

Neurite Extension of Chicken Peripheral Nervous System Neurons on Fibronectin: Relative Importance of Specific Adhesion Sites in the Central Cell-binding Domain and the Alternatively Spliced Type III Connecting Segment

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Abstract. Fibronectin contains at least two domains that support cell adhesion. One is the central cell-binding domain that is recognized by a variety of cell types, including fibroblasts. The second, originally identified by its ability to support melanoma cell adhesion, is located in the alternatively spliced type III connecting segment (IIICS). Using specific adhesive ligands and inhibitory probes, we have examined the role of each of these domains in fibronectin-mediated neurite extension of neurons from chick embryo dorsal root and sympathetic ganglia. In studies using explanted ganglia, both f13, a 75-kD tryptic fragment of human plasma fibronectin containing the central cell-binding domain, and CS1-IgG, a synthetic peptide-IgG conjugate containing the principal cell adhesion site from the IIICS, supported neurite outgrowth after adsorption onto the substrate. The maximal activities of f13 and CS1-IgG were 45–55% and 25–30% that of intact fibronectin, respectively. Co-coating of the substrate with f13 and CS1-IgG produced an additive stimulation of neurite outgrowth, the extent of which

approached that obtained with fibronectin. Similar results were obtained with purified neuronal cell preparations isolated by tryptic dissociation of dorsal root ganglia. In complementary studies, blockage of the adhesive function of either the central cell-binding domain (with mAb 333, an antiadhesive monoclonal antibody) or the IIICS (with CS1 peptide), resulted in ~60 or 30% reduction in fibronectin-mediated neurite outgrowth, respectively. When tested in combination, the inhibitory activities of mAb 333 and CS1 were additive. From these results, we conclude that neurons from the peripheral nervous system can extend neurites on both the central cell-binding domain and the IIICS region of fibronectin, and that these cells are therefore the first normal, embryonic cell type shown to adhere to the IIICS. These results suggest that spatiotemporal fluctuations in the alternative mRNA splicing of the IIICS region of fibronectin may be important in regulation of cell adhesive events during development of the peripheral nervous system.

DURING development of the embryonic nervous system and regeneration of damaged adult nerve fibers, innervation patterns are established via specific pathways (19, 65). The interaction of neuronal growth cones with their environment is considered to be of central importance for directing axons to their target sites. Thus, one of the principal goals in neuroscience is elucidation of the mechanisms that regulate axonal guidance.

The substrates encountered by elongating axons are a complex mixture of neuronal and nonneuronal cell surfaces, axonal processes, and fibronectin- and laminin-containing extracellular matrices, each of which has been implicated in the directed elongation of neuronal cells in vivo (7, 12, 32, 55,

56, 63, 66, 75). During development of the peripheral nervous system (PNS)¹, neuronal precursor cells originating from the neural crest travel long distances through intersomitic spaces that are rich in extracellular matrix (34, 62, 71, 77). Fibronectin is present along the pathways of neural crest cell migration (42, 48, 70) and in dermal and hypodermal tissues traversed by peripheral neurons (58), and both laminin and fibronectin have been found in association with

1. *Abbreviations used in this paper:* IIICS, type III connecting segment; CS-IgG, connecting segment peptide-IgG conjugate; DRG, dorsal root ganglion; PNS, peripheral nervous system; SPDP, succinimidyl 3-(2-pyridylidithio)-propionate.

perineurial basement membranes of peripheral nerves (15, 50, 51). Neuronal interactions with each of these adhesion proteins may therefore be an integral part of the development of the PNS.

In contrast, for development of the central nervous system, the interaction of neurons with nonneuronal cell surfaces appears to be of particular importance (9, 10, 27, 56). Fibronectin and laminin are present during development of the cerebral cortex (25, 37, 68), but they appear to be absent from the developing forebrain (37, 40, 47). Furthermore, the adult brain contains little organized extracellular matrix, and both fibronectin and laminin are restricted to meningeal cells and capillary basement membranes (37, 39, 40, 47, 50, 63). Laminin, however, has been reported to be expressed by astrocytes after brain injury and hence may play an active role in nerve regeneration (39).

In vitro assays examining neurite extension of explanted neuronal cells or ganglia have been shown to accurately reproduce many of the properties of elongating axons in vivo; for example, the degree of neurite extension correlates with substratum adhesiveness (22, 23, 35, 36), and the patterning obtained on substrates of varying adhesiveness mirrors the events that are thought to occur when neurons encounter developmental cues (22, 23). The spatiotemporal distribution of adhesion proteins may therefore be critical for controlling the directed migration of neuronal cells and their precursors. The ability of fibronectin and laminin to support neurite outgrowth of various types of explanted ganglia or individual neurons in vitro appears, in general, to parallel their tissue distribution; both central nervous system- and PNS-derived neurons extend neurites on laminin (1, 8, 18, 38, 41, 59, 67, 74), while fibronectin appears to be primarily a substrate for PNS neurons (1, 8, 13, 59, 61; but see reference 2).

There is currently great interest in identifying the sites on fibronectin and laminin that mediate neurite extension in order to obtain specific probes for evaluating the functional role of these molecules in neuronal development. For fibronectin, the central cell-binding domain containing the adhesive recognition tetrapeptide Arg-Gly-Asp-Ser (53, 79) has been shown to mediate neurite outgrowth of chick PNS neurons (60, 61) and human and rat neuroblastoma cells (73, 76); while synthetic Arg-Gly-Asp-Ser autoinhibited the fibronectin-mediated neurite extension of rat pheochromocytoma cells (3). In addition, neurite outgrowth-promoting activity for chick PNS neurons (60, 61), but not for human and rat neuroblastoma cells (73, 76), has been reported to reside in proteolytic fragments containing the COOH-terminal heparin-binding domain of fibronectin. The precise sequences required for the latter activity have not been described, and the relative importance of each of these domains of fibronectin for neuronal adhesion is currently unclear. Thus, it is also possible that further sites in the molecule await identification. For laminin, one major neurite outgrowth-promoting domain appears to be located at the end of the long arm of the molecule (16, 49, 57, 72).

