presence\ of\ inhibiting\ agent - growth\ on\ polylysine\ in\ the\ presence\ of\ inhibiting\ agent) / (growth\ on\ ECM\ molecule\ in\ the\ absence\ of\ inhibiting\ agent - growth\ on\ polylysine\ in\ the\ absence\ of\ inhibiting\ agent)]\}$.

Results

Collagen IV Promotes Neuritic Outgrowth in Sympathetic Neurons in Short-term Culture

Sympathetic neurons readily adhere to polylysine-coated coverslips with $\sim 80\%$ of the cells attaching within the first hour. Consistent with previous observations of PC12 cells (Turner et al., 1987), the attachment of sympathetic neurons to polylysine is not affected by reduced temperatures (4°C) (not shown) or by exposure to cycloheximide (10 $\mu\text{g/ml}$) (Lein and Higgins, 1991). Thus, adherence of sympathetic neurons to polylysine apparently does not require metabolic activity or protein synthesis.

Although sympathetic neurons attach to polylysine, their neuritic outgrowth on this substrate is extremely slow, and within the first 18 to 24 h in vitro, $<12\%$ of the neuronal population extend processes. Those neurites that are formed are very short (Fig. 1 A and Table I). Adsorption of collagen IV to polylysine does not affect the attachment of neurons to the substrate (Table I); it does, however, promote neuritic outgrowth. Thus, by 18 to 24 h, $\sim 90\%$ of all neurons grown on collagen IV (100 $\mu\text{g/ml}$) have a long, branched process (Fig. 1 B). A quantitative analysis of the differences between neurons grown in the absence or presence of collagen IV is summarized in Table I. After 18 h, collagen IV caused a 9-fold increase in the percentage of neurons with neurites, an 11-fold increase in the number of neurites extended, and a 6.5-fold increase in total neuritic length. This neurite-promoting activity of collagen IV was blocked by anticollagen IV, but not antilaminin antibodies (Table I). Neither of these antisera had a significant effect on attachment of neurons to substrates precoated with polylysine alone or polylysine and an ECM protein. The effects of collagen IV were also observed to be concentration dependent (Fig. 2). Maximal effects on the percentage of neurons with neurites were observed at concentrations greater than or equal to 100 $\mu\text{g/ml}$; half maximal effects were obtained at ~ 30 $\mu\text{g/ml}$.

Collagen IV's Neurite-promoting Activity Is Localized to the NCI Domain

The collagen IV molecule consists of three primary domains: a) the 7S region at the amino terminus; b) the central 330-nm of the molecule which represents the major triple helical portion; and c) the collagenase-resistant carboxyl-terminal globular domain termed NCI (Timpl, 1989). To determine whether collagen IV's neurite-promoting activity resided in one of these domains, the effects of intact collagen IV were

Table I. The Effects of Collagen IV and Laminin on the Neuritic Outgrowth of Sympathetic Neurons after 18 h In Vitro

Substrate	Sera added to culture medium	Number of neurons/culture	Neurons with neurites	Number of neurites/neuron	Total length of neuritic plexus/neuron*
			%		
Polylysine	none	1,103 \pm 71	10	0.13 \pm 0.05	116 \pm 19
	anti-CIV	1,020 \pm 105	12	0.13 \pm 0.05	63 \pm 7
	anti-LAM	1,136 \pm 63	8	0.08 \pm 0.03	141 \pm 36
	nonimmune	1,258 \pm 29	8	0.10 \pm 0.04	142 \pm 42
Polylysine + collagen IV	none	1,157 \pm 32	93	1.48 \pm 0.12	751 \pm 93
	anti-CIV	1,145 \pm 85	35	0.58 \pm 0.11	211 \pm 63
	anti-LAM	1,080 \pm 74	85	1.53 \pm 0.14	822 \pm 93
	nonimmune	1,063 \pm 37	85	1.57 \pm 0.15	865 \pm 125
Polylysine + laminin	none	1,192 \pm 77	100	3.32 \pm 0.16	1,734 \pm 127
	anti-CIV	1,143 \pm 71	98	3.22 \pm 0.16	1,668 \pm 107
	anti-LAM	1,207 \pm 98	40	0.70 \pm 0.13	123 \pm 25
	nonimmune	1,104 \pm 40	100	3.50 \pm 0.23	1,863 \pm 92

Polylysine-coated coverslips were precoated with either laminin at 10 $\mu\text{g}/\text{ml}$ or collagen IV at 100 $\mu\text{g}/\text{ml}$; anticollagen IV (*anti-CIV*), antilaminin (*anti-LAM*), and nonimmune sera were used at a 1/100 (vol/vol) dilution. The data in this table were obtained from a single dissection and the number of cells plated per coverslip was identical between culture conditions. Three coverslips were analyzed per culture condition to determine cell number. With respect to the other parameters analyzed, $n = 60$ neurons per culture condition, unless otherwise specified. Data are represented as the mean \pm SEM.

* $n = 30$ neurons per culture condition; only neurons with neurites were included in calculation of the mean and SEM.

compared to those of the 7S peptide, the NCI domain, or pepsinized collagen IV (PIV). PIV preparations are composed primarily of triple helix-rich fragments of collagen IV and are essentially devoid of the globular NCI domain (Timpl et al., 1983). The effects of these substrate-bound fragments on neuronal morphology after 18 h in vitro are depicted in Fig. 3. Neither PIV nor the 7S peptide had any significant effects on neuritic outgrowth at concentrations ranging from 10 to 100 $\mu\text{g}/\text{ml}$ or 10 to 50 $\mu\text{g}/\text{ml}$, respectively. In contrast, neurons grown on substrates precoated with NCI exhibited an enhanced rate of process growth similar to that observed with intact collagen IV. Furthermore, NCI's effects were concentration dependent over a range similar to that observed

for the intact collagen IV molecule (Fig. 3). Peptide binding studies indicated that the lack of neurite-promoting activity associated with the 7S and PIV fragments was not attributable to greatly decreased binding efficiencies of these peptides to polylysine-coated substrates relative to intact collagen IV or NCI at similar precoating concentrations (Table II). Results obtained using proteolytic fragments were corroborated using antisera specific for the 7S and NCI domains of collagen IV. Thus, anti-7S serum did not inhibit the neurite-promoting activity of intact collagen IV; however, antiserum specific for NCI inhibited the percent of neurons that ex-

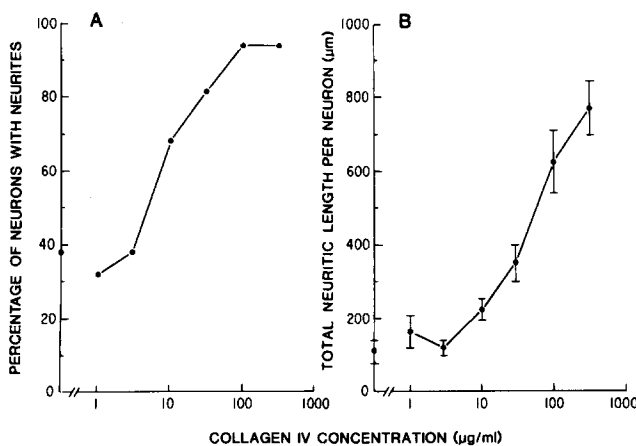


Figure 2. The effects of varying concentrations of collagen IV on the neuritic outgrowth of sympathetic neurons. Neuritic outgrowth was assessed in 18-h cultures of sympathetic neurons with respect to (A) the percentage of neurons with neurites ($n = 100$ per concentration); and (B) the mean length of the neuritic plexus extended by neurons ($n = 30$ per concentration). Error bars correspond to the SEM.

