Selective Interaction of Peripheral and Central Nervous System Cells with Two Distinct Cell-binding Domains of Fibronectin

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Abstract. Mechanisms of cell interaction with fibronectin have been studied with proteolytic fibronectin fragments that have well-defined ligand binding properties. Results of a previous study (Rogers, S. L., J. B. McCarthy, S. L. Palm, L. T. Furcht, and P. C. Letourneau, 1985, *J. Neurosci.*, 5:369–378) demonstrated that (*a*) central (CNS) and peripheral (PNS) nervous system neurons adhere to, and extend neurites on a 33-kD carboxyl terminal fibronectin fragment that also binds heparin, and (*b*) neurons from the PNS, but not the CNS, have stable interactions with a 75-kD cell-binding fragment and with intact fibronectin. In the present study domain-specific reagents were used in inhibition assays to further differentiate cell surface interactions with the two fibronectin domains, and to define the significance of these domains to cell interactions with the intact fibronectin molecule. These reagents are (*a*) a soluble synthetic tetrapeptide Arg-Gly-Asp-Ser (RGDS; Pierschbacher, M. D., and E. Ruoslahti, 1984, *Nature (Lond.)*, 309:30–33) representing a cell-binding determinant in the 75-kD fragment, and (*b*) an antibody raised against the 33-kD fragment that binds specifically to that fragment. Initial cell attachment to, and neurite extension upon, fibronectin and the two different fragments was evaluated in the presence and absence of the two reagents.

Attachment of both PNS and CNS cells to intact fibronectin was reduced in the presence of RGDS, the former more so than the latter. In contrast, the antibody to the 33-kD fragment did not affect attachment of PNS cells to fibronectin, but significantly decreased attachment of CNS cells to the molecule. RGDS inhibited attachment of both cell types to the 75-kD fragment to a greater degree than it did attachment to the intact molecule. Cell interaction with the 33-kD fragment was not affected by RGDS. Reduction of neurite lengths (determined after 24 h of culture) by the domain-specific reagents paralleled the reduction in initial adhesion to each substratum. Therefore, it appears that (*a*) both PNS and CNS cells have receptors for each cell-binding domain of fibronectin, (*b*) the receptor(s) for the two domains are distinct, with attachment to the 33-kD fragment being independent of RGDS, and (*c*) the relative importance of each domain to cell interaction with intact fibronectin is different for CNS and PNS cells.

NEURONS encounter extracellular matrix molecules during critical phases of embryonic differentiation and interact with a variety of these molecules in vitro. Definition of the molecular bases of these interactions is likely to be of major importance in understanding (a) extrinsic influences on neuronal differentiation, (b) cell type-specific responses to extracellular matrix components, and (c) mechanisms by which matrix molecules may support and possibly guide cell movements and nerve fiber elongation (Letourneau, 1975). The extracellular matrix glycoprotein fibronectin has been studied extensively with respect to both its chemical structure and its interactions with various types of cells (Furcht, 1983; Yamada, 1983; Hynes, 1986). Fibronectin mediates adhesion of many cells, including some neurons, to in vitro substrata, an interaction that may then lead to cell motility. Adhesion and motility of neuronal growth cones in response to fibronectin, as well as other matrix molecules, can result in neurite elongation in vitro and possibly in vivo.

Interactions of embryonic chick peripheral (PNS)[1] and central nervous system (CNS) cells with fibronectin and with proteolytic fragments of the molecule were investigated in earlier studies (Rogers et al., 1983, 1985). One of these fragments, with an approximate molecular mass of 75 kD, has been described by other laboratories and contains a cell-binding domain (Pierschbacher et al., 1981; Hayashi and Yamada, 1983) with a critical three amino acid sequence,

1. *Abbreviations used in this paper:* CNS, central nervous system; DRG, dorsal root ganglia; HSPG, heparan sulfate proteoglycan; PNS, peripheral nervous system; SC, spinal cord.

Arg-Gly-Asp (RGD; Pierschbacher and Ruoslahti, 1984*b*; Yamada and Kennedy, 1984). This sequence is involved in binding of fibronectin to a specific set of cell surface glycoproteins (Akiyama et al., 1985; Horwitz et al., 1985). A second cell-binding fragment, with an approximate molecular mass of 33 kD, was isolated and purified by virtue of its ability to bind to heparin (Smith and Furcht, 1982; McCarthy et al., 1986). It is located near the carboxyl terminus of fibronectin. Recently, other laboratories have described cell interactions with a heparin-binding domain of fibronectin (Izzard et al., 1986; Woods et al., 1986), but neither the cell-binding constituent of the fragment, nor the cell surface receptor for this constituent have yet been adequately defined.

Cells from the PNS attached to and extended neurites on intact fibronectin and both the 33- and 75-kD fragments. CNS cells attached to and extended neurites on the 33-kD heparin-binding fragment, but initial interactions of these cells with the 75-kD fragment and with intact fibronectin were not sustained.. Specifically, CNS neurons initiated neurites on the 75-kD fragment, but the neurites retracted and the cells detached after ~24 h in culture. Intact fibronectin supported attachment of fewer CNS cells than PNS cells, and neurites were not observed in these CNS cultures. These observations of cell behavior suggested that (*a*) the two cell-binding domains of fibronectin have distinct interactions with neuronal surfaces, (*b*) CNS and PNS cells have qualitative and/or quantitative differences in their binding sites for fibronectin, and (*c*) cells may have multiple interactions with fibronectin that are specific to cell type and/or activity.