In recent studies, we have identified an adhesion site that displays cell type specificity for melanoma cells in the alternatively spliced type III connecting segment (IIICS) of human plasma fibronectin (29, 30). This region is located close to the COOH terminus of fibronectin and lies between the COOH-terminal heparin- and fibrin-binding domains. Two active sites within this domain were localized to 20–25-mer

synthetic peptides termed CS1 and CS5 (29, 30). Since melanoma cells are of the same developmental lineage as PNS neurons (34), it was conceivable that neuronal cells might exhibit the same cell type-specific usage of this site. We have therefore examined whether the IIICS region is used by neurons from chick dorsal root and sympathetic ganglia for neurite extension, and have assessed quantitatively its contribution to the activity of the intact fibronectin molecule.

Materials and Methods

Materials

Fibronectin was purified from freshly frozen, citrated human plasma (National Institutes of Health Blood Bank, Bethesda, MD) by gelatin-affinity chromatography using elution with citric acid by a combination of published procedures (4, 46, 78). fl3, a 75-kD tryptic fragment containing the central cell-binding domain of fibronectin, was generated and purified as described (26). Fibronectin and fl3 were labeled with [³H]sodium borohydride as described previously (4, 69). Murine laminin and rabbit anti-mouse laminin IgG were purchased from Collaborative Research, Inc. (Waltham, MA) and Bethesda Research Laboratories (Gaithersburg, MD), respectively. The derivation, isolation, and characterization of mAb 333 (5) and mAb 304 (45) have been described previously. mAb 333 binds close to the Arg-Gly-Asp-Ser adhesive recognition signal in the central cell-binding domain of fibronectin, and is a potent inhibitor of both the direct binding of fibronectin to fibroblastic cells and of fibroblastic cell adhesion (5, 29), while mAb 304 binds specifically to the NH₂-terminal heparin/fibrin-binding domain of fibronectin (45). The synthesis, purification, and analysis of synthetic peptides spanning the IIICS region of human fibronectin (connecting segment [CS] peptides) were performed as described previously (29). CS peptides were synthesized with or without an NH₂-terminal cysteine residue for use either as inhibitory probes (no cysteine) or as adhesive ligands after conjugation to an inert protein carrier (cysteine-containing peptides). For conjugation, cysteine-containing CS peptides were covalently coupled to rabbit IgG to form CS-IgG conjugates, using the heterobifunctional cross-linker succinimidyl 3-(2-pyridylidithio)propionate (SPDP) (30).

Neurite Outgrowth

Preparation of Substrata. All neurite outgrowth assays were performed in 96-well tissue culture plates (Costar, Cambridge, MA). For preparation of substrates, ligands were diluted into Dulbecco's PBS, and then 100- μ l aliquots were incubated in the wells for 60 min at room temperature. Sites for nonspecific cell adhesion were then blocked by incubation with 100 μ l of 10 mg/ml heat-denatured BSA for 30 min at room temperature (29). For assays examining the effects of co-coating of fl3 and CS-IgG conjugates, substrates were first coated with fl3 for 60 min, and then recoated with CS-IgG for a further 60 min. The order of addition of fl3 and CS-IgG was necessary because of the large difference in protein concentration between the two reagents; simultaneous coating of fl3 and CS-IgG or precoating with CS-IgG was not possible, presumably because the high IgG concentration prevented the binding of fl3 to the plastic.

Isolation of Ganglia. Chains of sympathetic ganglia and individual dorsal root ganglia (DRG) were dissected from the lumbosacral region of 10–12-d chick embryos and transferred to calcium- and magnesium-free HBSS. For explant cultures, ganglia were freed from loosely attached connective tissue, and then in the case of sympathetic chains, the individual ganglia were dissected apart. Ganglia were then transferred to tissue culture plates containing preformed adhesive substrates and were incubated in Ham's F12 medium containing 2 mM glutamine, 25 U/ml penicillin, 25 μ g/ml streptomycin (all from Gibco, Grand Island, NY), and 20 ng/ml 7S-nerve growth factor (Collaborative Research, Inc., Waltham, MA; "supplemented Ham's F12 medium"). Nerve growth factor was included solely to augment neuronal survival, and in a control experiment was found to have no detectable effect on fibronectin-mediated neurite extension. To test the effects of peptides or antibodies on neurite outgrowth, these agents were added to the wells in a volume of 100 μ l, and then ganglia were added. All assays were performed in a final volume of 200 μ l. After overnight incubation at 37°C, ganglia were fixed by addition of 20 μ l 25% glutaraldehyde, and the radius of neurite outgrowth measured at four positions per ganglion (each at 90° to the others) using a phase-contrast microscope with a calibrated eyepiece. The radii of 6–10 ganglia were measured for each experimental point.

