NEURON-SPECIFIC INTERACTIONS WITH TWO NEURITE-PROMOTING FRAGMENTS OF FIBRONECTIN¹

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Abstract

Proteolytic fragments of fibronectin were used (1) to identify regions of the molecule that support neurite extension and (2) to investigate further the differential behavior of central and peripheral nervous system neurons in response to fibronectin (Rogers, S. L., P. C. Letourneau, S. L. Palm, J. B. McCarthy, and L. T. Furcht (1983) *Dev. Biol.* 98: 212–220). Fibronectin fragments with differing biological activities were produced by proteolytic digestion with trypsin and cathepsin D and sequential affinity chromatography on gelatin-agarose and heparin-Sepharose. The resulting fragments (described by Smith, D. E., D. F. Mosher, R. B. Johnson, and L. T. Furcht (1982) *J. Biol. Chem.* 257: 5831–5838; Smith, D. E., and L. T. Furcht (1982) *J. Biol. Chem.* 257: 6518–6523) included (1) an NH₂-terminal 27,000-dalton peptide that weakly binds heparin, (2) a 46,000-dalton gelatin-binding fragment, (3) a series of fragments (80,000 to 125,000 daltons) from the center of the molecule containing previously described cell-binding activity, (4) two major peptides of $M_r = 33,000$ and 66,000 that bind heparin strongly and are thought to originate from the A and B chains, respectively, of plasma fibronectin, and (5) a 31,000-dalton COOH-terminal peptide containing a free sulfhydryl from the A chain of the molecule. Tissue culture dishes were treated with these proteolytic preparations, and dissociated embryonic chick peripheral (PNS) and central nervous system (CNS) cells were cultured on each experimental substratum in serum-free medium. The fibronectin fragments were evaluated for ability to promote cell attachment, neurite initiation, and maintenance of neurite growth.

The 27,000-, 46,000-, and 31,000-dalton preparations did not promote cell attachment or neurite extension. Both PNS and CNS neurons attached to and extended stable neurites upon the COOH-terminal heparin-binding preparation containing the 33,000- and 66,000-dalton peptides. A differential response of the neurons to the 80,000- to 125,000-dalton “cell-binding” peptides was observed: whereas PNS neurons maintained neuritic growth on this preparation for at least 48 hr, CNS neurons extended neurites during the first 24 hr of culture but, by 48 hr, withdrew these neurites and became increasingly clumped. On the basis of (1) the observed neuronal responses to the heparin binding and “cell binding” regions, and (2) the different ligand-binding properties of these regions, we propose that cell attachment and neurite extension can be mediated and/or modulated by two separate regions of fibronectin and that cellular response to the intact molecule may involve multivalent interactions.

Neuronal interaction with extracellular matrix molecules is likely to be a critical factor in axonal guidance during devel-

opment and regeneration. Fibronectin (FN) is an adhesive matrix glycoprotein known to mediate attachment of mesodermal and some epithelial cells to collagen and other substrata (see Furcht, 1983, for review). Neurons also bind *in vitro* to FN-treated surfaces (Akers et al., 1981; Baron-VanEvercooren et al., 1982; Carbonetto et al., 1983; Rogers et al., 1983), and definition of this response is important to elucidation of (1) the potential role(s) of FN in axonal growth and (2) variations among cell types in FN-mediated behavior.

The behavior of neurons in response to FN appears to vary with culture conditions, developmental stage, and embryonic origin. FN promotes adhesion and directional motility of neural crest cells (Erickson and Turley, 1983; Rovasio et al., 1983). Peripheral sensory and sympathetic ganglion neurons, derived from neural crest, also adhere to and extend neurites on FN (Carbonetto et al., 1983; Rogers et al., 1983), as do neuroblastoma cells (Rauvala, 1984). In contrast, spinal cord neurons do

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not respond to FN bound to tissue culture plastic (Rogers et al., 1983) or when added to conditioned medium (Dribin and Barrett, 1980). Cerebellar cells adhere to FN substrata *in vitro*, but only after a specific developmental stage when FN is reported to appear along the path of granule cell migration (Hatten et al., 1982). Dissociated retinal neurons do not appear to respond to substratum-bound FN (Adler and Hewitt, 1983; Rogers et al., 1983; see also Thompson and Pelto, 1982), although aggregates of retinal cells may extend neurites on this glycoprotein (Akers et al., 1981).

Proteolytic fragments of FN have been used to map biologically active domains within the FN molecule (see Furcht, 1983, for review). Attachment and spreading of several cell lines, as well as fibroblast chemotaxis, seem to be promoted by a specific region of the molecule (Hahn and Yamada, 1979; Sekiguchi and Hakomori, 1980; Pierschbacher et al., 1981; Ruoslahti et al., 1981; Hayashi and Yamada, 1983). Recently, sensory neurons have also been reported to interact with a similar segment of FN (Carbonetto et al., 1983). We used peptide fragments similar to those studied by other investigators and characterized by Smith et al. (1982) and Smith and Furcht (1982) to better define possible mechanisms of neuron-FN interaction. In an earlier study (Rogers et al., 1983), we found that peripheral (PNS) but not central nervous system (CNS) neurons extended neurites on intact FN bound to plastic substrata. This behavioral difference between the two types of neuron might be due to differential binding affinity for FN *in vitro*, absence of CNS cell surface receptors for FN or inability of CNS cells to interact with particular conformations of FN. By testing the responses of these neurons to isolated portions of FN, we have (1) further defined differences between central and peripheral neurons in response to FN and (2) identified two apparently distinct regions of the FN molecule that can mediate and/or modulate cell attachment and neurite outgrowth.

Materials and Methods

Preparation of FN

Fibronectin (FN) was isolated from human plasma on DEAE-cellulose and gelatin affinity columns as previously described (Smith et al., 1982; Smith and Furcht, 1982).