Table II. Binding of ECM Proteins to Polylysine-coated Glass Coverslips

ECM protein	Concentration used to precoat substrate		
	$\mu\text{g}/\text{ml}$	ng/mm^2	fmol/mm^2
Collagen IV (intact)	100	1.5	2.7
	50	0.96	1.7
	10	0.48	0.87
PIV	100	1.4	3.5
	50	0.49	1.2
	10	0.19	0.47
7S	100	0.97	4.3
	50	0.48	2.1
	10	0.07	0.30
NCI	100	0.84	4.9
	50	0.62	3.6
	10	0.26	1.5
Laminin	10	1.7	1.8

ECM proteins were radiolabeled with ^{125}I and adsorbed to polylysine-coated coverslips as described in Materials and Methods. The bound protein was converted to molar values using the following molecular masses: laminin, 950 kD; collagen IV, 550 kD; PIV, 405 kD; 7S, 225 kD; NCI, 170 kD. These data were determined from duplicate samples which typically differed by <10% of the given value.

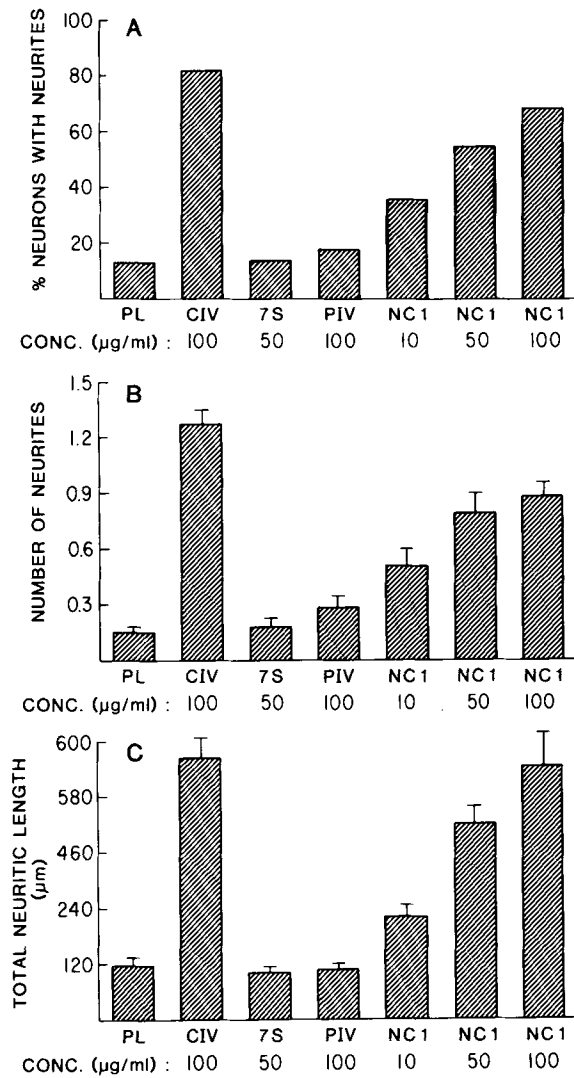


Figure 3. Neurite-promoting activity associated with various domains of collagen IV. Sympathetic neurons were grown on substrates precoated with polylysine alone (PL) or polylysine plus collagen IV (CIV), the 7S peptide, PIV, or the NC1 fragment. Neuronal morphology was analyzed 18 h after plating with respect to the following parameters: (A) the percentage of neurons with neurites ($n \geq 75$ per culture condition); (B) the number of neurites per neuron ($n \geq 75$ per culture condition); and (C) the total neuritic length per neuron ($n \geq 30$ per culture condition). These data are pooled from three separate dissections.

tended neurites on intact collagen IV by 79% (Table III). Those neurons that did respond to collagen IV in the presence of anti-NC1 serum exhibited a 92% inhibition of neuritic length relative to controls grown on collagen IV alone (Table III). Cell counts indicated that the inhibition of neuritic outgrowth by anti-NC1 serum was not due to decreased attachment of cells to mixed substrates of polylysine and collagen IV in the presence of anti-NC1 serum: the number of cells/coverslip ($n = 3$ coverslips/experimental condition) on collagen-IV substrates in the absence of any sera was $1,301 \pm 71$; in the presence of nonimmune sera, $1,038 \pm 41$; and in the presence of anti-NC1 serum, $1,184 \pm 111$. These data suggest that the neurite-promoting activity of collagen IV is mediated by the NC1 domain of the molecule.

Comparison of the Effects of Collagen IV and Other ECM Proteins on Neuronal Morphology

We next compared collagen IV's effects on the morphology of sympathetic neurons in short-term culture to those of other ECM proteins. Neurons did not respond to collagen I, fibronectin, or fibrinogen: after 18 to 24 h in vitro, the morphology of neurons grown on substrates precoated with these proteins at concentrations ranging from 10 to 100 µg/ml did not differ significantly from that of neurons grown on polylysine alone. Neurons did respond to laminin (at a maximally effective precoating concentration of 10 µg/ml) as evidenced by a significant increase in neuritic outgrowth in the absence of any changes in attachment to the substrate (Table I). However, laminin's neurite-promoting effects differed from those of collagen IV. First, laminin caused a significantly larger increase in total neuritic length; and, second, laminin induced the formation of a greater number (three to four) of neurites (Table I). Thus, although collagen IV and laminin both stimulate neuritic outgrowth in short-term culture, there are significant differences in their effects on neuronal morphology.

Previous studies have shown that proteins of the ECM can also exert long-term effects on the morphological differentiation of sympathetic neurons. Thus, laminin causes the stable expression (up to 30 d in vitro) of super-numerary axons (three to four per neuron), while an extract of basement membrane proteins (Matrigel) selectively promotes the extension of dendrites in the absence of any change in axon number (Lein and Higgins, 1989). In this study, we characterized the long-term effects of collagen IV on neurons by examining its effects on neuronal morphology after varying times in culture (from 3 to 28 d).

To verify that cells in long-term studies were continuously exposed to substrate-bound collagen IV, cultures were immunostained with antibody to collagen IV. An intense and homogenous staining of the substrate was observed at all times examined up to 28 d. Other studies indicated that exposing neurons plated on collagen IV-coated substrates to additional collagen IV in the culture medium (100 µg/ml) at every medium change did not affect neuronal morphology or cytochemistry. Under either paradigm of exposure to collagen IV, cell number remained essentially constant for up to 4 wk and the presence of the ECM protein did not alter cell survival. The morphology of neurons grown in the presence of collagen IV remained relatively constant between days 3 and 28, the only apparent change being a time-dependent increase in the density of the plexus formed by distal processes (Fig. 4 A). Lucifer yellow dye injections indicated that the number of neurites extended by these neurons did not change during this time: after 3, 14, and 28 d in culture, neurons grown on collagen IV had $1.6 (\pm 0.16)$, $1.70 (\pm 0.16)$, or $1.67 (\pm 0.12)$ neurites, respectively ($n = 30$ per experimental condition). These processes looked axonal in that they were long and thin ($< 1 \mu\text{m}$ in diameter) and of relatively constant diameter (Fig. 4 B). Dendrites were not observed on most neurons. After the third day in vitro, only 3% of the neuronal population had dendrites, and by day 21, just 17% of the population had formed dendrites ($n = 30$ per time point). The extent of dendritic growth observed in these cultures did not differ from that observed in sister cultures maintained on polylysine-coated coverslips in the absence of exogenous collagen IV (see also Lein and Higgins, 1989).