Preparation of Dissociated Neurons. For experiments involving dissociated neuronal cells, ganglia were disrupted with forceps, then digested at 100 ganglia/ml for 5 min at 37°C with 0.2–0.25% trypsin in calcium- and magnesium-free HBSS (Gibco). The reaction was stopped by addition of supplemented Ham's F12 medium containing 10% FCS (Gibco), and ganglia were then triturated by 10 passages through a fire-polished Pasteur pipette. Dissociated cells were collected gently by centrifugation at 100 g for 2 min, and were then washed and resuspended in supplemented Ham's F12 medium containing 1% FCS. Cell suspensions were first enriched for neuronal cells by preplating in 35 mm tissue culture dishes (Costar) for 30 min, and then unattached cells were added to tissue culture plates containing precoated adhesive substrates. Approximately 5×10^3 neurons were added to each well. After incubation for 24 h at 37°C, the number of neuronal cells extending neurites >1 cell diam was determined for 500 cells per experimental point.

Results

To obtain quantitative estimates of the contribution of each cell-binding domain of fibronectin to the neurite outgrowth-promoting activity of the intact molecule, a complementary approach was adopted based on promotion of neurite extension by defined proteolytic fragments and synthetic peptides, and inhibition of fibronectin-mediated neurite outgrowth by probes specific for abrogation of each adhesion site.

Role of the Central Cell-binding Domain in Promotion of Neurite Outgrowth by Fibronectin

Previous studies examining adhesion of fibroblastic cells have demonstrated that large proteolytic fragments of fibronectin containing the central cell-binding domain generally possess similar specific activity to the intact parent molecule in *in vitro* assays (17, 20, 26, 44). This retention of biological activity after proteolysis has been quite useful for structure-function analyses of fibronectin, since the properties of single functional domains can theoretically be studied in isolation. A 75-kD tryptic polypeptide, termed f13 according to the nomenclature of Hayashi and Yamada (26), is currently the smallest fragment reported to contain all of the adhesive activity of the central cell-binding domain. Therefore, the ability of explanted PNS ganglia to extend stable neurites on substrate-adsorbed f13 or fibronectin was compared to assess the contribution of the central cell-binding domain to the activity of the intact molecule.

As previously reported (13, 59–61), both dorsal root and sympathetic ganglia extended neurites on fibronectin in a time-dependent manner (throughout these studies, both types of ganglion yielded essentially the same results, and therefore for brevity only experimental data for DRG are presented). The rate of outgrowth on fibronectin was linear for at least 48 h (data not shown). The radius of neurite outgrowth 24 h after plating was dependent on the amount of fibronectin adsorbed onto the substrate; optimal outgrowth was obtained at a coating concentration of 10 $\mu\text{g}/\text{ml}$, and any further increase in adsorbed fibronectin had no further stimulatory effect (Fig. 1). The addition of 100 $\mu\text{g}/\text{ml}$ anti-laminin IgG to the assay medium had no effect on fibronectin-mediated outgrowth, whereas it completely blocked neurite extension on a laminin-coated substrate, suggesting that the activity of fibronectin may not be explained by endogenous synthesis of laminin by the neuronal cells (data not shown).

When the neurite outgrowth-promoting activity of f13 was compared to that of fibronectin, the intact molecule was ap-

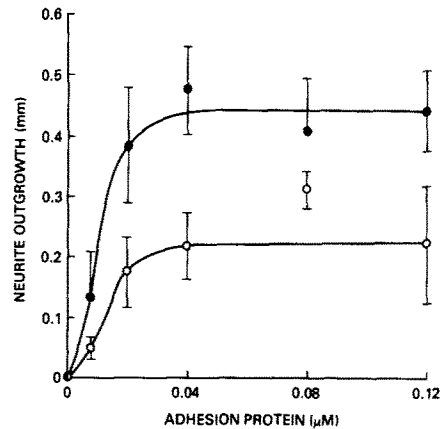


Figure 1. Dose dependence of neurite outgrowth on fibronectin (\bullet) and fragment f13 from the central cell-binding domain (\circ). Tissue culture wells were coated with the indicated concentrations of adhesion protein, and were then tested for their ability to promote neurite outgrowth from DRG. 0.12 μM is equivalent to 30 $\mu\text{g}/\text{ml}$ fibronectin or 8.3 $\mu\text{g}/\text{ml}$ f13. Bars, SEM.

proximately twice as active on a molar basis; the dose-response curve for f13 reached a plateau at a level 45–55% of that obtained with fibronectin (Fig. 1). To establish convincingly the difference in the response of DRG to fibronectin and f13, a further experiment was performed in which tissue culture dishes were coated either with 100 $\mu\text{g}/\text{ml}$ fibronectin, or its molar equivalent of f13. These coating concentrations were 10-fold higher than the levels of either fibronectin or f13 supporting a maximal response in Fig. 1. Even at such high levels of adhesion protein, the outgrowth-promoting activity of f13 was still only 45% that of fibronectin (data not shown), confirming the existence of a plateau in Fig. 1. As for fibronectin, the rate of outgrowth on f13 was also linear with time, demonstrating that the results in Fig. 1 were not due to a lag in neurite extension on this fragment (data not shown).

To rule out the possibility that the difference in activity between fibronectin and f13 might be due to preferential adsorption of fibronectin onto the substrate, the binding of each molecule to the tissue culture plate was examined. Using tritiated ligands, 39% of a 3.3- $\mu\text{g}/\text{ml}$ solution of f13 bound to the substrate after 1 h at room temperature compared to 35% for an equimolar 12- $\mu\text{g}/\text{ml}$ solution of fibronectin, indicating that the decreased activity of f13 in the neurite outgrowth assay was not due to its inability to bind to the substrate.