These hypotheses raised additional questions which are addressed in the present study. First, can the difference(s) between the two fragments be further defined with respect to their interactions with cell surfaces? Although fibronectin receptors have been isolated from several cell types (Horwitz et al., 1985; Pytela et al., 1985; Akiyama et al., 1986; Urushihara and Yamada, 1986), receptor isolation would be a difficult task with heterogeneous primary neuronal cultures. Fortunately, alternative approaches, such as using proteolytic fragments with adhesion-promoting activity, may also provide insights. Second, how do the interactions between neurons and isolated fibronectin fragments relate to neuronal interaction with the intact molecule? Although nervous system cells can interact with two distinct fibronectin domains, previous studies do not define the significance of each domain to neuronal interaction with intact fibronectin during cell attachment and/or neurite extension.

The development of reagents that interfere selectively with cell adhesion to cell-binding domains has permitted more refined approaches to the above questions. Attachment of some cells to fibronectin can be competitively inhibited by the synthetic tetrapeptide RGDS, representing a cell-binding determinant in the 75-kD fragment of fibronectin (Pierschbacher and Ruoslahti, 1984*a*; Yamada and Kennedy, 1984). To test the significance of the 33-Kd heparin-binding domain to cell interaction with fibronectin, a polyclonal antibody was raised against the 33-kD fragment. These two reagents and the highly purified fibronectin fragments were used in a variety of in vitro experiments to further probe interactions of nervous system cell interaction with fibronectin. The results reinforce the notion that different types of cells interact selectively with multiple cell-binding domains of extracellular matrix molecules.

Materials and Methods

Reagents

Human plasma fibronectin and fibronectin fragments were prepared as described previously (Smith and Furcht, 1982; Smith et al., 1982; Rogers et al., 1985; McCarthy et al., 1986). In the present study, only the two fragments that mediate cell–substratum binding were used. Briefly, a 75-kD fragment was isolated by trypsinization of fibronectin, followed by monoclonal antibody 180-8 affinity chromatography (McCarthy et al., 1986). This fragment does not bind heparin or gelatin. A 33-kD heparin-binding fragment from the carboxyl terminal region of fibronectin was purified from a 33/66 kD complex (Rogers et al., 1985; McCarthy et al., 1986) by affinity chromatography on monoclonal antibody 2-8 (Smith and Furcht, 1982; McCarthy et al., 1986). The 66-kD fragment binds the antibody, while the 33-kD peptide does not.

Laminin was isolated from mouse EHS tumor basement membrane as described previously (Palm and Furcht, 1983).

Antiserum was raised against the 33-kD fibronectin fragment by injection of the emulsified fragment into New Zealand white rabbits, and then purified on a 33-kD fragment affinity column. This antibody does not cross-react with any of the other fibronectin fragments as analyzed by immunoblot analysis.

The synthetic tetrapeptide Arg-Gly-Asp-Ser (RGDS), representing a cell-binding determinant of fibronectin, was purchased from Peninsula Laboratories, Inc., Belmont, CA. The hybridoma producing monoclonal antibody A2B5, raised against a chick retinal ganglioside (Eisenbarth et al., 1979), was purchased from American Type Culture Collection, Rockville, MD.

Preparation of Substrata

All proteins were diluted in 0.05 M carbonate buffer (pH 9.6). Concentrations for each experiment are indicated in the figure legends and discussed in Results. Diluted proteins were applied to 24-well plates (Costar, Cambridge, MA) in 300-μl aliquots and incubated overnight at 37°C. The wells were then rinsed twice with PBS, incubated for 2–3 h with BSA (5 mg/ml, fatty-acid free; Boehringer Mannheim Biochemicals, Indianapolis, IN) to reduce nonspecific cell attachment to plastic, and rinsed again with PBS before addition of cells. Approximately 10–25% of applied protein binds to plastic under the conditions described (Rogers et al., 1985).

Adhesion Assays

Dorsal root ganglia (DRGs) or spinal cords (SCs) were dissected from chick embryos at 10 or 6 d of incubation, respectively. Cells were dissociated with trypsin (0.25% Bactotrypsin; Difco Laboratories, Inc., Detroit, MI) and plated at 20,000 cells per well in serum-free Ham's F14 supplemented with insulin (5 μg/ml, Sigma Chemical Co., St. Louis, MO), transferrin (100 μg/ml), and sodium selenite (30 nM). DRG cultures were also supplemented with 10 ng/ml nerve growth factor (7S NGF; HyClone Laboratories, Logan, UT). Optimal times for adhesion assays were determined by trial and error for each cell type. At 2 h of incubation DRG cells attached to the fibronectin and laminin substrata, but not to BSA. For SC cells it was necessary to increase the assay time to 4 h to obtain approximately equal attachment to all of the coating proteins. After incubation at 37°C, the wells were rinsed twice with warm PBS to remove nonadherent cells, and the remaining cells were fixed with 2% glutaraldehyde. Cells were stained with Dif-Quick (Scientific Products) and counted with an Image System IV (Optomax, Hollis, NH) attached to an Olympus IMT-2 inverted microscope and to a computer (Apple Computer, Inc., Cupertino, CA) for statistical analysis of data. Duplicate wells were prepared for each experimental condition, and the average number of cells per field was computed from counts of cells in five fields per well.