Table III. The Effect of Antisera to Different Domains of the Collagen IV Molecule on the Neuritic Outgrowth of Sympathetic Neurons after 18 h In Culture

Substrate	Sera added to culture medium	Neurons with neurites	Number of neurites/neuron	Total length of neuritic plexus/neuron*
		%		μm
Experiment 1:				
Polylysine	none	17	0.19 ± 0.05	107 ± 17
Polylysine + collagen IV	none	79	1.12 ± 0.09	664 ± 83
	anti-NC1	30	0.43 ± 0.07	154 ± 50
	nonimmune	72	1.04 ± 0.08	462 ± 67
Experiment 2:				
Polylysine	none	12	0.12 ± 0.05	92 ± 12
Polylysine + collagen IV	none	88	1.46 ± 0.13	520 ± 52
	anti-7S	94	1.38 ± 0.11	518 ± 51

Polylysine-coated coverslips were precoated with collagen IV at $100 \mu\text{g/ml}$; all sera were used at a 1:100 (vol/vol) dilution. The addition of any of the above sera did not reduce cell attachment to the substrate relative to that observed in the absence of any sera. Unless otherwise specified, $n = 100$ (experiment 1) or 50 (experiment 2) neurons per culture condition. Data are represented as the mean \pm SEM.

* $n = 30$ neurons per culture condition; only neurons with neurites were included in calculation of the mean and SEM.

Additionally, to confirm our light microscopic identification of these processes as axons, long-term cultures grown in the presence of collagen IV were immunostained with antibodies known to react with axonal or dendritic antigens in situ. Nonphosphorylated forms of the M and H neurofilament subunits are localized primarily to the somata and den-

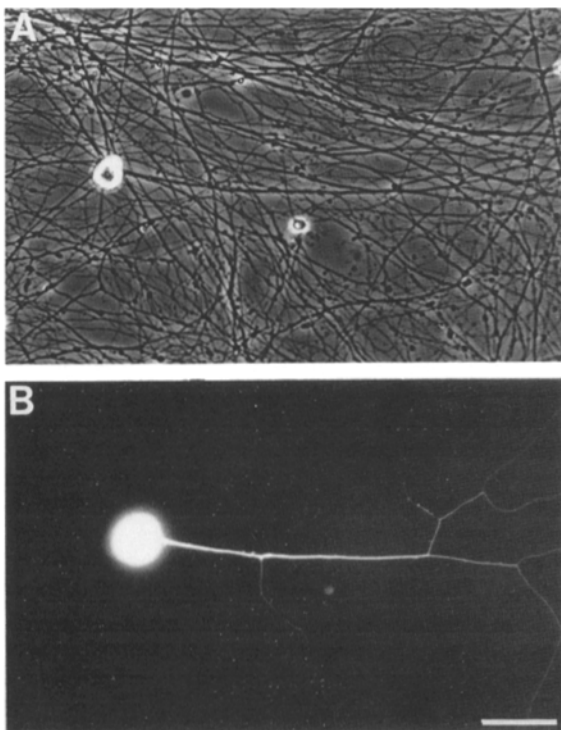


Figure 4. Effects of collagen IV on the morphology of sympathetic neurons in long-term culture. Phase-contrast (A) and fluorescence (B) micrographs of a neuron injected with Lucifer yellow. During the second week in vitro, neurons grown on coverslips precoated with collagen IV at $100 \mu\text{g/ml}$ typically had one long axon that branched frequently near the soma. Dendrites were not observed on most neurons grown under these culture conditions. Bar, $50 \mu\text{m}$.

rites of sympathetic neurons in situ (Carden et al., 1987) and in vitro (Bruckenstein and Higgins, 1988b; Tropea et al., 1988). Fig. 5, A and B are representative of the pattern of staining observed when neurons grown on collagen IV were reacted with mAb (SMI32) specific for the non-phosphorylated forms of these cytoskeletal proteins. Typically, the neuronal soma was intensely fluorescent but there was little or no staining of either proximal or distal processes. Quantitative analysis of cultures immunostained with mAb SMI32 after 18 d in vitro indicated that only 13% of the neuronal population exhibited SMI32-positive processes. These results are consistent with those obtained using dye injections to identify dendritic processes. The long, thin processes comprising the plexus of cultures grown on collagen IV could be stained by antibody (SY38) to the axon-specific antigen, synaptophysin (Fig. 5 C), an integral membrane protein of synaptic vesicles (Wiedenmann and Franke, 1985). When collagen IV cultures were reacted with antibody (NE14) specific for another axon-selective antigen, the phosphorylated H neurofilament subunit (Carden et al., 1987; Bruckenstein and Higgins, 1988a,b), all thin processes were again intensely stained (Fig. 5 D).

These observations suggest that collagen IV enhances the outgrowth of one to two neurites per neuron which eventually assume axonal characteristics. These long-term effects of collagen IV on neuronal morphology differ from those induced by laminin and other ECM components. Next, to further explore the differences in neuronal response to these ECM proteins, we compared the ability of antibodies to various integrin subunits to block collagen IV and laminin-induced neuritic outgrowth.

Comparison of Mechanisms Mediating Neuritic Outgrowth on Collagen IV and Laminin

Anti-ECMR serum has been shown to selectively interfere with the function of integrins in both nonneuronal and neuronal tissues (Tomaselli et al., 1987; Sutherland et al., 1988; Albelda et al., 1989). In PC12 cells, this antiserum immunoprecipitates integrins of the β_1 family (Tomaselli et al., 1987, 1988). As indicated in Table IV, a 0.5% concentra-