Role of the IIICS in Promotion of Neurite Outgrowth by Fibronectin

Recently, the alternatively spliced IIICS region of human fibronectin has been identified as a cell type-specific adhesion site for melanoma cells (29, 30). By comparing the adhesion-promoting activity of overlapping synthetic peptides that together spanned the entire 120-amino acid IIICS, activity was narrowed down to two sequences represented by the synthetic peptides CS1 and CS5 (29, 30). Since PNS neurons are ultimately from the same original developmental lineage as melanocytes/melanoma cells (34), we examined the role of the IIICS in neurite extension of DRG.

Table I. Neurite Outgrowth-promoting Activity of CS-IgG Conjugates

Substrate	Neurite outgrowth	Relative activity
	$\mu\text{m} \pm \text{SEM}$	%
BSA	12 \pm 12	—
IgG	26 \pm 12	—
CS1-IgG	169 \pm 24*	21
CS2-IgG	38 \pm 22	1.8
CS3-IgG	33 \pm 28	1.0
CS4-IgG	10 \pm 6	0
CS5-IgG	64 \pm 36	5.7
CS6-IgG	43 \pm 27	2.5
Fibronectin	696 \pm 54*	100

Cysteine-containing CS peptides were covalently coupled to rabbit IgG and then used to coat tissue culture wells at IgG concentrations of 180–1,200 $\mu\text{g}/\text{ml}$ (coating concentrations were corrected for the number of peptide molecules coupled to each molecule of IgG in each conjugate, so that equal numbers of peptide molecules were added to each well). Wells were also coated with 1,200 $\mu\text{g}/\text{ml}$ unconjugated IgG or 30 $\mu\text{g}/\text{ml}$ fibronectin as controls. All wells were then incubated with heat-denatured BSA to reduce the background level of neurite extension. BSA indicates uncoated plastic blocked with BSA. The activity of each CS-IgG conjugate is also expressed relative to the activity of fibronectin after subtraction of the background due to IgG. The sequences of the CS peptides are as follows: CS1, DELPQLVTLPHPNLHGPEILDVPST; CS2, PSTVQKTPFVTHPGYDTGNGIQLPG; CS3, LPGTSGQQPSVGGQMFEEHGFRR; CS4, FRRTTPPTATPIRHRPRYPNVGEE; CS5, GEEIQIGHIPREDDVYHLYP; CS6, LYPHGPGLNPNAST.

* Significantly different from IgG control at $P < 0.001$ using the Mann-Whitney U-test. The activity of CS5-IgG was not significantly different from IgG ($P = 0.4$).

Each CS peptide was covalently coupled to IgG via an NH_2 -terminal cysteine residue using the heterobifunctional cross-linker, SPDP, and the resulting conjugates were tested for their ability to promote neurite outgrowth after adsorption onto the substrate. As presented in Table I, only CS1-IgG (residues 1–25 of the IIICS) was active; CS2–CS6 supported only a minimal degree of outgrowth similar to underivatized IgG, which was itself similar to BSA-blocked, uncoated plastic. CS2–CS6 therefore serve as negative controls for the activity of CS1. The inactivity of CS5 contrasts with its ability to promote melanoma cell adhesion, but as discussed in detail below, this probably reflects sequence differences between the IIICS in human and chicken fibronectins. The outgrowth-promoting activity of the CS1 peptide conjugate was dose dependent (Fig. 2) and plateaued at a level 25–30% that of intact fibronectin; i.e., approximately half the level obtained with fl3 (similar results were obtained in six independent experiments). This high level of retention of activity in such a short synthetic peptide is perhaps surprising, but agrees well with the data obtained in the melanoma cell system, where CS1 was only 2.5-fold less active than fibronectin on a molar basis (30).

Phase-contrast micrographs showing the relative activities of fibronectin, fl3, and CS1-IgG in the neurite outgrowth assay are shown in Fig. 3. It is notable that, in addition to the neurite outgrowth induced by substratum-bound fibronectin, fl3, and CS1-IgG, migration of nonneuronal cells out from the ganglia was also observed on all three substrates. This finding suggests that these glial cells are also able to use the IIICS region of fibronectin for adhesion and migration.

Dissociated Neurons

To test whether single cell preparations of neurons exhibited

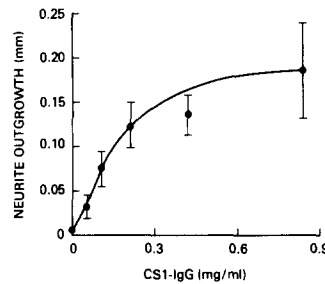


Figure 2. Dose dependence of neurite outgrowth on the synthetic peptide conjugate CS1-IgG. Wells were coated with the indicated concentrations of CS1-IgG and were then tested for their ability to promote neurite outgrowth from DRG. In this experiment, the radius of neurite outgrowth on a substrate coated with 20 $\mu\text{g}/\text{ml}$ fibronectin was 0.692 ± 0.076 mm. Bars, SEM.

the same substrate specificity for neurite extension as intact ganglia, DRG were dissociated with trypsin, and then the ability of purified neuronal cells to extend on various substrates was tested. As shown in Table II, ~30% of the neuronal cells extended neurites on intact fibronectin, a result in quantitative agreement with previous studies (59, 61). As for intact ganglia, both fl3 and CS1-IgG supported neurite outgrowth, but at a level lower than that of fibronectin (Table II). None of the other CS-IgG conjugates were able to support neurite extension, demonstrating the specificity of the CS1-IgG effect (data not shown).

In a control experiment, chick embryo fibroblasts were found to adhere equally well to fl3 and fibronectin, but were completely unable to spread on CS1-IgG. As found originally in melanoma cells, the recognition of the IIICS region is therefore cell type specific, rather than species specific.