When the synthetic tetrapeptide RGDS was used in adhesion assays, it was added to wells along with the cell suspension. For assays with the 33-kD fragment antibody, substrata were preincubated with a 10-fold excess of antibody for 2 h, the wells were rinsed, and additional antibody was added to the cell suspension.

Neurite Extension Assays

DRG cells were allowed to attach to the various substrata for ~16 h, by which time many neurons had extended short neurites. RGDS or antibody to the 33-kD fragment was then added to the cultures. Neurite elongation was allowed to proceed for an additional 8 h and the cells were then fixed

with 2% glutaraldehyde. Neurites were measured with a digitizing tablet attached to the image analysis system described above.

Immunocytochemistry

To stain cells with the A2B5 monoclonal antibody, adhesion assays were performed with cells cultured on coverslips that had been treated with the substratum proteins and placed in 24-well plates. Living cells were treated with A2B5 in PBS containing 5 mg/ml BSA (1:10 dilution of A2B5 ascites fluid) for 15 min. The coverslips were then rinsed with PBS, fixed with 4% paraformaldehyde, rinsed, treated with rhodamine isothiocyanate goat anti-mouse IgG (1:100), rinsed again, and mounted on slides. Cells were examined with a Zeiss IM35 microscope equipped with fluorescence optics.

Results

Characterization of Fibronectin Fragments

The two fibronectin fragments used in this study have been described in detail in a recent report by McCarthy et al. (1986). The 75- and 33-kD fragments are distinct by electrophoretic and immunochemical criteria, have different ligand-binding properties, and can mediate different types of cell behavior. Location of these fragments in the fibronectin molecule is shown in Fig. 1. Relevant to the present study is the ability of the 33-kD fragment to bind heparin, and the location of a cell-binding determinant, Arg-Gly-Asp-Ser (RGDS), in the 75-kD fragment.

Domain-specific Inhibition of Cell Adhesion to Fibronectin Substrata

The absolute number of cells that attached to any given substratum varied as experiments were repeated. However, within each experiment the number of cells that attached to control substrata (i.e., in the absence of competing reagents) was relatively consistent. These values are presented in the figure legends. For each substratum, counts of cells attached in the presence of competing reagents were compared with control values, and the relative values expressed as percentage of control cells attached (mean number of experimental cells/mean number of control cells $\times$ 100 $\pm$ SEM).

Concentrations of the substratum proteins in the coating solutions were 1×10^{-7} M fibronectin and the two fragments (20 μg/ml fibronectin, 7 μg/ml 75-kD fragment, and 3 μg/ml 33-kD fragment) for most experiments. In addition, some experiments were performed using 5×10^{-7} M 75- and 33-kD fragments. Since cell attachment did not differ significantly (by *t* test analysis) among experiments using different coating concentrations, values are presented for one concentration of each extracellular matrix protein in each experiment. Those concentrations are indicated in the figure legends. Laminin was diluted to 2×10^{-8} M (20 μg/ml). Laminin-treated substrata provided a positive control, as cell binding to this glycoprotein has not been reported to be RGDS-dependent.

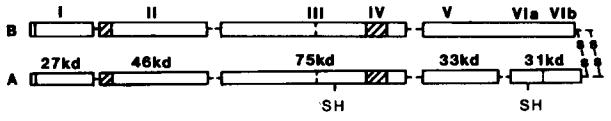

Figure 1. A model of the fibronectin molecule (adopted from McCarthy et al., 1986) illustrating the positions of the two proteolytic fragments used in this study, as well as other biologically active domains (Furcht, 1983). At the amino terminus of the molecule, a 27-kD fragment binds staph, heparin (weak activity), and fibrin. Collagen-binding activity resides in domain II, in a 46-kD fragment. The 75-kD fragment, first described by Hayashi and Yamada (1983), contains a free sulfhydryl (domain III) and a cell-binding determinant (Arg-Gly-Asp-Ser) in domain IV (*hatched area*; Pierschbacher and Ruoslahti, 1984*a*). The 33-kD heparin-binding fragment (domain V) also binds to cell surfaces, as described in detail in the text. Domain VIa represents a free sulfhydryl, and domain VIb, at the carboxyl terminus of fibronectin, contains interchain disulfide bonds.

The synthetic tetrapeptide RGDS was used at a concentration of 10^{-3} M (0.44 mg/ml) in all experiments reported. Because inhibition of cell attachment was incomplete at this concentration, repeated attempts were made to increase inhibition of attachment with a higher dose of RGDS and/or lower concentrations of substratum proteins. Although 2×10^{-3} M RGDS (1 mg/ml) inhibits attachment of normal rat kidney (NRK) cells (Pierschbacher and Ruoslahti, 1984*b*) and spreading of baby hamster kidney (BHK) and B16-F10 melanoma cells (Humphries et al., 1986), increased inhibition was not observed in the primary cultures used in the present study. In addition, lowering the concentration of fibronectin to 5×10^{-9} M did not increase inhibition at any dose of RGDS. Possible explanations for this incomplete inhibition will be discussed below.

Attachment to Nonfibronectin Substrata. Laminin was used as a control extracellular matrix glycoprotein to ensure that effects of the competing reagents were not due to toxicity or to other nonspecific events. Neither RGDS nor the 33-kD antibody had significant effects on cell attachment to laminin.