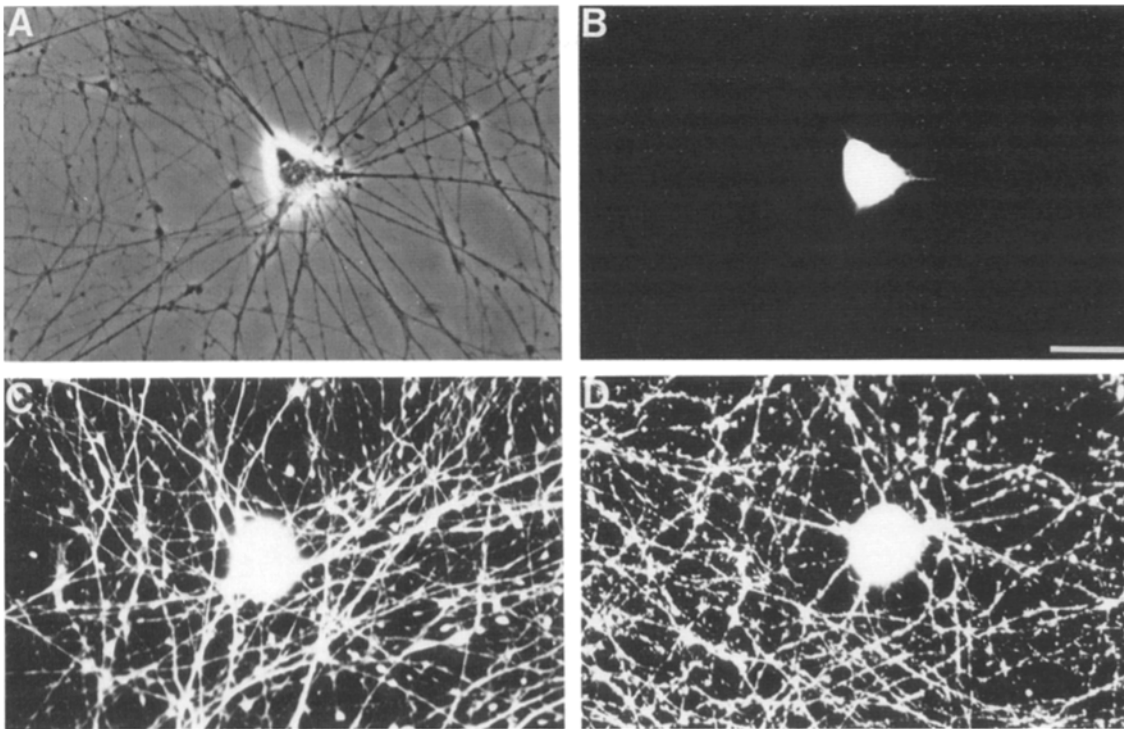


Figure 5. The processes of sympathetic neurons grown on collagen IV in long-term culture have the cytochemical characteristics of axons. Phase-contrast (*A*) and fluorescence (*B–D*) micrographs of sympathetic neurons (10–14 d) exposed to collagen IV-coated (100 $\mu\text{g}/\text{ml}$) substrates. *B* illustrates the pattern of staining typical of cultures reacted with antibody specific for the nonphosphorylated forms of the M and H neurofilament subunits, which are localized primarily to the somata and dendrites of sympathetic neurons in situ. There is intense staining of the somata, but little or no immunoreactivity is observed in neuronal processes. In contrast, the neuronal processes of sister cultures are intensely stained after reaction with antibody directed against the axon-specific antigen, synaptophysin (*C*). These processes also reacted with antibody directed against another axonal antigen, the phosphorylated forms of the H neurofilament subunit (*D*). Bar, 25 μm .

tion of this antiserum markedly inhibited neuritic outgrowth on both collagen IV and laminin. Similar results were obtained using a 0.2% concentration of the antiserum. Control serum at a 0.5% concentration had no effect on process

growth. Attachment of sympathetic neurons to polylysine alone or to polylysine coated with collagen IV or laminin was not affected by anti-ECMR serum (Table IV). The effects of anti-ECMR serum on process outgrowth on either colla-

Table IV. The Effect of Anti-ECMR Sera on the Neuritic Outgrowth of Sympathetic Neurons after 18 h in Culture

Substrate	Sera added to culture medium	Concentration of sera added	Number of neurons/ coverslip	Neurons with neurites	Number of neurites/ neuron	Total length of neuritic plexus/ neuron*
				%		
Polylysine	none	—	2,092 \pm 80	7	0.07 \pm 0.03	69 \pm 15
	nonimmune	0.5	1,860 \pm 124	9	0.12 \pm 0.05	107 \pm 26
	anti-ECMR	0.5	1,898 \pm 6	1	0.01	46
	anti-ECMR	0.2	—	5	0.07 \pm 0.03	45 \pm 7
Polylysine + collagen IV	none	—	1,715 \pm 33	87	1.23 \pm 0.09	683 \pm 76
	nonimmune	0.5	1,493 \pm 94	83	1.16 \pm 0.09	653 \pm 73
	anti-ECMR	0.5	1,538 \pm 54	11	0.11 \pm 0.04	90 \pm 19
	anti-ECMR	0.2	—	21	0.21 \pm 0.05	135 \pm 22
Polylysine + laminin	none	—	1,758 \pm 44	100	3.25 \pm 0.17	1,121 \pm 109
	nonimmune	0.5	1,642 \pm 50	100	3.12 \pm 0.15	1,597 \pm 115
	anti-ECMR	0.5	1,660 \pm 33	23	0.28 \pm 0.07	117 \pm 27
	anti-ECMR	0.2	—	33	0.39 \pm 0.07	188 \pm 36

Polylysine-coated coverslips were precoated with either laminin at 10 $\mu\text{g}/\text{ml}$ or collagen IV at 100 $\mu\text{g}/\text{ml}$. Unless otherwise specified, $n = 75$ per culture condition. Two coverslips per culture condition were analyzed to determine cell numbers. The % inhibition of growth in the presence of antibody (Ab) was calculated as $100 \times [1 - \{(\text{growth on ECM molecule in presence of Ab} - \text{growth on polylysine in presence of Ab}) / (\text{growth on ECM molecule in absence of Ab} - \text{growth on polylysine in absence of Ab})\}]$. Data are represented as the mean \pm SEM.

* $n = 30$ neurons per culture condition; only neurons with neurites were included in the calculation of the mean and SEM for this morphological parameter.

Table V. The Effect of mAb 3A3 on Neuritic Outgrowth from Sympathetic Neurons in 18-h Cultures

Substrate	Concentration of mAb used	Neurons with neurites	Number of processes/neuron	Total length of neuritic plexus/neuron*
	$\mu\text{g/ml}$	%		μm
Polylysine	0	9	0.11 \pm 0.04	75 \pm 11
	10	9	0.11 \pm 0.04	69 \pm 11
	30	11	0.13 \pm 0.05	89 \pm 11
Polylysine + collagen IV	0	83	1.25 \pm 0.10	667 \pm 78
	10	37	0.51 \pm 0.08	151 \pm 21
	30	19	0.24 \pm 0.06	118 \pm 20
Polylysine + laminin	0	96	2.96 \pm 0.19	1,465 \pm 82
	10	91	2.87 \pm 0.20	1,650 \pm 81
	30	95	2.89 \pm 0.17	1,558 \pm 91

Polylysine-coated coverslips were precoated with laminin at 10 $\mu\text{g/ml}$ or with collagen IV at 100 $\mu\text{g/ml}$. Unless otherwise noted, $n = 75$ per culture condition; data are represented as the mean \pm SEM. The % inhibition of growth in the presence of antibody (Ab) was calculated as $100 \times \{1 - [(growth\ on\ ECM\ molecule\ in\ presence\ of\ Ab) / (growth\ on\ ECM\ molecule\ in\ absence\ of\ Ab)]\}$.

* $n = 30$ per culture condition; neurons without neurites were excluded from calculation of the mean and SEM for this morphological parameter.

gen IV or laminin were reversible after removal of the antibody (not shown). To substantiate that anti-ECMR serum was inhibiting neuritic growth by blocking the function of β_1 integrins, we also examined the effects of an antiserum that specifically recognizes the rat β_1 integrin subunit (Gullberg et al., 1989). This antiserum (at 400 $\mu\text{g/ml}$) also reduced the number of processes extended on both collagen IV and laminin by 99 and 92%, respectively.

mAb 3A3, which specifically recognizes the $\alpha_1\beta_1$ integrin (Ignatius et al., 1990; Tawil et al., 1990), was also assessed for its effects on collagen IV and laminin-induced neuritic outgrowth. The presence of this mAb at 30 $\mu\text{g/ml}$ caused a significant inhibition of process growth on collagen IV: the number of neurons with neurites was decreased by 89% and both the number of processes and neuritic length were reduced by approximately ninefold (Table V). Furthermore, these inhibitory effects were concentration dependent, with 63% inhibition observed at 10 $\mu\text{g/ml}$. mAb 3A3 at 10 $\mu\text{g/ml}$ also blocked neuritic outgrowth promoted by the NCI fragment of collagen IV by 67%. In contrast, mAb 3A3 had no effect on neuritic outgrowth on laminin or on polylysine (Table V), a finding which suggests that this mAb interfered specifically with process growth on collagen IV. Similar results were observed in cultures of dorsal root sensory neurons (examined 10 h after plating). Antibody 3A3 (30 $\mu\text{g/ml}$) decreased the number of neurons with neurites on collagen IV by 56%, but had no effect on the number of neurons extending processes on polylysine or laminin. To confirm that the antigen(s) recognized by 3A3 in sympathetic neurons was the $\alpha_1\beta_1$ integrin, cultures were surface labeled with ^{125}I by the lactoperoxidase method, solubilized in a detergent buffer, and immunoprecipitated using mAb 3A3. SDS-PAGE analysis of the immunoprecipitate indicated that 3A3 specifically recognized two surface-iodinated proteins: a strong band of M_r 180,000 and a weaker band of 120,000 under nonreducing conditions.