Site-directed Inhibition of Fibronectin-mediated Neurite Outgrowth

Specific inhibitors of the adhesive function of the central cell-binding domain and the IIICS region of human fibronectin were tested for their effects on fibronectin-mediated neurite extension of DRG. mAb 333, which binds close to the Arg-Gly-Asp-Ser sequence in the central cell-binding domain, has been shown previously to completely inhibit the adhesion of cell types that use this region of fibronectin, but to have no detectable effect on cell types that use the IIICS (29). Conversely, the CS1 peptide autoinhibits the function of the IIICS region without affecting adhesion to the central cell-binding domain (30).

mAb 333 caused a dose-dependent inhibition of the fibronectin-mediated neurite outgrowth of DRG that plateaued at ~60% (Fig. 4 A). In a parallel experiment, 20 $\mu\text{g}/\text{ml}$ of mAb 333 was found to be sufficient to completely block the spreading of baby hamster kidney (BHK) fibroblasts on fibronectin, whereas a 25-fold higher concentration of mAb 333 still produced only 60% inhibition of DRG neurite outgrowth. As expected, however, the neurite outgrowth of DRG on substrate-bound fl3 was almost completely sensitive to inhibition by mAb 333, while extension on CS1-IgG was not significantly affected (Table III). These results indicate that ~60% of the outgrowth-promoting activity of fibronectin is contributed by the central cell-binding domain.

To specifically abrogate the function of the IIICS region, CS1 and the other five CS peptides were tested for their ability to inhibit fibronectin-mediated neurite outgrowth. 25–

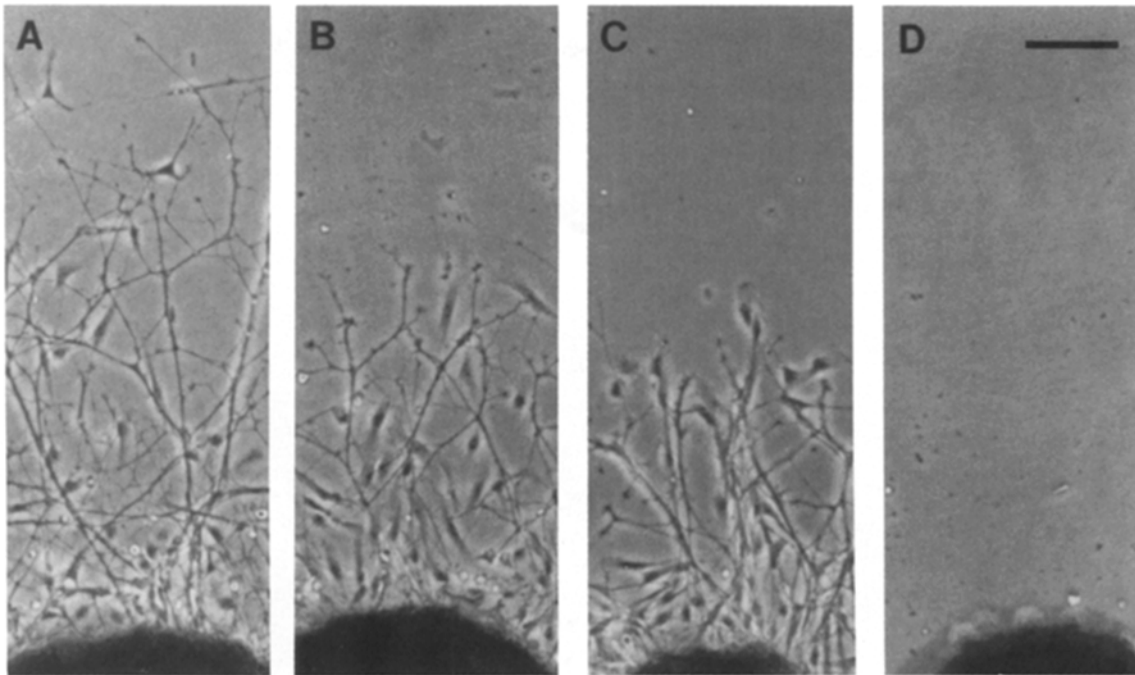


Figure 3. Phase-contrast microscopy of the neurite outgrowth of DRG on substrates coated with either 20 µg/ml fibronectin (A), 5.5 µg/ml f13 (B), or 500 µg/ml CS1-IgG (C), or on BSA-blocked, uncoated plastic (D). Note the presence of migrating glial cells in A–C. Bar, 100 µm.

30% inhibition of neurite extension was observed in five independent experiments with CS1, whereas the degree of outgrowth observed in the presence of CS2–CS6 was generally $\pm 10\%$ of the fibronectin control (Table IV). Inhibition by CS1 was dose dependent and plateaued at $\sim 30\%$ (Fig. 4 B). Further inhibition was observed at high concentrations of CS1, but this was apparently due to nonspecific toxicity since other CS peptides were also inhibitory and the inhibition was not reversible (data not shown). Based on the data in Fig. 4, the relative contribution of the central cell-binding domain and the IIICS to the activity of intact fibronectin appears to be $\sim 2:1$.

Complementation Analyses

In an attempt to reconstitute the activity of intact fibronectin, both the additivity of neurite outgrowth-promoting activity of f13 and CS1-IgG and, in a complementary assay, the inhibitory activity of mAb 333 in combination with CS1 on fibronectin-mediated neurite outgrowth were examined.

The stimulatory activities of CS1-IgG and f13 were additive when both were co-coated onto the substrate; an activity $\sim 75\%$ that of intact fibronectin was obtained (Fig. 5). As described below, the difference in activity between fibronectin and co-coated f13/CS1-IgG may be explained either by the incomplete retention of biological activity in the CS1 peptide, or alternatively by the existence of a third contributory site to the activity of fibronectin. Similar results to those in Fig. 5 were obtained with dissociated neuronal cells; when co-coated onto the substratum, the neurite outgrowth-promoting activity of f13 and CS1-IgG was additive, and in this experiment their combined activity approached the level obtained with intact fibronectin (Table II).