Preparation of proteolytic fragments of FN

FN fragments were purified according to the general scheme of Smith et al. (1982) and Smith and Furcht (1982) with some modifications in the sequence of proteolytic digestion and affinity chromatography. Figure 1 summarizes the protocol used in this study and described below. Tables I and II summarize the binding characteristics of the fragments produced by this protocol. FN was digested with trypsin for 2 min and chromatographed on gelatin-agarose. The bound and unbound fragments were then processed as follows. (1) The gelatin-binding tryptic fragments were chromatographed on heparin-sepharose, and heparin-bound fractions were obtained. These gelatin- and heparin-binding tryptic fragments of plasma FN had estimated M_r of 190,000 and 200,000. The 190,000- and 200,000-dalton fragments were further digested with cathepsin D and sequentially chromatographed on gelatin-agarose and heparin-sepharose. This procedure resulted in a tryptic/catheptic 46,000-dalton gelatin-binding fragment (Fig. 2, lane b), 66,000-, 43,000-, and 33,000-dalton heparin-binding fragments (Fig. 2, lane d), and a series of fragments of $M_r = 80,000$ to 125,000 that bound neither heparin nor gelatin (Fig. 2, lane c). All three of these preparations were tested for neurite-promoting activity. (2) Tryptic fragments that did not bind gelatin were chromatographed on heparin-sepharose yielding an amino-terminus 27,000-dalton fragment that weakly bound heparin (Fig. 2, lane a) and a 31,000-dalton peptide, previously shown to contain a free sulfhydryl, that did not bind heparin but did bind monoclonal antibody 2-8 (Smith et al., 1982; see below for further explanation) (Fig. 2, lane e). These two fragments were assayed for neurite-promoting activity.

Tryptic/catheptic FN fragments were further characterized with monoclonal antibodies 180-8, 2-8 (Smith et al., 1982; Smith and

Figure 1. Preparation of FN fragments: Summary of the proteolytic digestions and sequential affinity chromatography used to produce FN fragments for this study. The apparent molecular weights of the fragments analyzed for cell attachment and neurite extension are indicated in boxes.

TABLE I
Binding properties of FN fragments

Major Fragments Bind to:	Preparation ^a					
	FN	a (27 Kd) ^b	b (46 Kd)	c (80-125 Kd)	d (33/66 Kd)	e (31 Kd)
Heparin	+	+	—	—	+	—
		(weak)			(strong)	
Gelatin	+	—	+	—	—	—
Cells ^c	+	—	—	+	+	—
Monoclonal 180-8	+	—	—	+	—	—
Monoclonal 2-8	+	—	—	—	+	+
Monoclonal 3E3 ^e	+	—	—	+	—	—

^a Refer to Figure 2, lanes a to e.

^b Kd, kilodaltons.

^c Several types of non-neuronal cells (see the text for references) and neurons (this study) have been reported to adhere to regions of FN that align with the 80,000- to 125,000-dalton series of fragments.

^d We report in this study that PNS and CNS neurons bind to, and extend neurites upon, an FN proteolytic preparation that contains 33,000- and 66,000-dalton fragments.

^e Monoclonal antibodies 180-8 and 2-8 have been described by Smith et al. (1982) and Smith and Furcht (1982). Monoclonal antibody 3E3 was kindly provided by Dr. M. Pierschbacher and has been reported to bind to the cell-binding determinant of FN (Pierschbacher et al., 1981).

Furcht, 1982), and 3E3 (kindly provided by Dr. Michael Pierschbacher, La Jolla Cancer Research Foundation) by Western analysis (J. B. McCarthy, S. Hagen, and L. T. Furcht, submitted for publication). The binding site of monoclonal antibody 180-8 is located within a 35,000-dalton region, at least 66,000 daltons from the COOH terminal of the FN subunit (Furcht, 1983). This site is contained within the series of non-heparin/non-gelatin fragments (80,000 to 125,000 daltons). Monoclonal antibody 2-8 recognizes a determinant close to the COOH terminal; large fragments (i.e., 66,000 daltons) from this region bind heparin, whereas smaller fragments such as the terminal 31,000-dalton peptide do not bind heparin. Monoclonal antibody 3E3 has previously been reported to bind the cell attachment fragment of FN (Pierschbacher et al., 1981). This antibody recognizes a determinant within the 80,000- to 125,000-dalton peptides, but not those in any of the other fragment preparations.

Figure 2. SDS-polyacrylamide gel of FN fragments. Lanes a to e represent the proteolytic preparations analyzed for cell attachment and neurite extension activity, containing peptides with approximate molecular weights of 27,000 (lane a), 46,000 (lane b), 80,000 to 125,000 (lane c), 33,000 and 66,000 (lane d), and 31,000 (lane e).

Polyacrylamide gel electrophoresis (PAGE)

Preparations of FN fragments were tested for purity by sodium dodecyl sulfate (SDS)-PAGE as previously described (Smith and Furcht, 1982).

Protein-binding assay

FN and affinity-purified proteolytic fragments were labeled with tritium by the reductive methylation procedure of Jentoft and Dearborn (1979). Free radioactivity was removed by extensive dialysis against phosphate-buffered saline (PBS). Fragments were assessed qualitatively for radioactivity by autoradiography of an En^3Hance (New England Nuclear, Boston, MA)-treated 10% SDS-polyacrylamide gel. The ^3H fragments were adjusted to appropriate protein concentrations based on absorbance at 280 nm (see below) in carbonate buffer and dispensed into 24-well tissue culture plates. Plates were incubated overnight at 37°C in a humid atmosphere. Wells were then washed three times with PBS, and bound radioactivity was solubilized with 0.25 ml of 1% SDS in 0.5 N NaOH. This solution was mixed with 3 ml of Aquasol II (New England Nuclear), and bound radioactivity was quantified with a Beckman LS250 liquid scintillation counter. Calculations of molar equivalents for fragment preparations containing more than one fragment were made using an average molecular weight for these fragments.