For adhesion assays, wells coated with fibronectin, the two fragments, or laminin were subsequently treated with BSA to prevent nonspecific binding of cells to tissue culture plastic. In all experiments reported, a negligible number of cells attached to BSA-treated wells. This prompted the assumption that the cell attachment observed was in response to the extracellular matrix proteins adsorbed to plastic.

Attachment to Fibronectin Fragments. Inhibition of cell attachment to the two fibronectin fragments by RGDS and the 33-kD fragment antibody occurred in a domain-specific manner. Attachment of both DRG and SC cells to the 75-kD fragment was reduced by RGDS (Fig. 2). While this inhibition is somewhat greater than that observed for attachment to fibronectin, these differences are not statistically significant. In contrast, the 33-kD fragment antibody did not significantly affect cell attachment to the 75-kD fragment (Fig. 3). The small effect observed is probably nonspecific, as there is a similar effect of the antibody on cell attachment to laminin.

Attachment of DRG and SC cells to the 33-kD fragment was reduced to 35–40% of control values by the antibody raised against the 33-kD fragment (Fig. 3). Significantly, RGDS did not affect interaction of either cell type with this fragment (Fig. 2; $P < 0.01$).

Attachment to Intact Fibronectin. RGDS reduced the number of both DRG and SC cells attached to intact fibronectin (Fig. 2). For DRG cells the percentage of cells attached to fibronectin in the presence of RGDS was significantly smaller than that for attachment to laminin or to the

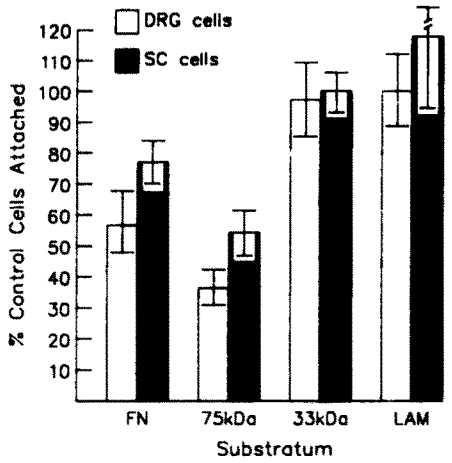

Figure 2. Effect of the fibronectin tetrapeptide RGDS on attachment of DRG and SC cells to fibronectin substrata and laminin. Cells were plated in tissue culture wells treated with fibronectin (FN), the two FN fragments (75 and 33 kD) or laminin (LAM), in the presence (experimental) and absence (controls) of RGDS (10^{-3} M). Concentrations of proteins in the coating solutions were 1×10^{-7} M for FN and the two fragments and 2×10^{-8} M for LAM. After 2 (DRG cells) or 4 (SC cells) h of incubation, nonadherent cells were removed by rinsing and the remaining cells fixed and counted. The percentage of cells attached in the presence of RGDS (experimental values) was expressed as a percentage of control values (without RGDS), calculated as the number of experimental cells divided by the number of control cells × 100. Error bars = SEM. Means of the absolute numbers of control cells attached (mean number of cells per field ± SEM): (DRG cells) FN = 79 ± 24; 75 kD = 66 ± 15; 33 kD = 47 ± 10; LAM = 51 ± 15; (SC cells) FN = 20 ± 9; 75 kD = 30 ± 11; 33 kD = 22 ± 6; LAM = 92 ± 46.

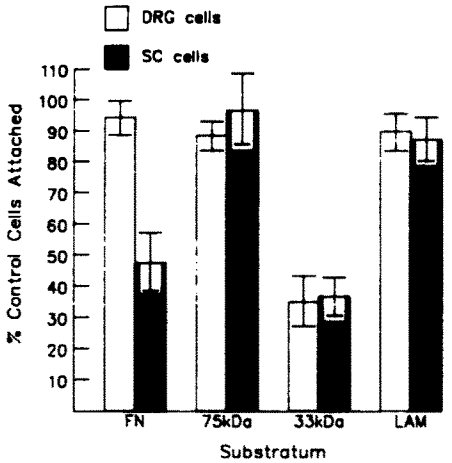

Figure 3. Attachment of DRG and SC cells in the presence of polyclonal antibody raised against the 33-kD heparin-binding fragment. Wells coated with each substratum protein were preincubated with a 10-fold excess of antibody before addition of cells, and additional antibody was added to the cell suspension. Concentrations of proteins in the coating solutions were: FN, 1×10^{-7} M; 75- and 33-kD fragments, 5×10^{-3} M; LAM, 2×10^{-8} M. Attached cells were fixed and counted as described for Fig. 2. Means of the absolute numbers of control cells attached (mean number of cells per field ± SEM): (DRG cells) FN = 60 ± 8; 75 kD = 82 ± 4; 33 kD = 51 ± 10; LAM = 45 ± 27; (SC cells) FN = 40 ± 21; 75 kD = 17 ± 3; 33 kD = 33 ± 3; LAM = 96 ± 39.

33-kD fragment ($P < 0.05$). There was less inhibition of SC cell attachment to fibronectin by RGDS, and this value (76.7% control cells attached) falls between values for SC cell attachment to the 75- and 33-kD fragments (i.e., it is not significantly different from either, $P > 0.05$ for each comparison). While the difference between the effects of RGDS on SC and DRG attachment to fibronectin was not quite statistically significant ($P = 0.156$), a significant difference might be revealed with a larger sample size (Cohen, 1977).