Consistent with the results in Fig. 1, the specific inhibition of the function of the central cell-binding domain and the IIICS with either mAb 333 or CS1 peptide was also additive (Fig. 6, see shaded bars). Coincubation of mAb 333 and CS1 resulted in 80% inhibition of fibronectin-mediated neurite outgrowth. Controls in which mAb 333 was coincubated with an inactive peptide (CS4) or CS1 was coincubated with a control mAb directed against the NH_2 -terminal domain of fibronectin (mAb 304) showed no further inhibitory activity above that obtained with mAb 333 or CS1 alone (Fig. 6). These results are consistent with the hypothesis that the central cell-binding domain and the IIICS function independently within fibronectin and that both domains are important for full activity of the parent molecule.

Table II. Additivity of the Neurite Outgrowth-promoting Activity of f13 and CS1-IgG for Purified Neuronal Cells

Substrate	Neurons with neurites % \pm SEM
BSA	2.3 \pm 0.3
f13	12.7 \pm 0.9
CS1-IgG	15.3 \pm 1.6
f13 + CS1-IgG	31.0 \pm 2.8
Fibronectin	32.7 \pm 2.7

Neuronal cells were isolated from DRG by trypsinization and preplating, and then incubated on substrates coated with either 5 µg/ml fibronectin, 1.4 µg/ml f13, 500 µg/ml CS1-IgG, or co-coated with f13 and CS1-IgG as described in Materials and Methods. After 24 h, the percentage of neuronal cells extending neurites >1 cell diam was determined by counting 500 cells/well. BSA indicates an uncoated plastic substrate blocked with heat-denatured BSA.

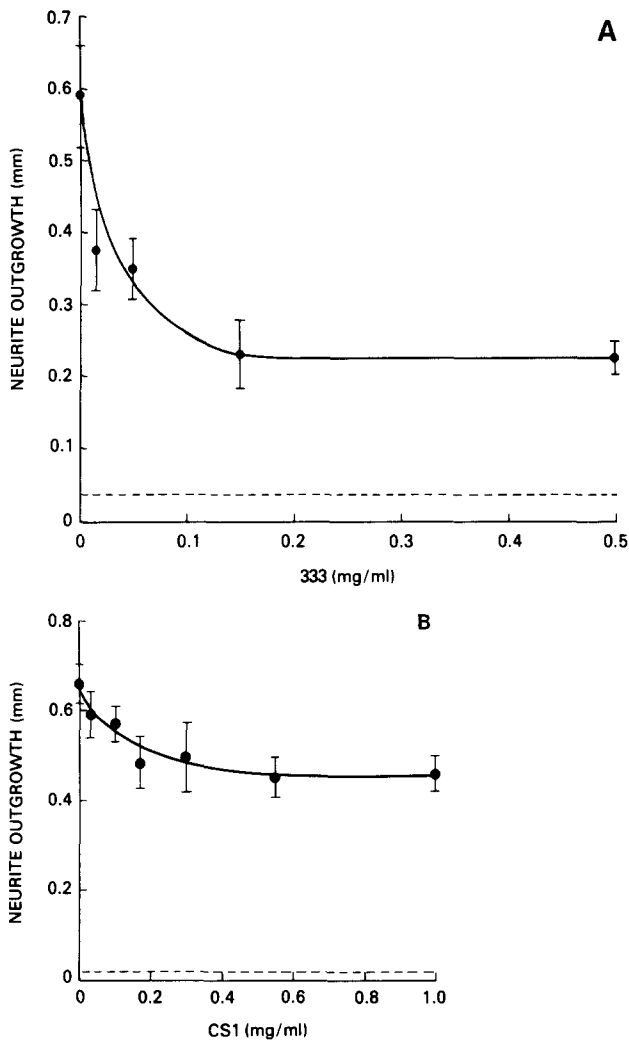


Figure 4. Effect of mAb 333 and CS1 peptide on fibronectin-mediated neurite outgrowth. Tissue culture wells were coated with 20 μ g/ml fibronectin and blocked with heat-denatured BSA, and then the effects of coincubation with the indicated concentrations of mAb 333 (A) or CS1 (B) on the neurite extension of DRG were examined. The dashed line denotes the background level of neurite outgrowth on uncoated plastic blocked with heat-denatured BSA. Bars, SE.

Discussion

Our major conclusions from these studies are as follows: (a) neurons from the PNS can extend neurites on both the central cell-binding domain and the IIICS region of fibronectin; (b) the relative contribution of the central cell-binding domain to the activity of whole fibronectin is approximately twice that of the IIICS; (c) the active site within the IIICS has been narrowed down to a defined amino acid sequence represented by the CS1 peptide; and (d) PNS neurons are the first normal cell type shown to recognize the IIICS.