Preparation of substrata

Plastic tissue culture dishes (35 mm, Corning Glass Works, Corning, NY) were treated for 4 to 6 hr with FN or proteolytic FN fragments in 0.05 M carbonate buffer (pH 9.6). Concentrations of the fragments were equalized to molar equivalents corresponding to 200, 100, and 20 $\mu\text{g}/\text{ml}$ of intact FN, except in the case of the 80,000- to 125,000-dalton non-heparin/non-gelatin-binding preparation. Preliminary experiments indicated that very low concentrations of the 80,000- to 125,000-dalton preparation supported cell attachment and neurite extension, and it was therefore possible to use lower molar equivalents of 100, 10, and 1 $\mu\text{g}/\text{ml}$ of FN to analyze the neuronal response. Unbound protein was removed from the culture dishes by rinsing twice with serum-free

medium. Because the neurons used in this study extend neurites on untreated tissue culture plastic when cultured in serum-free medium, dishes were treated overnight with bovine serum albumin (BSA; 1 mg/ml; Miles Laboratories, Elkhart, IN) following FN coating to prevent this nonspecific response. Dishes were rinsed twice with serum-free medium prior to addition of cells.

Cell culture

Neurons from embryonic chickens were dissected and dissociated with trypsin as previously described (Rogers et al., 1983). Cultures of PNS neurons consisted of dorsal root (DRG) or sympathetic ganglion cells from 9- to 11-day embryos. Cultures of CNS neurons were from ventral spinal cord (SC; day 6) or neural retina (day 8). Culture medium in all experiments was serum-free Ham's F12 (GIBCO, Grand Island, NY) supplemented with insulin (5 $\mu\text{g}/\text{ml}$), transferrin (100 $\mu\text{g}/\text{ml}$), and sodium selenite (30 nM), according to the method of Bottenstein and Sato (1979). Nerve growth factor (10 ng/ml; a gift from Dr. E. Shooter, Stanford University) was also added to PNS cultures.

Analysis of neuronal interaction with prepared substrata

Cell-substratum adhesion. To examine the correspondence between initial cell-substratum adhesion and neurite initiation on the substrata tested, adhesion assays were performed with DRG and SC cells. Adhesion of total cell populations, not neurons alone, was evaluated. Tissue culture wells (16 mm; Costar, Cambridge, MA) were treated with FN or FN fragments as described above, and dissociated cells were plated at a density of 10,000 to 20,000 cells/well in 0.5 ml of supplemented serum-free F12. After 1 or 3 hr incubation at 37°C for DRG and SC cells, respectively, wells were rinsed with serum-free medium and the cells were fixed in 2% glutaraldehyde in PBS. The number of attached cells in 5 fields/well was determined at a magnification of $\times 200$. Counts were performed in duplicate and averaged for two separate experiments.

Neurite initiation. DRG and SC cells were cultured in 35-mm tissue culture dishes treated with the experimental preparations for 24 or 48 hr, respectively. These times were chosen because (1) the rapid production of neurites by DRG neurons makes individual DRG neurites difficult to discern after more than 24 hr of culture, and (2) in our cultures, the rate of neurite initiation and extension by CNS neurons was considerably slower than for PNS neurons. The percentage of cells (including neurons and non-neuronal cells) with neurites more than one cell body in length was determined for 9 fields/dish at a magnification of $\times 200$. Neurons with neurites were also subdivided into those with cell bodies attached directly to the substratum versus those with the soma in contact with other cells.

Neurite length. Measurements of neurites were made using an Apple II computer equipped with a Digisector (Microworks, Del Mar, CA). The computer was connected to an Olympus inverted microscope, and the image was transmitted to a separate video monitor. Nine representative areas of each culture dish were selected for analysis, and neurons were chosen within each area for measurement of neurite length. From the video monitor, neurite lengths were computed at a magnification of $\times 200$ and statistically analyzed with software written for our laboratory by Mr. James Borchart.

Results

FN fragments. The procedure for generating proteolytic fragments of the FN molecule is based on the work of Smith et al. (1982) and Smith and Furcht (1982). This scheme of enzyme digestion and purification permitted mapping of the resulting peptides within the intact molecule. The biological domains of FN represented by these fragments have also been reviewed recently (Furcht, 1983) and are summarized in Figure 3.

Brief tryptic digestion of intact human plasma FN generates small fragments from either end of the molecule. The first of these peptides, with approximate $M_r = 27,000$, binds heparin weakly and represents the amino terminal domain I of FN. This domain contains additional biological activities of staphylococcal binding and fibrin cross-linking. The second fragment, with $M_r = 31,000$, contains a free sulfhydryl and the antigenic determinant recognized by antibody 2-8, and includes domain VI. It is apparently generated by selective cleavage of

Figure 3. Model of the FN subunit. This model, adapted from Furcht (1983) shows the locations of the proteolytic fragments used in this study. Mapping of the fragments by ligand- and monoclonal antibody-binding properties has been described by Smith et al. (1982) and Smith and Furcht (1982). The various functional domains of FN (i.e., heparin binding, cell attachment, etc.) are indicated by Roman numerals I to VI. Work by Hayashi and Yamada (1983) shows that the A and B chains of FN differ with respect to a tryptic site present in the A chain between domains V and VI that is not present in the B chain. Therefore, the 66,000-dalton catheptic carboxyl heparin-binding fragment presumably originates solely from the B chain, and the 33,000-dalton fragment originates from the A chain (see "results"). The minor 43,000-dalton heparin-binding fragment is apparently a degradation product of the 66,000-dalton fragment.