In contrast to RGDS, the 33-kD fragment antibody strikingly inhibited SC cell attachment to fibronectin, reducing the number to <50% of control cells attached (Fig. 3). This value is not different statistically from the percentage of SC cells attached to the 33-kD fragment in the presence of antibody ($P > 0.05$). Attachment of DRG cells to fibronectin, on the other hand, was not affected by the 33-kD antibody. This difference in cell interaction with fibronectin is emphasized by the fact that attachment of both DRG and SC cells to the 33-kD fragment was similarly reduced by the antibody. These observations suggest that (*a*) DRG and SC cells interact differently with these two cell-binding domains of intact fibronectin, (*b*) the 33-kD heparin-binding domain is more important to SC than to DRG cell attachment to the intact molecule, and (*c*) the cell-binding determinant in the 33-kD domain is accessible to cells in intact fibronectin.

To investigate further whether cells can use both the 33- and 75-kD domains in intact fibronectin, or interact with them in an alternate manner, an additional experiment was performed. Substrata coated with fibronectin (1×10^{-7} and 2.5×10^{-8} M) were treated with the 33-kD fragment antibody as described above and RGDS was added to the DRG cell suspension in some of the wells. When combined, these two reagents have an additive effect, reducing the number of cells attached to 20% of control values in one experiment, and to 37% in another experiment (data not shown). This indicates that DRG cells can interact with both the 33- and 75-kD domains in intact fibronectin, and supports the possibility that cell interaction with the 33-kD domain may at least partially account for the incomplete inhibition of cell attachment by RGDS.

Demonstration of the Presence of Neurons in Adhesion Assays

A potential problem in interpretation of the adhesion assays described above is the heterogeneity of the cell populations. In assays that are only a few hours in length it is impossible to determine accurately, at least by morphological criteria, what cell types are present. During this period nonneuronal cells may not spread, and, consequently, they may resemble phase-bright neuronal somata. Also, most neurons have not yet begun to extend neurites and cannot, therefore, be identified on this basis. The monoclonal antibody A2B5 (Eisenbarth et al., 1979) recognizes a neuronal cell surface ganglioside and was used to identify neurons in DRG cell adhesion assays. (This antibody was not useful in differentiating cell types in SC cultures, as it also recognizes a class of CNS glia (Schnitzer and Schachner, 1982).

In cultures of DRG cells, A2B5 stains 3% or fewer cells that are nonneuronal in appearance (i.e., flattened or spread). After staining cultures with the antibody, the percentage of fluorescent cells was determined for each experimental con-

dition (cells were counted in 10 fields for each condition). At least 100 cells per condition were counted. Both A2B5-positive cells and cells with nonneuronal morphology attached to the three fibronectin substrata in 2-h adhesion assays (Fig. 4). The percentage of neurons in the heterogeneous cell populations plated on fibronectin and the two fragments was nearly identical (~33%), and demonstrated that neurons participate in the interactions described above. Thus, differential attachment of neuronal and nonneuronal populations to the three substrata was not observed. (It should be noted that this proportion of neurons approximates that found in intact dorsal root ganglia at this embryonic stage.) Addition of RGDS did not significantly alter the proportion of neurons attached to each substratum, indicating that two major subpopulations, at least in these cultures, interact with the RGDS determinant of fibronectin to a similar extent. There was a reduction in the percentage of neurons attached to the three substrata in the presence of 33-kD fragment antibody, but the significance of this observation is not yet clear.

Domain-specific Inhibition of Neurite Extension on Fibronectin Substrata

Measurements of neurite length were obtained for DRG neurons only, as SC neurons extend neurites on fibronectin and the 75-kD fragment only transiently. Neurite elongation by DRG neurons paralleled initial cell adhesion to the substrata, in terms of (*a*) the domain-specific effects of RGDS and the 33-kD fragment antibody, and (*b*) the relative impact of each reagent on DRG neurite extension on intact fibronectin. The values reported in Tables I and II represent mean lengths of all neurites extended by each neuron sampled.

Neurites elongated in response to both high and low coating concentrations of the substratum proteins (as discussed in the section on adhesion assays), but they were shorter when the lower concentration was used. Therefore, values are reported for experiments using coating concentrations that yielded similar neurite lengths under control conditions (see Tables). Addition of RGDS to cultures at 16 h resulted in significantly shorter neurites on substrata treated with fibronectin and the 75-kD fragment (Table I). The reduction in neurite length by RGDS was consistent for these two substrata, regardless of concentration of the substratum proteins. During the 8 h of culture after adding RGDS, neurite elongation appeared to be slowed rather than halted, judged by