The response of sympathetic neurons to collagen IV and laminin was also differentially affected by an inhibitor of protein synthesis, cycloheximide. When morphology was examined 6 h after plating, cycloheximide was observed to inhibit

collagen IV-induced neuritic outgrowth, causing an 88% reduction in the number of neurons with neurites, as well as significant decreases in the number of neurites extended per neuron and in total neuritic length (Table VI). In contrast, cycloheximide had only a slight effect on the neuritic outgrowth of neurons grown on laminin. These neurons exhibited a 14% decrease in the number of neurons with neurites, and no significant differences with respect to the number of neurites or neuritic length (Table VI). After 20 h, the marked inhibition of process growth on collagen IV was still apparent, as evidenced by a 94% reduction in the number of neurons with neurites and a 14-fold decrease in neuritic length. Process outgrowth on laminin was also inhibited after 20 h of exposure to cycloheximide, but not to the same extent as that observed on collagen IV. The number of neurons with neurites was decreased by 18% and neuritic length was decreased by eightfold. To confirm that cycloheximide's effects on neuritic outgrowth resulted from an inhibition of protein synthesis and not from a deleterious effect on neuronal viability, these parameters were examined in sympathetic neurons exposed to the inhibitor for 20 h. Cycloheximide at 10 $\mu\text{g/ml}$ decreased ^3H -leucine incorporation into cellular protein by 88% and had no effect on neuronal viability (>95% viable) as determined by trypan blue dye exclusion.

Discussion

We observed that (a) collagen IV selectively enhanced axonal but not dendritic growth in sympathetic neurons; and (b) this axon-promoting activity was associated primarily with the NCI domain. Further studies using antisera against integrins suggest that collagen IV's effects on axonal growth are mediated primarily by the $\alpha_1\beta_1$ integrin. Moreover, an mAb (3A3) that retards sciatic nerve regeneration in situ (Toyota et al., 1990) was found to selectively inhibit the response of sympathetic and dorsal root sensory neurons to collagen IV, but not to laminin. These data suggest that collagen IV may play a significant role in the development and regeneration of axons in the peripheral nervous system.

Table VI. The Role of Protein Synthesis in Laminin and Collagen IV-induced Neuritic Outgrowth during the First 6 h In Vitro

Substrate	Cycloheximide concentration	Neurons with neurites	Number of neurites/neuron	Total length of neuritic plexus/neuron*
	$\mu\text{g/ml}$	%		μm
Polylysine	0	5	0.05 ± 0.03	51 ± 10
	10	5	0.05 ± 0.03	32 ± 4
Polylysine + collagen IV	0	63	1.09 ± 0.14	158 ± 16
	10	12	0.23 ± 0.09	80 ± 16
Polylysine + laminin	0	83	3.04 ± 0.26	443 ± 46
	10	72	2.56 ± 0.23	460 ± 43

Polylysine-coated coverslips were precoated with laminin at 10 $\mu\text{g/ml}$ or collagen IV at 300 $\mu\text{g/ml}$. Unless otherwise noted, $n = 75$ neurons per culture condition; data are expressed as the mean \pm SEM.

* $n = 30$ neurons per culture condition; only neurons with neurites were included in the calculation of the mean and SEM for this morphological parameter.

The NC1 Domain of Collagen IV Promotes Neuritic Outgrowth in Sympathetic Neurons

Collagen IV promoted the growth of processes in short-term cultures of embryonic rat sympathetic neurons. This was evidenced as a decrease in time to initial process extension and increases in the number of primary processes and total neuritic length. It is important to note that the presence of collagen IV in this culture system did not affect cell number or viability; thus, the collagen IV-induced changes in cellular morphology can not be explained as selective survival of neuronal subpopulations. Moreover, collagen IV-stimulated neuritic outgrowth appears to be independent of either endogenous laminin production by neurons (see also Lein and Higgins, 1989) or laminin contamination within our preparations since (a) anticollagen IV, but not antilaminin antibodies blocked neuritic growth on collagen IV; (b) a purified proteolytic fragment of collagen IV with neurite-promoting activity was identified; and (c) collagen IV but not laminin-induced process growth could be selectively inhibited by mAb 3A3.

The collagen IV molecule is composed of three distinct domains (Timpl, 1989), and our data suggest that the intact molecule's neurite-promoting activity is mediated predominantly by the NC1 domain. Purified NC1 elicited neuritic outgrowth comparable to that observed over a similar range of concentrations of the intact collagen IV molecule. Moreover, the neurite-promoting activity of NC1 was inhibited by mAb 3A3. In contrast, neither the 7S domain nor the triple helix-rich fragment (PIV) of collagen IV had any significant effects on process growth. Since binding of 7S and PIV fragments to the substrate was comparable to that of both intact collagen IV and NC1, the lack of neurite-promoting activity observed in the former is not due to their inability to adsorb to polylysine. Additional experiments demonstrated that anti-NC1 antibodies inhibited process growth on collagen IV by 79%, whereas anti-7S antibodies had negligible effects. The large fractional inhibition observed with anti-NC1 antibodies suggests that the axon-promoting activity of collagen IV is mediated primarily by the NC1 domain. However, since complete inhibition was not obtained, and since effects of antibodies against the PIV region were not examined, it is possible that some activity is associated with other do-

main of the intact molecule. Although enhanced neurite growth was not observed in our experiments with purified 7S or PIV fragments, it is possible such activity was lost during the proteolytic preparation of these peptides. This is especially true of PIV, since pepsin treatment can disrupt the interruptions in the triple helical region.

The identification of specific domains within collagen IV that mediate its effects on cellular function has been reported in nonneuronal cell systems. Thus, attachment and migration of human fibrosarcoma cells (Aumailley and Timpl, 1986), aortic endothelial cells (Herbst et al., 1988), and melanoma cells (Chelberg et al., 1989) on collagen IV are differentially mediated by the triple helix-rich and NC1 domains. These observations, together with our demonstration of the domain specificity of collagen IV's neurite-promoting activity, suggest that collagen IV is similar to laminin (Sephel et al., 1989) and fibronectin (Rogers et al., 1989) in that it contains multiple distinct sites which mediate cell attachment, motility, and neurite outgrowth.