the A chain of plasma FN (Hayashi and Yamada, 1983). The location of this fragment on the molecule was established using monoclonal antibody and affinity chromatography. The two high molecular weight ($M_r = 190,000$ and $200,000$) tryptic fragments of FN were purified by binding first to gelatin and then to heparin. Cathepsinization of these fragments for 15 min was performed to generate the remaining peptides. The location of these catheptic peptides along the intact FN molecule was also established with monoclonal antibody and affinity chromatography. The 46,000-dalton fragment, containing domain II, binds to gelatin and is immediately adjacent to the 27,000-dalton tryptic fragment (domain I). The series of fragments ranging in M_r from 80,000 to 125,000 contain domains III and IV. Domain IV represents a region of the molecule with $M_r = 11,500$, which has been shown by Pierschbacher et al. (1981) to mediate fibroblast attachment to FN. The localization of domain IV was verified in the two high molecular weight bands in this preparation by reactivity with monoclonal antibody 3E3. Finally, two major (33,000- and 66,000-dalton) peptides and one minor (43,000-dalton) peptide that all bind heparin strongly were isolated from the catheptic digest. The 33,000- and 66,000-dalton heparin-binding fragments originate from the A and B chains of the FN molecule, respectively (Hayashi and Yamada, 1983; J. B. McCarthy, S. Hagen, and L. T. Furcht, unpublished observations). The 33,000-dalton fragment contains domain V, while the 66,000-dalton fragment contains both domains V and VI. This assertion is based on the reactivity of monoclonal antibody 2-8 with the 66,000- and not the 33,000-dalton heparin-binding fragment. The minor 43,000-dalton fragment presumably represents a breakdown product of the 66,000-dalton fragment, since it also binds monoclonal antibody 2-8. Trypsin apparently releases domain VI from the A chain but not the B chain (Hayashi and Yamada, 1983), possibly as a result of the insertion of an A chain-specific amino acid sequence between domains V and VI (Schwarzbauer et al., 1983). Importantly, domain IV, which has been shown to mediate cell attachment and binds monoclonal antibody 3E3, is absent from this series of heparin-binding fragments.

Protein-binding assay. As previously described, intact FN binds to tissue culture plastic in a concentration-dependent manner (Palm and Furcht, 1983). Radioactively labeled FN fragments also bound to plastic (Table II). The amount of each fragment preparation that bound (Table II, column 1) greatly exceeds its contribution to the amount of intact FN bound.

TABLE II
Binding of FN and FN fragments to tissue culture plastic

Concentration of FN or FN Fragment ($\mu\text{g/ml}$)	1 Amount Bound to Well (μg)	2 Molar Ratio of Fragment to Intact FN ^a	3 Calculated Molar Equivalent Bound Relative to Intact FN (μg) ^b
Intact FN (20)	2.5	1.00	2.5
27,000 daltons (10)	2.7	0.12	22.5
46,000 daltons (45)	2.2	0.20	10.5
80,000 to 125,000 daltons (25)	4.9	0.50	9.8
33,000/66,000 daltons (15)	3.1	0.23	13.5
31,000 daltons (20)	1.8	0.14	12.8

^a Calculated on the basis of an M_r for monomeric FN of 220,000. The molecular weight used to calculate molar equivalents for the 80,000- to 125,000-dalton and the 33,000/66,000-dalton fragments was an average value of 110,000 and 50,000 daltons, respectively.

^b Calculated by dividing the actual amount bound to the well (column 1) by the molar ratio for each fragment (column 2).

This is demonstrated by calculating the molar equivalent to intact FN of each bound fragment (Table II, column 3).

Neurite extension: Summary of response. In a previous study (Rogers et al., 1983), we reported that PNS but not CNS neurons, cultured in serum-supplemented medium, extended neurites in response to substratum-bound FN. This was further examined in the present study with serum-free conditions. PNS neurons consistently formed neurites on FN when cultured without serum (Fig. 4A, Table III). Occasionally, neurites formed in serum-free CNS cultures during the first 24 hr on intact FN, but only at the highest concentration of FN tested ($200 \mu\text{g/ml}$). The CNS cells tended to be more clumped than DRG cells grown on FN, raising the possibility that the observed neurites were initiated by interactions among CNS cells rather than supported by the FN substratum alone. Neurites were not observed in CNS cultures on intact FN at 48 hr (Fig. 4E, Table III).

The responses of PNS and CNS neurons to the preparations of FN and affinity-purified FN fragments are summarized in Table III. None of the cells studied extended neurites on the amino-terminal heparin-binding fragment (Fig. 2, lane a), the

Figure 4. Neuronal response to FN and FN fragments. *A* to *D*, DRG cells; *E* to *H*, SC cells. *A* and *E*, Intact FN (100 $\mu\text{g}/\text{ml}$). Neurites extended by DRG neurons at 24 hr (*A*, arrows) continued to grow through 48 hr of culture. SC cells were clumped (*E*, arrows) and neurites were not observed at 48 hr. *B* and *F*, "Cell-binding" FN fragments, 80,000 to 125,000 daltons at 24 hr of culture. Arrows, neurites. *C* and *G*, "Cell-binding" fragments, 80,000 to 125,000 daltons, at 48 hr of culture. Neurites remain extended from DRG neurons (*C*, arrows), but most SC cells have clumped (*G*, arrows) and withdrawn their neurites. The few SC neurites that remain at 48 hr extend between clumps of cells (*G*, arrowheads). *D* and *H*, COOH-terminal heparin-binding preparation, 33,000 and 66,000 daltons. Both DRG (*D*) and SC (*H*) neurons extend and maintain neurites (arrows) at 24 and 48 hr, respectively. Magnification $\times 200$ (*A* to *H*).