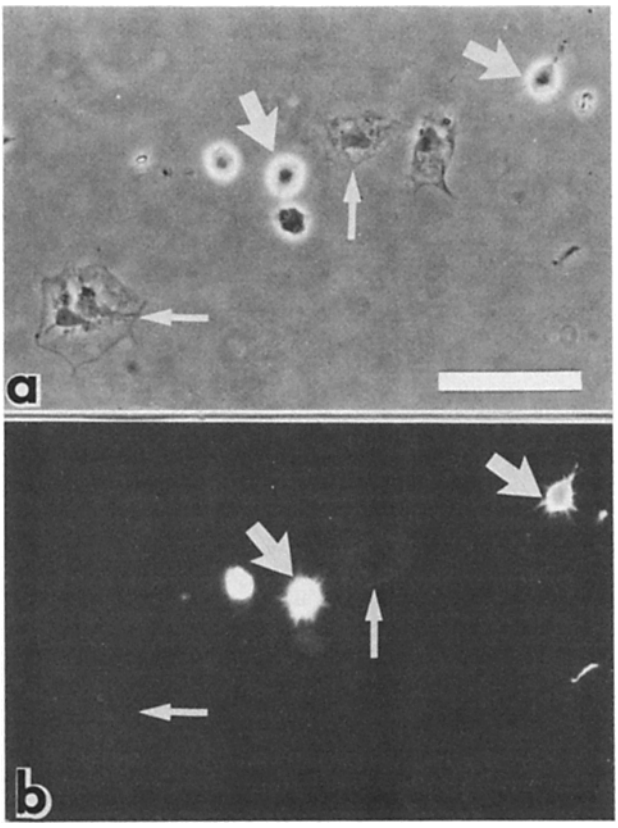

Figure 4. Dorsal root ganglion cells plated on fibronectin for 2 h. (*a*) Both flattened cells (*small arrows*) and phase-bright cells that appear to be neurons (*large arrows*) have attached to the substratum. Phase optics. Bar, 50 μm. (*b*) Same cells as in *a*, viewed with fluorescence optics. Cultures have been stained with the neuron-specific monoclonal antibody A2B5, confirming the identity of the nonflattened cells in *a* as neurons.

Table I. Effect of RGDS on DRG Neurite Lengths

Substratum	Average neurite length: Control	Average neurite length: +RGDS	P‡
	μm ± SEM (No. neurons)*	*μm ± SEM* (No. neurons)*	
Fibronectin	280 ± 34 (10)	163 ± 34 (10)	<0.05
75 kD	279 ± 23 (19)	166 ± 17 (19)	<0.001
33 kD	271 ± 22 (12)	237 ± 42 (12)	NS

* Neurite lengths represent the average total neurite length per neuron, measured after 24 h of culture. The values reported are from experiments using 10^{-7} M and 5×10^{-7} M 75- and 33-kD fragments to coat substrata.
‡ Significance levels for two sample *t* tests comparing neurite lengths in the presence and absence of antibody. The differences in neurite lengths on fibronectin and the 75-kD fragment are significant.

Table II. Effect of 33-kD Fragment Antibody on DRG Neurite Lengths

Substratum	Average neurite length: Control	Average neurite length: +33-kD Antibody	P‡
	μm ± SEM (No. neurons)*	*μm ± SEM* (No. neurons)*	
Fibronectin	247 ± 19 (40)	242 ± 19 (40)	NS
75 kD	357 ± 42 (24)	263 ± 23 (24)	0.057
33 kD	249 ± 20 (30)	104 ± 11 (30)	<0.0001

* Neurite lengths represent the average total neurite length per neuron, measured after 24 h of culture. The values reported are from experiments using 10^{-7} M fibronectin and 5×10^{-7} M 75- and 33-kD fragments to coat substrata.
‡ Significance levels for two sample *t* tests comparing neurite lengths in the presence and absence of antibody. The difference in neurite lengths on the 33-kD fragment is highly significant.

time-lapse video observations of growth cone activity (Letourneau et al., 1986). Neurite formation on the 33-kD fragment was not significantly affected by RGDS. This indicates that binding of RGDS to growth cone surfaces does not, in itself, slow neurite elongation, but that RGDS blocks interaction with a specific determinant of fibronectin.

Antibody against the 33-kD fragment had no effect on neurite elongation on intact fibronectin, but caused a highly significant reduction of length on the 33-kD fragment (Table II). The reduction in average neurite length on the 75-kD fragment is significant ($P = 0.057$), but may be a nonspecific effect, as a slight inhibition of initial attachment to the 75-kD fragment and to laminin was also observed (Fig. 3). Although neurite elongation occurs on both fibronectin fragments, these experiments suggest that DRG growth cones, like DRG cell bodies, interact preferentially with the fibronectin molecule via the RGDS determinant.

Discussion

Complex interactions of cells with components of extracellular matrices may be mediated by cell binding to specific molecular domains. In previous studies, it was demonstrated that CNS and PNS cells interact differently with the fibronectin molecule (Rogers et al., 1983, 1985). Both DRG cells from the PNS, and SC cells from the CNS adhered to a 33-kD heparin-binding fragment, and neurons in the cell populations extended neurites in response to it. Intact fibronectin and a 75-kD cell-binding fragment supported these responses by PNS cells, but interactions of CNS cells with these proteins were not sustained. However, the mechanisms underlying these differential responses were not clear. The experiments reported here demonstrate that (*a*) distinct molecular mechanisms are responsible for cell interactions with the two regions of fibronectin, and (*b*) the relative importance of each domain to interaction with intact fibronectin is different for the two cell populations.