The 75-kD tryptic fragment, fl3, and the synthetic peptide-protein conjugate, CSI-IgG, have been characterized previously as adhesive ligands that contain the principal active sites from the central cell-binding domain and the IIICS region of human fibronectin, respectively (26, 30). Dose-response analyses of the neurite outgrowth-promoting activity

Table III. Substrate Specificity of the Inhibitory Activity of mAb 333 on Neurite Outgrowth

Substrate	Inhibitor	Neurite outgrowth	
		$\mu\text{m} \pm \text{SEM}$	%
BSA	—	26 ± 12	—
Fibronectin	—	722 ± 191	—
Fibronectin	mAb 333	240 ± 70	69
fl3	—	405 ± 47	—
fl3	mAb 333	16 ± 13	100
CSI-IgG	—	236 ± 32	—
CSI-IgG	mAb 333	212 ± 46	11

Wells were coated with either 20 μ g/ml fibronectin, 5.5 μ g/ml fl3, or 500 μ g/ml CSI-IgG, and then the effect of 300 μ g/ml mAb 333 on the neurite outgrowth of DRG was examined for each ligand. BSA indicates an uncoated plastic substrate blocked with heat-denatured BSA. The percent inhibition by mAb 333 was calculated after subtraction of the background level of neurite extension on BSA from each experimental value.

Table IV. Inhibition of Neurite Outgrowth on Fibronectin by CS Peptides

Inhibitor	Neurite outgrowth	
	$\mu\text{m} \pm \text{SEM}$	%
None	411 ± 75	—
CS1	$281 \pm 67^*$	32
CS2	398 ± 72	3.2
CS3	364 ± 84	11
CS4	463 ± 111	(13)
CS5	373 ± 59	9
CS6	418 ± 87	(2)

Tissue culture wells were coated with 20 μ g/ml fibronectin and blocked with heat-denatured BSA, and then the effects of 1 mg/ml of each CS peptide on the neurite outgrowth of DRG was examined. The radius of neurite outgrowth on uncoated plastic blocked with heat-denatured BSA was 2 ± 2 μ m. This value was subtracted from all experimental points before calculation of percent inhibition.

* Significant at $P = 0.1$ using the Mann-Whitney U-test.

of both fl3 and CSI-IgG were carried out to evaluate the relative contribution of each region to the activity of whole fibronectin. These studies indicated that the maximal extent of outgrowth on fl3 was 45–55% that of fibronectin, while CSI-IgG was 25–30% as active as the parent molecule. For fl3, the difference in activity could not be attributed either to a lag in the extension of neurites or to defective binding of the polypeptide to plastic. For CSI-IgG, neurite outgrowth-promoting activity was highly specific, since five other control peptides taken from the IIICS were inactive.

In complementary studies, the inhibitory effects of probes specific for inhibition of the function of each adhesive domain were examined. Abrogation of the function of the central cell-binding domain with mAb 333 resulted in a 60% decrease in neurite outgrowth on fibronectin, while blockage of the function of the IIICS with CS1 inhibited the activity of fibronectin by 30%. In each case, a plateau of inhibitory activity was obtained, suggesting that the observed levels of inhibition reflected the contribution of each domain to the activity of whole fibronectin. Taken together, these results indicate that both cell-binding regions of fibronectin are recognized by neurons from the PNS, and that each can function independently to stimulate neurite extension.

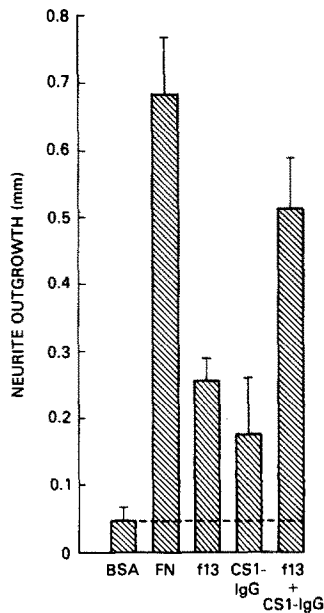


Figure 5. Additivity of the neurite outgrowth-promoting activity of f13 and CS1-IgG. Substrates were coated either with 20 $\mu\text{g/ml}$ fibronectin, 5.5 $\mu\text{g/ml}$ f13, or 500 $\mu\text{g/ml}$ CS1-IgG, or were co-coated with f13 and CS1-IgG as described in Materials and Methods. All substrates were blocked with heat-denatured BSA and were then tested for their ability to support neurite outgrowth from DRG. The dashed line denotes the level of neurite outgrowth on uncoated plastic blocked with heat-denatured BSA. Bars, SEM.

As reported previously for B16-F10 murine melanoma cells (30), the CS1 peptide was the principal active site in the IIICS region for neurite extension. In contrast to melanoma cells, however, neither dorsal root nor sympathetic ganglia were able to extend neurites onto the CS5-IgG conjugate. Although less active than CS1, the CS5 peptide possessed significant adhesive activity for melanoma cells (30). The active site within the CS5 peptide appeared to be the tetrapeptide Arg-Glu-Asp-Val (29). The apparent discrepancy in the activity of CS5 for these two cell types may be explained by differences in the amino acid sequence of the IIICS region between chicken and human fibronectins. Recent sequencing of genomic DNA clones for chicken fibronectin has shown that the Arg-Glu-Asp-Val motif is completely absent from the chicken fibronectin gene (48a), suggesting that in the chicken there may be only one active site in the IIICS (equivalent to CS1).