TABLE III
Summary of neurite extension on FN and FN fragments

Cell Type	Protein Preparation ^a					
	FN	^a (27 Kd)	^b (46 Kd)	^c (80-125 Kd)	^d (33/66 Kd)	^e (31 Kd)
PNS neurons (24 or 48 hr)	+	—	—	+	+	—
CNS neurons (48 hr)	—	—	—	—	+	—

^a Dissociated neurons were cultured in serum-free medium on FN and proteolytic FN fragments bound to tissue culture plastic. The molecular weights (in kilodaltons, Kd) refer to the major fragments in each proteolytic preparation of FN (see Fig. 2, lanes a to e, and the text for concentrations used).

46,000-dalton gelatin-binding fragment (Fig. 2, lane b), or the COOH-terminal 31,000-dalton free sulfhydryl-containing fragment (Fig. 2, lane e) of FN. DRG neurons extended and maintained neurites on the central non-heparin/non-gelatin-binding fragments (Fig. 2, lane c) containing regions reported by other investigators to mediate attachment of various cell types (Yamada, 1983). All concentrations of this preparation used here (6 to 60 $\mu\text{g}/\text{ml}$) were effective in eliciting the DRG response, and neurites were present in these cultures through 48 hr (Fig. 4, B and C). The 33,000/66,000-dalton COOH-terminal heparin-binding preparation also supported DRG neurite outgrowth. The highest concentration (60 $\mu\text{g}/\text{ml}$) of this preparation was consistently effective (Fig. 4D), and one-half this concentration was effective in most experiments, but the lowest concentration (6 $\mu\text{g}/\text{ml}$) never supported neurite extension. The responses of dissociated sympathetic ganglion cells to the above preparations were similar to those of DRG cells.

Spinal cord neurons exhibited a complex response to the 80,000- to 125,000-dalton "cell-binding" preparation. During the first 24 hr of culture, neurites extended from many SC cells that were attached either individually or in clumps to the substratum (Fig. 4F). This response was noted only when at least 60 $\mu\text{g}/\text{ml}$ of this preparation was used. By 48 hr, the SC neurites had retracted, and cells were predominantly clumped and often completely detached from the substratum (Fig. 4G). This was true even when the concentration of the fragments was increased to 120 $\mu\text{g}/\text{ml}$. The time course of these events was similar in several separate experiments. In contrast, SC neurites were extended and maintained for at least 48 hr on the 33,000/66,000-dalton heparin-binding fragments (Fig. 4H). Only the highest concentration of this preparation was consistently effective in supporting SC neurite outgrowth. Retinal cells exhibited responses similar to those of the SC cells to the 80,000- to 125,000- and the 33,000/66,000-dalton preparations.

Cell-substratum adhesion. Optimal times for the cell adhesion assays were selected, after several trials with each cell type, on the basis of cells remaining attached in the uncoated wells (positive control) but predominantly unattached in the bovine serum albumin (BSA)-treated wells (negative control) following rinsing. We do not yet know which cell types in the heterogeneous DRG or SC cell populations adhered in these assays, but the pattern of cell adhesion to the fragments (see below) does correlate with ability to support neurite outgrowth. This suggests that neurons do adhere during early phases of culture. Also, since non-neuronal cells are evident in longer-term cultures, it may be that all cells within these populations have relatively similar adhesive properties with respect to FN.

Compared to BSA-treated plastic, intact FN, the 80,000- to 125,000-dalton series of "cell-binding" fragments, and the 33,000/66,000-dalton heparin-binding fragments supported adhesion of relatively large numbers of DRG cells (Fig. 5A).

Figure 5. Adhesion assays of DRG (A) and SC (B) cells to FN and FN fragments at 1 and 3 hr of culture, respectively. Tissue culture wells were treated with the appropriate FN preparation for 4 to 6 hr, rinsed, treated overnight with 1% BSA, and rinsed again prior to addition of 10,000 to 20,000 cells/well. Cells were counted in 5 random fields/well and averages were obtained from 4 wells (two experiments)/experimental substratum. BSA-treated wells and uncoated plastic served as negative and positive controls, respectively.

Each protein concentration that promoted DRG cell adhesion also supported neurite extension (see Fig. 4 and summary, above). In most cases, attachment at each of these concentrations exceeded attachment to untreated plastic. In contrast to the active preparations, few DRG cells attached to the 27,000-dalton heparin-binding, 46,000-dalton gelatin-binding, or 31,000-dalton sulfhydryl-containing fragments. The diminished attachment to these fragments is not simply due to a small amount of protein bound to the plastic substrata (see Table II and "Protein-binding assay," above).

Like DRG cells, SC cells attached initially to the 80,000- to 125,000-dalton fragments (all concentrations) and to the 33,000/66,000-dalton region (two highest concentrations) in substantially greater numbers than to the 27,000-, 40,000-, and 31,000-dalton fragments (Fig. 5B). Attachment to the latter three preparations did not exceed attachment to the BSA control. In contrast to the DRG response, there was little adhesion of SC cells to intact FN. Adhesion of SC cells to intact FN and FN fragments was consistently lower than that of DRG cells and required a longer period of time. That this lower rate of adhesion was specific to the SC cell-FN response, rather than a general property of these cells, is indicated by the rapid and substantial adhesion of SC cells to untreated plastic.

Neurite initiation. The extent to which DRG and SC cells initiated neurites on different substrata was examined by counting the number of total cells, rather than the number of neurons, with neurites. This was necessary for comparison of DRG and SC cells: whereas DRG neurons are relatively easy to distinguish from non-neuronal cells, SC neurons without neurites are not.