The synthetic tetrapeptide RGDS represents a cell-binding determinant in a 75-kD fragment of fibronectin (Pierschbacher and Ruoslahti, 1984*a*, *b*). When added to cell suspension, RGDS binds to a cell surface receptor and inhibits cell interaction with that sequence, if present, in substratum-bound molecules. Experiments in which RGDS reduced cell attachment to intact fibronectin and to the 75-kD fibronectin fragment indicate that both SC and DRG cells have receptors for RGDS and that they can bind to this determinant in the substratum-bound proteins. Further, this interaction is distinct from cell attachment to the 33-kD domain, as RGDS had no effect on attachment to the 33-kD fragment. Somewhat less RGDS inhibition of SC cell adhesion to fibronectin and the 75-kD fragment, compared with DRG cell adhesion, was observed, and might partially explain weaker interaction of SC cells with intact fibronectin. The defect(s) in this interaction is not limited to short term adhesion assays, as neurite extension in response to fibronectin and the 75-kD fragment is also defective (Rogers et al., 1985). RGDS-mediated binding to fibronectin may be critical for stable cell interaction with the molecule, and SC cells may have insufficient numbers of RGDS receptors and/or may lack additional surface constituents necessary for stable interaction. Interestingly, SC cells stain with the CSAT monoclonal antibody, which recognizes a receptor for fibronectin and laminin (Horwitz et al., 1985), but this staining is less intense than CSAT staining of DRG cells (Letourneau, P., unpublished observations).

Inhibition by RGDS of cell attachment to fibronectin substrata was incomplete in all experiments, and several potential explanations for this observation should be considered. First, binding of RGDS to cell surfaces appears to be of low affinity (Yamada, 1983; Akiyama et al., 1985, 1986), which might allow some cells to make adhesive contacts with the substratum that are stable to the rinses used in the adhesion assays. Second, soluble RGDS may not be able to compete effectively with more stable interactions of cells with substratum-bound fibronectin peptides. Stability of these interactions might be imparted by sequences adjacent to RGDS, obviously not present in the soluble, isolated tetrapeptide (Ruoslahti et al., 1985). Third, incomplete inhibition by RGDS might be explained simply by alternative cell interactions with non-RGDS sites in intact fibronectin and possibly in the 75-kD fragment (Urushihara and Yamada, 1986). While each of these phenomena may contribute to the incomplete inhibition observed, the heterogeneous nature of the primary cultures used in this study is also likely to be a critical factor. Studies using homogeneous cell lines to evaluate the effects of RGDS on attachment to fibronectin have obtained complete, or nearly complete inhibition of attachment (Pierschbacher and Ruoslahti, 1984*b*; Yamada and Kennedy, 1984; Akeson and Warren, 1986; McCarthy et al., 1986). In fact, complete inhibition of cell attachment was observed with doses of RGDS identical to those used in this study (Pierschbacher and Ruoslahti, 1984; Humphries et al., 1986). In heterogeneous DRG and SC cultures, different cell types (i.e., neurons, fibroblasts, glia) may have different affinities for RGDS, may bind preferentially to non-RGDS binding sites in the substratum-bound proteins, and/or may produce their own attachment factors. It is important to note, however, that neurons (identified as A2B5-positive cells) in the DRG populations were shown to be affected by RGDS to the same extent as the population as a whole. Further work is necessary to more precisely characterize interactions, independent of RGDS, of nonneuronal nervous system cells with fibronectin.

The antibody to the 33-kD fragment interfered with DRG and SC cell binding to that fragment, but had no effect on cell attachment to the 75-kD fragment. The location of the site in the 33-kD fragment with which nervous sytem cells interact is not yet clear, but it appears to be separate from an alternatively spliced cell-binding site in this region of fibronectin, described recently by Humphries et al. (1986). The 33-kD fragment antibody had strikingly different effects on DRG and SC cell attachment to intact fibronectin. Compared with a negligible effect on attachment of DRG cells, the antibody reduced SC cell adhesion to intact fibronectin by an average of 55% (in contrast to only 24% inhibition by RGDS). This experiment demonstrates preferential adhesion of SC cells to the 33-kD domain of fibronectin, as was suggested previously (Rogers et al., 1985). However, the question then arises as to why SC cells have stable interactions (i.e., neurite extension) with the 33-kD fragment of fibronectin but not with the intact fibronectin molecule. This question will be difficult to address until more is known about (*a*) exposure of the 33-kD domain during conformational alterations of fibronectin under various conditions of binding to in

vitro substrata (Grinnell and Field, 1981; Tooney et al., 1983), and (*b*) possible remodeling of substratum-bound fibronectin by cells in the process of attachment and/or migration. It seems likely that multivalent interactions with fibronectin are complex (see Decker et al., 1984), involving even more cell-binding determinants in the molecule and/or cell surface constituents than have been evaluated to date.

The identity of cell surface receptors for fibronectin is still under active investigation. Receptors have been isolated by several groups using a variety of methods (Brown and Juliano, 1985; Hasegawa et al., 1985; Horwitz et al., 1985; Pytela et al., 1985; Akiyama et al., 1986; Urushihara and Yamada, 1986). Most of these studies have identified a complex of three glycoproteins ranging in apparent molecular size from 120 to 155 kD, referred to as a 140-kD complex, which apparently interacts with the RGDS sequence of fibronectin (Akiyama et al., 1986). The CSAT monoclonal antibody recognizes this 140-kD complex (Knudsen et al., 1985) and also interacts with neuronal surfaces (Bozyczko and Horwitz, 1986). Therefore, this complex of glycoproteins may be involved in RGDS-mediated neuronal adhesion to fibronectin. However, at least one additional glycoprotein class appears to be involved in cell–fibronectin adhesion (Urushihara and Yamada, 1986); gangliosides (Yamada et al., 1983) and proteoglycans (Perkins et al., 1979) are fibronectin receptor candidates as well.