A second difference between the adhesive activity of neurons and melanoma cells is their relative usage of the central cell-binding domain and the IIICS. For B16-F10 melanoma cells, ~90–95% of the activity of whole fibronectin is contained within the IIICS, while for DRG this figure is ~25–30%. This difference may reflect the relative numbers of cell surface receptors for each site on the two cell types and suggests that the use of the different adhesion sites in fibronectin may be regulated at the receptor level. At present, the identity of the receptor for the IIICS is unknown, but a recently characterized 140-kD complex appears to function as the receptor for the central cell-binding domain (6, 28, 54). It is notable that antiadhesive mAbs (CSAT and JG22) directed against the chicken 140-kD complex have been reported to inhibit the neurite extension of neural retina cells, DRG, and ciliary ganglia on a fibronectin-coated substrate (11, 21, 74). Moreover, in preliminary studies, we have found that the neurite outgrowth of DRG on fibronectin is completely sensitive to the effects of JG22E, a subclone of the original JG22 antibody (24), permitting the tentative conclusion that the receptors for the central cell-binding domain

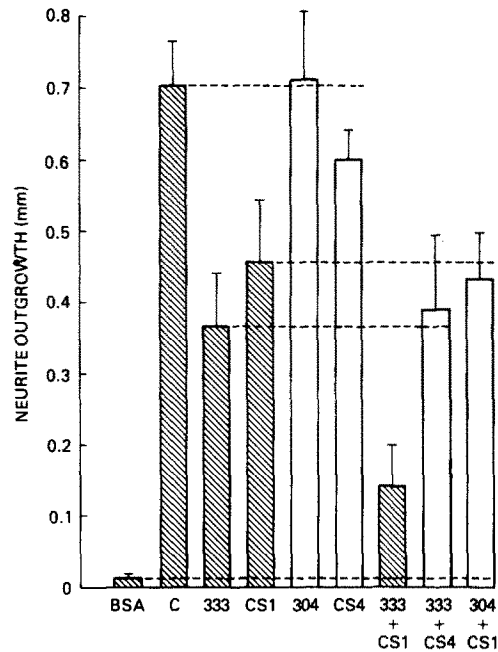


Figure 6. Additivity of the inhibitory activities of mAb 333 and CS1 peptide on fibronectin-mediated neurite outgrowth. Substrates were coated with 20 $\mu\text{g/ml}$ fibronectin and blocked with heat-denatured BSA. The effects of coincubation of DRG with various combinations of mAb 333 (300 $\mu\text{g/ml}$), mAb 304 (300 $\mu\text{g/ml}$), CS1 peptide (1 mg/ml), and CS4 peptide (1 mg/ml) on neurite extension was examined. Dashed lines are used to compare the levels of neurite outgrowth obtained under various experimental conditions with the appropriate control. Shaded bars are used to highlight the additive effects of coincubated mAb 333 and CS1. Bars, SEM.

and the IIICS may be related (e.g., by sharing a common beta subunit).

To examine whether the combined activities of the central cell-binding domain and the IIICS might be sufficient to account for all of the activity of intact fibronectin, we tested the effects of co-coating of f13 and CS1-IgG on neurite extension, and of coincubation of mAb 333 and CS1 on inhibition of fibronectin-mediated outgrowth. A co-coated substrate of f13 and CS1-IgG possessed ~75% of the activity of fibronectin, while coincubation of mAb 333 and CS1 blocked ~80% of the activity of the parent molecule. There may be several explanations for the inability of the central cell-binding domain and the IIICS to account for the entire activity of fibronectin. For example, the CS1 peptide may not represent 100% of the activity of the IIICS. For B16-F10 melanoma cells, which recognize the IIICS region almost exclusively, CS1-IgG was found to be only 40% as active as fibronectin on a molar basis (30). Thus, although this degree of retention of biological activity is surprisingly high for a synthetic peptide, the activity of CS1 may not be optimal. It is conceivable that other regions of the IIICS or neighboring domains in fibronectin may determine the fully active conformation of the CS1 sequence and that the inclusion of CS1 within the polypeptide framework of the fibronectin molecule may significantly affect its activity. Alternatively, the IIICS may require positioning at a specific distance from the central cell-binding domain for a full combined activity. In the fu-

ture, cDNA cloning and expression studies should be able to answer some of these questions. Nonetheless, from these studies, it is now apparent that the central cell-binding domain and the IIICS are the two most active cell interaction sites within fibronectin for neuronal cells. This result suggests therefore that both the neurite outgrowth- and melanoma cell adhesion-promoting activities which have been localized previously to the COOH-terminal heparin-binding domain of fibronectin (43, 60, 61) may in fact be explained by the presence of the CS1 sequence in the heparin-binding fragments used for these studies.

Two very interesting aspects of the cell adhesive activity of the IIICS are the potential for regulation of its activity through alternative splicing of precursor mRNA molecules and the cell type specificity of its usage. Since it can be specifically excised from the parent molecule, the IIICS is ideally suited to function as a modulatory adhesion site that could augment the activity of other nonregulatable sites such as the central cell-binding domain. The CS1 sequence represents one entire spliced segment of the IIICS that is completely conserved between rat and human fibronectins (31, 33, 64). Splicing of the IIICS is complex, with either three (rat), five (human), or two (chicken) potential variants in different species (31, 33, 48a, 64). Either one (rat and chicken) or two (human) of these products contains the CS1 peptide or a sequence homologous to it. It is conceivable, therefore, that programmed fluctuations in the splicing of the IIICS may regulate the adhesive activity of neuronal cells. A precedent for the regulated splicing of the IIICS already exists for hepatocytes, platelets (52), and certain transformed cells (14). Since melanoma cells and peripheral neurons both arise from the neural crest during development (34), it may also be speculated that neural crest derivatives in general may be specialized for recognition of the IIICS. It will therefore be instructive to examine whether other neural crest-derived cell populations are able to adhere to the IIICS, or to determine whether it is recognized by a wider range of cell types. Furthermore, in the future, it will be valuable to examine the oncocodevelopmental usage of the IIICS *in vivo*, to test the effects of superimposition of the activity of the IIICS on the baseline activity of the central cell-binding domain, and to determine whether the presence or absence of the CS1 sequence has any effect on substrate guidance during migration of embryonic cell populations *in vivo*.

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