The percentage of DRG cells with neurites was relatively consistent on FN, the 80,000- to 125,000-dalton non-heparin/non-gelatin-binding fragments, and the 33,000/66,000-dalton heparin-binding fragment (Table IV). Since approximately one-half of the cells in the DRG preparation are neurons, about 2 times the percentages in Table IV, column 1 would represent the number of neurons with neurites. Approximately one-fourth to one-half of cells with neurites were in contact with other cell bodies (Table IV, column 2), raising the possibility that, in these instances, neurite initiation was supported by an adjacent cell surface rather than by the prepared substratum. However, in all other cases neurite initiation appeared to result from direct neuron-FN or neuron-FN fragment interaction. A somewhat lower incidence of cell-cell interaction on the 33,000/66,000-dalton fragment (Table IV, column 2) was observed compared to that on the other fragments, but the biological significance of this is not yet clear.

TABLE IV

Neurite initiation by DRG cells at 24 hr in vitro

Dissociated DRG cells were cultured in serum-free medium on FN, proteolytic FN fragments, or BSA-treated plastic for 24 hr. The percentage of total cells (between 200 and 300 cells, from two experiments, were counted for each group) with neurites more than one cell body in length was determined for each substratum tested (column 1). Column 2 indicates the percentage of neurite-producing cells that initiated neurites on the experimental substrata rather than on the surface of adjacent cells. The 27,000-, 46,000-, and 31,000-dalton preparations did not support cell attachment (see Fig. 5) or neurite extension.

Preparation	1 Percentage of Cells with Neurites	2 Percentage of Neurite- producing Cells with Cell Bodies not Contacting Another Cell
Intact FN		
200 μ g/ml	12	44
100 μ g/ml	11	48
80,000 to 125,000 daltons		
60 μ g/ml	16	42
30 μ g/ml	15	62
33,000/66,000 daltons		
60 μ g/ml	12	65
30 μ g/ml	12	74
27,000 daltons	0	0
46,000 daltons	0	0
31,000 daltons	0	0
BSA-treated plastic	0	0

TABLE V

Neurite initiation by spinal cord cells at 48 hr in vitro

Dissociated spinal cord cells were cultured in serum-free medium on FN, FN fragments, or BSA-treated plastic. At 48 hr, neurites were present only on the 66,000-dalton heparin binding preparation. At a protein concentration of 60 μ g/ml, a substantial number of cells extended neurites (column 1), and most neurites were initiated directly on the protein rather than on other cells (column 2) ($N = 590$). Fewer cells extended neurites on 30 μ g/ml of this preparation (column 1), and many appeared to be initiated upon adjacent cell surfaces (column 2) ($N = 95$). The other fragment preparations, as well as intact FN, did not support neurite growth at 48 hr.

Preparation	1 Percentage of Cells with Neurites	2 Percentage of Neurite- producing Cells with Cell Bodies not Contacting Another Cell
33,000/66,000 daltons		
60 μ g/ml	35	87
30 μ g/ml	8	25
FN	0	0
27,000 daltons	0	0
46,000 daltons	0	0
80,000 to 125,000 daltons	0	0
31,000 daltons	0	0
BSA-treated plastic	0	0

TABLE VI

DRG neurite lengths

To compare the relative effectiveness of intact FN, the 80,000- to 125,000-dalton "cell-binding peptides, and the 66,000-dalton COOH-terminal heparin-binding region in supporting neurite growth, lengths of DRG neurites at 24 hr *in vitro* were computed. Although values varied somewhat between experiments, all three substrata supported roughly equivalent rates of neurite extension. The large standard deviations for average neurite length are due to asynchronous production of neurites within each culture.

Substratum	Average Neurite Length (μ m + SD) ^a
FN	
200 μ g/ml	221 $\pm$ 131
	411 $\pm$ 320
100 μ g/ml	228 $\pm$ 189
	382 $\pm$ 248
80,000 to 125,000 daltons	
60 μ g/ml	283 $\pm$ 189
	266 $\pm$ 164
30 μ g/ml	247 $\pm$ 194
	220 $\pm$ 167
33,000/66,000 daltons	
60 μ g/ml	395 $\pm$ 275
	232 $\pm$ 153
30 μ g/ml	274 $\pm$ 194

^a Average neurite lengths were calculated for two experiments per substratum, with the exception of the low concentration of the 66,000-dalton fragment, where values represent only one experiment. $N = 20$ cells/experiment.

Substantial numbers of SC cells initiated and maintained neurites on the highest concentration (60 μ g/ml) of the 33,000/66,000-dalton heparin-binding fragments (Table V, column 1). A large portion of these cells were attached directly to the substratum-bound protein rather than to adjacent cells (Table V, column 2). At a lower concentration (30 μ g/ml) of these peptides, fewer cells formed neurites, and those that did were more often associated with other cells from the dissociated

spinal cords than at the higher concentration (Table V, column 2).

Neurite length. In order to compare the relative effectiveness of intact FN, the centrally located "cell-binding" fragments, and the 33,000/66,000-dalton heparin-binding fragments in support of neurite outgrowth, DRG neurites on each preparation were measured. The neurite lengths provided an indication of the rate of growth promoted by each experimental substratum. As Table VI illustrates, each preparation supported an approximately equivalent rate of neurite extension, although values varied somewhat between experiments. In other words, once initiated, DRG neurites grow equally well on each substratum. The very high standard deviations for average neurite length are due to the asynchronous nature of neurite initiation within each culture.