The Differing Effects of Collagen IV and Laminin on Axonal Morphology

ECM molecules can regulate the morphogenesis of sympathetic neurons in vitro by selectively modifying axonal or dendritic growth (Lein and Higgins, 1989). Thus, when these neurons are exposed to an extract of basement membrane they form multiple dendrites; however, when grown in the presence of laminin alone, they extend only axons. Our data show that collagen IV resembles laminin in that it also selectively promotes axonal growth. The mechanism by which these proteins confer process-specific information is unknown, but a testable hypothesis is that receptors for ECM molecules with axon-specific effects are expressed on axons but not dendrites. Although qualitatively similar, collagen IV's effects on axonal morphology differed quantitatively from laminin's in that (a) collagen IV caused a significantly smaller increase in total neuritic length (approximately half that on laminin); and (b) collagen IV promoted the growth of a single axon, whereas laminin caused the formation of multiple axons. These differences in axonal morphology persisted even after long-term exposure to individual ECM proteins. Similar results have been observed in

cultures of retinal ganglion cells (Hall et al., 1987). The smaller amount of neuritic outgrowth elicited by collagen IV can not be attributed to decreased binding of this protein to the substrate relative to that of laminin since precoating with maximally effective concentrations of either protein resulted in greater amounts of collagen IV bound. Possible explanations for the differences between collagen IV's and laminin's effects on axonal morphology include (a) these proteins are acting through the same receptor(s) but with different efficacy; or (b) collagen IV's and laminin's effects are mediated via different receptors. Results obtained using antibodies to various integrin subunits suggest the latter may be true in sympathetic neurons.

Collagen IV and Laminin Modulate Process Growth via Immunologically Distinct Integrins

Integrins are a superfamily of transmembrane glycoproteins that mediate many of the effects of ECM on cellular function (Hynes, 1987; Reichardt et al., 1989). Each receptor is a dimer in which a specific β subunit can associate with one of various α subunits to form a functional receptor. Antiserum specific for the rat β_1 integrin subunit (Gullberg et al., 1989) or anti-ECMR antiserum (Knudsen et al., 1981), which selectively inhibits β_1 integrin-mediated functions in both neuronal (Tomaselli et al., 1987; Tomaselli et al., 1988) and nonneuronal cells (Sutherland et al. 1988; Albelda et al., 1989), caused an almost total inhibition of neuritic outgrowth in sympathetic neurons grown on collagen IV or laminin. That this inhibition resulted from a specific disruption of β_1 integrin function is supported by observations that antisera had negligible effects on cell attachment to mixed substrates of polylysine and ECM proteins. Moreover, these inhibitory effects on process growth were reversible.

mAb 3A3, specific for the $\alpha_1\beta_1$ integrin (Ignatius et al., 1990; Tawil et al., 1990), inhibited neuritic outgrowth on collagen IV but not laminin in both sympathetic and dorsal root sensory neurons. The antigen recognized by 3A3 in sympathetic neurons was identified as the $\alpha_1\beta_1$ integrin by immunoprecipitation of detergent extracts of radiolabeled cells followed by SDS-PAGE. As has been observed using PC12 cells (Tomaselli et al., 1990) and several nonneuronal rat tissues (Clyman et al., 1990; Gullberg et al., 1990; Tawil et al., 1990), mAb 3A3 specifically precipitated two surface-labeled proteins whose molecular masses under nonreducing conditions were consistent with those expected for the α_1 (180 kD) and β_1 subunits (120 kD). These data indicate that (a) the $\alpha_1\beta_1$ integrin functions as a selective collagen IV receptor in sympathetic and sensory neurons; (b) the $\alpha_1\beta_1$ integrin is quantitatively the most important receptor mediating the neurite-promoting activity of collagen IV in peripheral neurons; and (c) the effects of laminin are mediated by a β_1 integrin immunologically distinct from $\alpha_1\beta_1$.

Antibody 3A3 has been reported to cause retraction of neurites extended by PC12 cells on collagen or laminin (Turner et al., 1989), suggesting that the $\alpha_1\beta_1$ integrin in this cell type mediates neuritic growth on both ECM proteins. Subsequently, Tomaselli et al. (1990) observed that although 3A3 inhibited both collagen and laminin-induced neurite outgrowth in PC12 cells, these effects were quantitatively different in that there was much less inhibition of the latter. Several explanations for the discrepancy between the above data and our data are plausible: (a) recent observa-

tions suggest that the ligand specificity of the $\alpha_2\beta_1$ integrin is modulated by cell type-specific factors (Elices and Hemler, 1989; Kirchofer et al., 1990), and similar modulation may occur with the neuronal $\alpha_1\beta_1$ receptor; and (b) the relative expression of $\alpha_1\beta_1$ and laminin-binding integrin(s) not recognized by 3A3 may differ quantitatively between neuronal cell types. The validity of either of these possibilities is dependent upon the expression by peripheral neurons of at least one other β_1 integrin that interacts with laminin. In this respect, it is important to note that (a) at least four integrins of the β_1 family have been demonstrated to bind laminin (Gehlsen et al., 1988; Sonnenberg et al., 1988; Elices and Hemler, 1989); (b) three of these laminin-binding integrins have been identified in sympathetic and sensory neurons (Tomaselli et al., 1990); (c) the $\alpha_3\beta_1$ integrin is involved in laminin-induced process outgrowth in a neuroblastoma cell line (Clegg et al., 1989) and in PC12 cells (Tomaselli et al., 1990); and (d) the CSAT mAb which inhibits laminin-induced neuritic outgrowth in chick retinal ganglia cells (Hall et al., 1987) has recently been shown to immunoprecipitate the β_1 subunit complexed to α_3 and α_5 subunits (Hynes et al., 1989). These data suggest that (a) the neurite-promoting activities of collagen IV and laminin can be mediated via different receptors; and (b) laminin-induced process growth may be regulated by multiple β_1 integrins, the relative roles of which may be determined by cell-specific factors.

Cycloheximide markedly inhibited process formation and growth on collagen IV. Observations in other cell systems have indicated that cell attachment to collagen IV, specifically that mediated by the NC1 domain, is also inhibited by cycloheximide (Herbst et al., 1988; Chelberg et al., 1989). In contrast, cycloheximide had no effect on initial process formation on laminin, although by 24 h some inhibition of neuritic growth was observed. Similarly, it has been reported that cycloheximide did not block laminin-mediated attachment and neuritic outgrowth in a neuronal cell line (Luckenbill-Edds and Kleinman, 1988). Thus, the neurite-promoting activity of collagen IV, but not that of laminin, is partially dependent upon de novo protein synthesis. Although the cause of this differential dependence is unknown, these data lend further support to the hypothesis that the neuritic response of sympathetic neurons to collagen IV and laminin is mediated by different receptors.

In summary, laminin has been shown to participate in both the formation and regeneration of the peripheral nervous system. The role of collagen IV in these processes is less clearly defined. This study demonstrates that collagen IV exerts an axon-specific effect on the morphogenesis of sympathetic neurons. Moreover, peripheral neurons express an $\alpha_1\beta_1$ integrin that serves as the dominant receptor mediating collagen IV-induced neuritic outgrowth but does not play a functional role in the response of these neurons to laminin. Since an mAb specific for this receptor retards nerve regeneration *in situ* (Toyota et al., 1990), these data raise the possibility that collagen IV and laminin may operate in a coordinated fashion to regulate axonal growth in the peripheral nervous system. Interestingly, studies of retinal ganglia cells have demonstrated that at the time these neurons innervate their targets, the function of integrins that recognize laminin is downregulated yet the activity of integrins that interact with collagen IV is maintained (Cohen et al., 1987; Hall et al., 1987). Similar studies of the developmental expression of

laminin and collagen IV receptors in sympathetic neurons may allow assessment of their relative roles in the morphological development of axons in this class of neurons.

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References

- Albelda, S. M., M. Daise, E. M. Levine, and C. A. Buck. 1989. Identification and characterization of cell-substratum adhesion receptors on cultured human endothelial cells. *J. Clin. Invest.* 83:1992-2002.
- Aumailley, M., and R. Timpl. 1986. Attachment of cells to basement membrane collagen type IV. *J. Cell Biol.* 103:1569-1575.
- Bornstein, M. B. 1958. Reconstituted rat-tail collagen used as substrate for tissue cultures on coverslips in Maximov slides and roller tubes. *Lab. Invest.* 7:134-137.
- Bruckenstein, D., and D. Higgins. 1988a. Morphological differentiation of embryonic rat sympathetic neurons in tissue culture. I. Conditions under which neurons form axons but not dendrites. *Dev. Biol.* 128:324-336.
- Bruckenstein, D. A., and D. Higgins. 1988b. Morphological differentiation of embryonic rat sympathetic neurons in tissue culture. II. Serum promotes dendritic growth. *Dev. Biol.* 128:337-348.
- Bunge, M. B., R. P. Bunge, D. J. Carey, C. J. Cornbrooks, C. F. Eldridge, A. K. Williams, and P. M. Wood. 1983. Axonal and nonaxonal influences on Schwann cell development. In *Developing and Regenerating Vertebrate Nervous Systems*. P. W. Coates, R. R. Markwald, and A. D. Kenney, editors. Alan R. Liss, Inc., New York. 71-105.
- Bunge, M. B., R. P. Bunge, N. Kleitman, and A. C. Dean. 1989. Role of peripheral nerve extracellular matrix in Schwann cell function and in neurite regeneration. *Dev. Neurosci.* 11:348-360.
- Carbonetto, S., M. M. Gruver, and D. C. Turner. 1983. Nerve fiber growth in culture on fibronectin, collagen and glycosaminoglycan substrates. *J. Neurosci.* 3:2324-2335.
- Carden, J. J., J. Q. Trojanowski, W. W. Schlaepfer, and V. M.-Y. Lee. 1987. Two-stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. *J. Neurosci.* 7:3489-3504.
- Carey, D. J., C. F. Eldridge, C. J. Cornbrooks, R. Timpl, and R. P. Bunge. 1983. Biosynthesis of type IV collagen by cultured rat Schwann cells. *J. Cell Biol.* 97:473-479.
- Chelberg, M. K., E. C. Tsilibary, A. R. Hauser, and J. B. McCarthy. 1989. Type IV collagen-mediated melanoma cell adhesion and migration: involvement of multiple, distinct domains of the collagen molecule. *Cancer Res.* 49:4796-4802.
- Chen, J.-M., and C. D. Little. 1987. Cellular events associated with lung branching morphogenesis including the deposition of collagen type IV. *Dev. Biol.* 120:311-321.
- Clegg, D. O., E. A. Wayner, J. Snyder, D. B. Gervin, A. Bradshaw, G. Cann, E. S. Choi, P. D. Sullivan, and D. M. Eckley. 1989. Evidence for involvement of the integrin alpha 3 subunit in process outgrowth on laminin. *Soc. Neurosci. Abstr.* 15:575.
- Clyman, R. I., D. C. Turner, and R. H. Kramer. 1990. An $\alpha_1\beta_1$ -like integrin receptor on rat aortic smooth muscle cells mediates adhesion to laminin and collagen types I and IV. *Atherosclerosis.* 10:402-409.
- Cohen, J., J. F. Burne, J. Winter, and P. Bartlett. 1987. The role of laminin and the laminin/fibronectin receptor complex in the outgrowth of retinal ganglion cell axons. *Dev. Biol.* 122:407-418.
- Elices, M. J., and M. E. Hemler. 1989. The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. *Proc. Natl. Acad. Sci. USA.* 86:9906-9910.
- Foidart, J. M., E. W. Bere, Jr., M. Yaar, S. I. Rennard, M. Guillino, G. R. Martin, and S. I. Katz. 1980. Distribution and immunoelectron microscopic localization of laminin, a noncollagenase basement membrane glycoprotein. *Lab. Invest.* 42:336-345.
- Gehlsen, K. R., L. Dillner, E. Engvall, and E. Ruoslahti. 1988. The human laminin receptor is a member of the integrin family of cell adhesion receptors. *Science (Wash. DC).* 24:1228-1229.
- Gullberg, D., L. Terracio, T. K. Borg, and K. Rubin. 1989. Identification of integrin-like matrix receptors with affinity for interstitial collagens. *J. Biol. Chem.* 264:12686-12694.
- Gullberg, D., D. C. Turner, T. K. Borg, L. Terracio, and K. Rubin. 1990. Different β_1 integrin receptors on primary hepatocytes and cardiac fibroblasts. *Exp. Cell Res.* 190:254-264.
- Haas, A. L., and P. M. Bright. 1985. The immunochemical detection and quantitation of intracellular ubiquitin-protein conjugates. *J. Biol. Chem.* 260:12464-12473.
- Hall, D. E., K. M. Neugebauer, and L. F. Reichardt. 1987. Embryonic neural retinal cell response to extracellular matrix proteins: developmental changes and effects of the cell substratum attachment antibody (CSAT). *J. Cell Biol.* 104:623-634.
- Hammarback, J. A., J. B. McCarthy, S. L. Palm, L. T. Furcht, and P. C. Letourneau. 1988. Growth cone guidance by substrate-bound laminin pathways is correlated with neuron-to-pathway adhesivity. *Dev. Biol.* 126:29-39.
- Herbst, T. J., J. B. McCarthy, E. C. Tsilibary, and L. T. Furcht. 1988. Differential effects of laminin, intact type IV collagen, and specific domains of type IV collagen on endothelial cell adhesion and migration. *J. Cell Biol.* 106:1365-1373.
- Higgins, D., A. Waxman, and G. Banker. 1988. The distribution of microtubule-associated protein 2 changes when dendritic growth is induced in rat sympathetic neurons *in vitro*. *Neuroscience.* 24:583-592.
- Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549-554.
- Hynes, R. O., E. E. Marcantonio, M. A. Stepp, L. A. Urry, and G. H. Yee. 1989. Integrin heterodimer and receptor complexity in avian and mammalian cells. *J. Cell Biol.* 109:409-420.
- Ignatius, M. J., T. H. Large, M. Houde, J. W. Tawil, A. Barton, F. Esch, S. Carbonetto, and L. F. Reichardt. 1990. Molecular cloning of the rat integrin α_1 -subunit: a receptor for laminin and collagen. *J. Cell Biol.* 111:709-720.
- Kirchhofer, D., L. R. Languino, E. Ruoslahti, and M. D. Pierschbacher. 1990. $\alpha_1\beta_1$ integrins from different cell types show different binding specificities. *J. Biol. Chem.* 265:615-618.
- Kleinman, H. K., D. H. Rohrbach, V. P. Terranova, H. H. Varner, A. T. Hewitt, G. R. Grotendorst, C. M. Wilkes, G. R. Martin, H. Seppa, and E. Schiffman. 1982a. Collagenous matrices as determinants of cell function. In *Immunocytochemistry of the Extracellular Matrix*. Vol. 2. H. Furthmayr, editor. CRC Press, Inc., Boca Raton, FL. 151-174.
- Kleinman, H. K., M. L. McGarvey, L. A. Liotta, P. Gehron Robey, K. Trygvason, and G. R. Martin. 1982b. Isolation and characterization of type IV procollagen laminin and heparan sulfate proteoglycan from the EHS sarcoma. *Biochemistry.* 21:6188-6193.
- Knudsen, K. A., P. E. Rao, C. H. Damsky, and C. A. Buck. 1981. Membrane glycoproteins involved in cell-substratum adhesion. *Proc. Natl. Acad. Sci. USA.* 78:6071-6075.
- Laemmli, U. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680-685.
- Lein, P. J., and D. Higgins. 1989. Laminin and a basement membrane extract have different effects on axonal and dendritic outgrowth from embryonic rat sympathetic neurons *in vitro*. *Dev. Biol.* 136:330-345.
- Lein, P. J., and D. Higgins. 1991. Protein synthesis is required for the initiation of dendritic growth in embryonic rat sympathetic neurons *in vitro*. *Dev. Brain Res.* In press.
- Liotta, L., R. H. Goldfarb, R. Brundage, G. P. Siegal, V. P. Terranova, and S. Garbisa. 1981. Effect of plasminogen activator (urothane), plasmin and thrombin on glycoproteins and collagenous components of basement membrane. *Cancer Res.* 41:4629-4636.
- Luckenbill-Edds, L., and H. K. Kleinman. 1988. Effect of laminin and cytoskeletal agents on neurite formation by NG108-15 cells. *J. Neurosci. Res.* 19:219-229.
- Markwell, M. A. K. 1982. A new solid-state reagent to iodinate proteins. I. Conditions for efficient labeling of antiserum. *Anal. Biochem.* 125:427-432.
- Peng, I., L. I. Binder, and M. M. Black. 1986. Biochemical and immunological analyses of cytoskeletal domains of neurons. *J. Cell Biol.* 102:252-262.
- Politis, M. J. 1989. Exogenous laminin induces regenerative changes in traumatized sciatic and optic nerve. *Plastic Reconstruct. Surg.* 83:228-235.
- Reichardt, L. F., J. L. Bixby, D. E. Hall, M. J. Ignatius, K. M. Neugebauer, and K. J. Tomaselli. 1989. Integrins and cell adhesion molecules: neuronal receptors that regulate axon growth on extracellular matrices and cell surfaces. *Dev. Neurosci.* 11:332-347.
- Rogers, S. L., P. C. Letourneau, and I. V. Pech. 1989. The role of fibronectin in neural development. *Dev. Neurosci.* 11:248-265.
- Sandrock, A. W., and W. D. Mathew. 1987. An *in vitro* neurite-promoting antigen functions in axonal regeneration *in vivo*. *Science (Wash. DC).* 237:1605-1608.
- Sanes, J. R. 1989. Extracellular matrix molecules that influence neural development. *Annu. Rev. Neurosci.* 12:491-516.
- Sephel, G. C., G. A. Burrous, and H. Kleinman. 1989. Laminin neural activity and binding proteins. *Dev. Neurosci.* 11:313-331.
- Shaw, G., M. Osborn, and K. Weber. 1986. Reactivity of a panel of neurofilament antibodies to phosphorylated and dephosphorylated neurofilaments. *Eur. J. Cell Biol.* 42:1-9.
- Sonnenberg, A., P. W. Modderman, and F. Hogervorst. 1988. Laminin receptor on platelets is the integrin VLA-6. *Nature (Lond.).* 360:487-489.
- Sternberger, L. A., and N. H. Sternberger. 1983. Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments *in situ*. *Proc. Natl. Acad. Sci. USA.* 80:6126-6130.
- Sutherland, A. E., P. G. Carlarco, and C. H. Damsky. 1988. Expression and function of cell surface extracellular matrix receptors in mouse blastocyst attachment and outgrowth. *J. Cell Biol.* 106:1331-1348.
- Tawil, N. J., M. Houde, R. Blacher, F. Esch, L. F. Richards, D. C. Turner, and S. Carbonetto. 1990. The integrin heterodimer $\alpha_1\beta_1$ functions as a dual