Discussion

The FN fragments used in this study have been extensively characterized in previous reports (Smith et al., 1982; Smith and Furcht, 1982) and again for analysis of neuronal interaction with the FN molecule. The predominant peptides in each of the five preparations studied here are unique in terms of molecular weight and ligand-binding properties. A 27,000-dalton peptide generated from the amino terminus binds heparin weakly; an adjacent 46,000-dalton fragment binds gelatin but not heparin, and a 31,000-dalton carboxyl terminus peptide contains a free sulfhydryl but does not bind either ligand. These three peptides do not support adhesion of neurons or other cell types (Furcht, 1983). The two remaining peptide preparations are also distinct. (1) The 33,000- and 66,000-dalton fragments both bind heparin strongly, whereas the 80,000- to 125,000-dalton fragments do not. (2) The 80,000- to 125,000- but not the 33,000/66,000-dalton fragments contain the antigenic determinant recognized by monoclonal antibody 3E3, which has been reported to inhibit cell attachment to intact FN (Pierschbacher et al., 1981). (3) There are no co-migrating peptides when visualized by SDS-PAGE. In addition to the different ligand- and monoclonal antibody-binding properties of the 33,000/66,000- and 80,000 to 125,000-dalton fragment populations, the cell behavior reported here strongly suggests that each of these two fragments contain regions that can mediate or modulate cell attachment and neurite extension, possibly by different mechanisms.

CNS and PNS neurons differ in their responses to the FN molecule. In summary, peripheral neurons bind to and extend neurites on (1) intact FN, (2) tryptic/catheptic fragments (80,000 to 125,000 daltons) that contain previously described cell-binding activity (Furcht, 1983), and (3) 33,000- and 66,000-dalton COOH-terminus fragments with strong heparin-binding activity. CNS cells bind to both the 80,000- to 125,000- and the 33,000/66,000-dalton fragments but maintain neurite growth only on the 33,000- and 66,000-dalton fragments. Both adhesion and neurite formation by CNS cells in response to intact FN was minimal. These responses raise three related questions concerning neuron-FN interactions: (1) Why do CNS cells interact with two proteolytic FN fragments, but not with intact FN? (2) What is the nature of the difference between central and peripheral neurons? and (3) What is the significance of each active region with respect to neuronal interaction with the intact FN molecule?

Initial adhesion of CNS cells to the 33,000/66,000- and 80,000- to 125,000-dalton fragments, in comparison with FN, suggests exposure of binding sites on these peptides that are not available in intact FN when it is bound to plastic. FN has been shown to change shape (Tooney et al., 1983) and binding properties (Grinnell and Feld, 1981) with variations in pH and surface conditions, and isolated peptides of FN also have unique configurations (Odermatt et al., 1982). Thus, each active region

of the fragments may be (1) only partially exposed in intact FN or (2) in a different (i.e., more biologically active) conformation when isolated. The lower adhesive affinity of CNS, compared to PNS, cells for the fragments might then be related to low binding of CNS cells to intact FN. Variations in conformation of bound FN under varying culture conditions might also account for discrepancies in reports of neuronal response to FN *in vitro*. In particular, the ability of retinal neurons to extend neurites on FN bound at low pH may result from exposure of the heparin-binding domain of FN (Akers et al., 1981). It must be emphasized that we also do not know the precise conformation of FN *in vivo*. Although it appears to be globular at neutral pH (Tooney et al., 1983), the shape of FN in extracellular matrices is not clear: the complexity and flexibility of the molecule suggest that its configuration may vary with its roles in cell-cell interaction, or in cell binding to collagen or other extracellular materials. Therefore, it is possible that CNS as well as PNS neurons do interact with FN during certain events *in vivo*.

In addition to the marked difference in response of CNS versus PNS cells to intact FN, adhesive interactions with the 80,000- to 125,000-dalton fragments also differ. Although CNS cells attached to, and many extended neurites on, these centrally derived peptides, neurite withdrawal beginning at approximately 24 hr was rapid and complete, possibly due to loss of neurite adhesivity (Letourneau, 1975). Decreased cell-substratum adhesivity was also indicated by a marked clumping of cells when neurite retraction occurred. Cells commonly clump when seeded onto a poorly adhesive substratum. One explanation for this alteration in adhesivity is that the neurite growth cones, and possibly other CNS cells, may degrade the FN fragments, eventually depleting the protein so that cell-substratum adhesion is greatly diminished. An important alternative is that these cells initially have the necessary binding sites for attachment to this domain of FN, but as the surface area is expanded through neurite growth, binding sites are not maintained in sufficient quantity, proper orientation, or required combination with other molecules to sustain neurite adhesivity.

The isolation of two different fragments of FN which promote neurite outgrowth, and the differential response of CNS neurons to the fragments, suggest the existence of multiple interactions of the cell surface with intact FN. Three types of interaction can be hypothesized. First, adhesive interaction of the neuronal cell surface with particular conformations of *both* the centrally located "cell-binding" and the COOH-terminal heparin-binding domains might be required for successful neurite adhesion to intact FN. Second, cells might bind initially to one region of intact FN, with subsequent interaction with the other region modulating or stabilizing their behavior. The latter interaction might lead to detachment of CNS cells from the molecule. Both of these models imply that there are two separate and distinct sites on FN that interact with cells. A third model involves a cell-binding site common to both the 33,000/66,000- and the 80,000- to 125,000-dalton fragments, with additional activity present in the conformation of the latter peptides that promotes detachment of CNS cells. Although our data on ligand-binding and neuronal activity do not support the existence of such a common region, until we know the precise amino acid sequences of the active regions of these FN fragments we cannot rule out this possibility.

It still might be argued that the "cell-binding" regions of each fragment are essentially identical and that the active sequences of the 80,000- to 125,000-dalton fragments (domain IV) are also present in the 33,000/66,000-dalton preparation but in smaller amounts (i.e., contamination). This might then account for the fact that higher concentrations of the 33,000/66,000-dalton peptides (molar equivalents to 100 to 200 $\mu\text{g}/\text{ml}$ of intact FN) compared to the 80,000- to 125,000-dalton peptides (molar

equivalents to 1 to 100 $\mu\text{g/ml}$ of intact FN) were needed to elicit the observed PNS cell behavior. However, this explanation is unlikely because (1) CNS cells would not be expected to exhibit a greater, more stable response to the "contaminant" in the 33,000/66,000-dalton preparation than to the active portions of the 80,000- to 125,000-dalton fragments, and (2) CNS cells should respond to high levels of the 80,000- to 125,000-dalton fragments at least as well as to equivalent levels of the 33,000/66,000-dalton peptide, but, in fact, they do not. Although our observations probably do not, therefore, reflect a dose response, it is still possible that sites within each preparation are similar, with additional activity in the 33,000/66,000-dalton peptide that stabilizes the CNS cells. If this were the case, a fundamental difference between the two fragment populations, in terms of interaction with cells, would still exist.