- laminin/collagen receptor in neural cells. *Biochemistry*. 29:6540-6544.
- Terranova, V. P., D. Rohrbach, and G. R. Martin. 1980. Role of laminin in the attachment of PAM 212 cells to basement membrane collagen. *Cell*. 22:719-726.
- Timpl, R. 1989. Structure and biological activity of basement membrane proteins. *Eur. J. Biochem.* 180:487-502.
- Timpl, R., H. Weidmann, V. van Delden, H. Furthmayr, and K. Kuhn. 1981. A network for the organization of type IV collagen molecules in basement membrane. *Eur. J. Biochem.* 120:203-211.
- Timpl, R., S. Johansson, V. van Delden, I. Oberbaumer, and M. Hook. 1983. Characterization of protease resistant fragments of laminin mediating attachment and spreading of rat hepatocytes. *J. Biol. Chem.* 258:8922-8927.
- Timpl, R., I. Oberbaumer, H. von der Mark, W. Bode, G. Wick, S. Weber, and J. Engel. 1985. Structure and biology of the globular domain of basement membrane type IV collagen. *Ann. N.Y. Acad. Sci.* 460:58-72.
- Tomaselli, K. J., C. H. Damsky, and L. F. Reichardt. 1987. Interactions of a neuronal cell line (PC12) with laminin, collagen IV, and fibronectin: identification of integrin-related glycoproteins involved in attachment and process outgrowth. *J. Cell Biol.* 105:2347-2358.
- Tomaselli, K. J., C. H. Damsky, and L. F. Reichardt. 1988. Purification and characterization of mammalian integrins expressed by a rat neuronal cell line (PC12): evidence that they function as α/β heterodimeric receptors for laminin and type IV collagen. *J. Cell Biol.* 107:1241-1252.
- Tomaselli, K. J., D. E. Hall, L. A. Flier, K. R. Gehlsen, D. C. Turner, S. Carbonetto, and L. F. Reichardt. 1990. A neuronal cell line (PC12) expresses two β_1 -class integrins— $\alpha_1\beta_1$ and $\alpha_3\beta_1$ that recognize different neurite outgrowth-promoting domains in laminin. *Neuron*. 5:651-661.
- Toyota, B., S. Carbonetto, and S. David. 1990. A dual laminin/collagen receptor acts in peripheral nerve regeneration. *Proc. Natl. Acad. Sci. USA*. 87:1319-1322.
- Tropea, M., M. E. Johnson, and D. Higgins. 1988. Glial cells promote dendritic development in rat sympathetic neurons *in vitro*. *Glia*. 1:380-392.
- Tsilibary, E. C., and A. S. Charonis. 1986. The role of the main noncollagenous domain (NC1) in type IV collagen self-assembly. *J. Cell Biol.* 103:2467-2473.
- Turner, D. C., L. A. Flier, and S. Carbonetto. 1987. Magnesium-dependent attachment and neurite outgrowth by PC12 cells on collagen and laminin substrata. *Dev. Biol.* 121:510-525.
- Turner, D. C., L. A. Flier, and S. Carbonetto. 1989. Identification of a cell-surface protein involved in PC12 cell-substratum adhesion and neurite outgrowth on laminin and collagen. *J. Neurosci.* 9:3287-3296.
- Wiedenmann, B., and W. W. Franke. 1985. Identification and localization of synaptophysin, an integral membrane glycoprotein of M_r 38,000 characteristic of presynaptic vesicles. *Cell*. 41:1017-1028.