Multiple interactions of cells with FN suggest multiple types of cell surface-binding sites that might be used during different types or phases of cell attachment and movement. It seems likely that a heparan sulfate proteoglycan is involved in cell interaction with the heparin-binding domain of the FN molecule. Cell attachment and neurite extension on proteoglycan-binding proteins has shown that cell surface proteoglycans can be utilized for these activities (Chernoff and Culp, 1983; Laterra et al., 1983a, b). Importantly, heparan sulfate proteoglycan has been reported to be a transmembrane component capable of interacting with the cytoskeleton (Rapraeger and Bernfield, 1982) and thereby influencing cell behavior.

Since intact FN binds heparin, another approach to examining the importance of this activity to cell-FN interaction has been to competitively inhibit this interaction with exogenous heparin. Carbonetto et al. (1983) report that added heparin does inhibit neurite extension on FN, implying involvement of cell surface heparan sulfate, but our attempts to utilize this approach have been inconclusive (unpublished observations). In both cases, the effects of heparin may be at least partially independent of the presence of FN, as heparin-treated surfaces are highly anionic and thus unattractive for cells. Furthermore, if the heparin-binding region of FN is cryptic when certain binding conditions are used, as our data and others' (Gold et al., 1983; Sekiguchi et al., 1983) suggest, heparin inhibition of cellular activity would be nonspecific. It will be important to test the involvement of cell surface heparan sulfate (which is complexed with protein) using a heparan sulfate proteoglycan or an antibody to the molecule, rather than heparin, to interfere with cell-FN and cell-FN fragment interaction. Indeed, one report shows that cell interaction with FN is specifically inhibited by heparan sulfate proteoglycan but not by heparin (Klebe and Mock, 1982).

It should be noted that there is evidence to suggest that heparan sulfate may also directly mediate cell attachment and neurite extension via a FN-independent mechanism(s). For example, the substratum-bound factor from conditioned medium described by Lander et al. (1982) appears to contain a proteoglycan that promotes neurite extension. It is conceivable that, in our culture system, the substratum-bound heparin-binding FN fragments bind extracellular heparan sulfate, which in turn binds neuronal cell surfaces. Such binding could involve specific cell surface polysaccharide receptors and/or intercalation of proteoglycan core proteins into the plasma membrane (Kjelln et al., 1980), thus facilitating cell association with extracellular matrix glycosaminoglycans or proteoglycans. Clearly, precise localization of these molecules and their relationship to cell surface-mediated behavior will require further investigation.

Interaction of neurons and other cell types with a non-heparin/non-gelatin-binding (i.e., "cell binding") region of FN (Ruoslahti et al., 1981; Albini et al., 1983; Carbonetto et al., 1983; Yamada, 1983; J. B. McCarthy, S. Hagen, and L. T.

Furcht, unpublished observations) implicates cell surface molecules in addition to heparan sulfate proteoglycan that will mediate cell attachment. The manner in which cells utilize the two regions of the FN molecule may vary significantly with cell type and activity as well as with FN conformation. For example, spreading and formation of specialized adhesive contacts by non-neuronal cells appear to require interaction with *both* cell-binding and heparin-binding domains (Izzard and Culp, 1983; Laterra et al., 1983a, b). On the other hand, our results indicate that neurons can use the heparin-binding domain alone for adhesion and extension of neurites, although interaction with intact FN may involve multiple cell surface constituents. Such different responses to FN might reflect differences between cell types in modes of locomotion. Whereas formation of highly adherent focal contacts on heparin-binding proteins (Laterra et al., 1983a, b) may actually impede locomotion of cells that must also break these contacts in order to move, neuronal growth cones move very well over extremely adhesive surfaces (Letourneau, 1975). The heparin-binding region of FN apparently provides such a surface, but initial cell adhesion to the cell-binding region is even greater when the two are compared on a molar basis. It is still unclear how each region interacts with cell surfaces to promote cell-specific behavior, and it will be helpful when the membrane molecules involved, as well as their interaction with cytoskeletal components, are more fully understood. It is plausible that domain-specific and cell-specific multivalent interactions will account for variations in cell behavior mediated by FN.

In summary, the responses of PNS and CNS neurons to isolated regions of FN may involve different adhesive molecules on the surfaces of these cells. While one heparin-binding region of FN binds to and promotes neurite outgrowth from both types of neurons, a second region supports sustained neurite growth only from PNS neurons. Stable interaction of CNS neurons with a heparin-binding domain but not with the previously reported "cell-binding" region or with the intact FN molecule suggests (1) absence, or insufficient numbers, on CNS cells of an "FN receptor" unrelated to heparin-binding, (2) insufficient or improper accessibility of the heparin-binding region in the nonfragmented molecule, and/or (3) multiple cell surface interactions with intact FN that modulate adhesive contacts. We do not yet know how the two regions described here, either individually or in combination with one another, are involved in neuronal interaction with intact FN. Such information will require further studies involving antibodies against each active site, isolation of smaller peptides from these regions, and inhibition of cell behavior with molecules that compete with cell surface-binding sites. Finally, understanding of the various roles of FN in development and regeneration may require consideration of multiple mechanisms by which this molecule mediates cellular adhesion and locomotion.

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