Cell surface heparan sulfate proteoglycan (HSPG) is an obvious possibility as a receptor for the 33-kD heparin-binding domain of fibronectin (Laterra et al., 1983; Woods et al., 1984). Evidence demonstrating that the 33-kD fragment binds HSPG (McCarthy, J. B., unpublished observations) is highly suggestive, but does not in itself prove that cell surface HSPG binds to the fibronectin domain. Two recent studies show that fibroblasts (Izzard et al., 1986; Woods et al., 1986) require both the cell-binding and a heparin-binding domain to exhibit a spreading response complete with focal contacts and stress fibers. Although direct evidence is lacking that HSPG is involved in these responses, the heparan sulfate binding protein, platelet factor 4, in combination with the cell-binding fibronectin fragment, elicits the same complete spreading response as does intact fibronectin (Izzard et al., 1986). Some neurons express HSPG on their surfaces (Ratner et al., 1985) and may use the molecule to adhere to in vitro substrata (Cole et al., 1985; Matthew et al., 1985; Ratner et al., 1985; Schubert and LaCorbiere, 1985; Tobey et al., 1985). It is important that the consequences for cytoskeletal organization of cell binding to fibronectin via HSPG appear to be very different from those resulting from adhesion via the 140-kD complex mediated by RGDS (Izzard et al., 1985). It is plausible that HSPG-mediated cell binding to fibronectin, or other matrix molecules, stabilizes cells (some cells attach to, but do not migrate on the 33-kD fragment [McCarthy et al., 1986]) and/or modulates interaction with the other cell-binding determinant(s). Similar mechanisms for modulation by HSPG of cell adhesion, shape, and/or locomotion might operate for fibroblasts, neuronal cell bodies, and neuronal growth cones. But additional tools, such as antibodies that block the HSPG-binding capacity of fibronectin or recognize neuronal cell surface HSPG, are needed to evaluate potential roles of this proteoglycan more precisely.

Adhesion and motility of DRG growth cones on substrata treated with fibronectin and the two fibronectin fragments paralleled the responses of neuronal cell bodies during initial adhesion. Shorter neurite lengths on fibronectin and the 75-kD fragment in the presence of RGDS were due to slower rates of growth cone migration, rather than complete cessation of activity (Letourneau et al., 1986). Continued growth cone activity might be explained by low affinity binding of RGDS to growth cone surfaces, or to alternative growth cone adhesion to the 33-kD heparin-binding domain (in the case of migration on intact fibronectin). The results indicate that growth cones of DRG neurons can migrate in response to fibronectin via at least two separate mechanisms, one of which does not involve the RGDS determinant. However, interaction with the 33-kD domain does not appear to be essential for growth cone migration on intact fibronectin, as in the results of assays for initial adhesion of DRG cell bodies.

In conclusion, several specific questions raised by our previous work have been at least partially answered in this report. First, we have demonstrated that attachment of CNS and PNS cells to the 75-kD fragment of fibronectin involves a cell surface receptor for the RGDS sequence, while attachment to the 33-kD heparin-binding fragment is independent of RGDS. Second, the role that each cell-binding domain plays in cell attachment to the intact fibronectin molecule can vary with the cell population. Specifically, DRG cells can interact with fibronectin via the RGDS determinant when the 33-kD domain is blocked, while binding of SC cells to intact fibronectin depends to a greater degree on interaction with the 33-kD domain. In addition, it appears that nervous system cells can have multivalent interactions with fibronectin and that these interactions may involve a number of binding determinants in fibronectin and/or several cell surface receptors for the molecule.

It will now be important to test the significance of the two cell-binding domains of fibronectin (*a*) to growth cone migration through complex extracellular matrices and (*b*) during embryonic differentiation of PNS neurons. Populations of neural crest cells can use fibronectin as a substratum for migration (Weston, 1980; Rovasio et al., 1983; Bronner-Fraser, 1985) and it appears that differentiation of these cells into a variety of phenotypes can also be influenced by exposure to the molecule (Loring et al., 1982). An intriguing possibility is that differentiation of individual neural crest cells into subpopulations of PNS cells may be mediated by specific fibronectin cell-binding domains. As a result, the ability of PNS cells to interact in a stable manner with two domains of fibronectin might reflect their developmental history. Comparisons between neural crest-derived and non-neural crest-derived cells (such as those from the CNS) may provide clues to the significance of multivalent cell–extracellular matrix interactions.

The authors wish to thank Susan Alexander, Joy Bennin, and Scott Hagen for their assistance with various technical aspects of this study. Ms. Kathy Hsi and Dr. Dorothy Pathak (Family, Community, and Emergency Medicine, University of New Mexico) are gratefully acknowledged for performing and interpreting the statistical analyses.

This research was supported by National Institutes of Health grants HD19950 (P. C. Letourneau), NS23360 (S. L. Rogers), CA39510 (J. B. McCarthy), and CA21463 and CA29995 (L. T. Furcht) and by grants from the National Science Foundation (PCM8203855) and the Spinal Cord Soci-

ety (P. C. Letourneau). L. T. Furcht is Stone Professor of Pathology at the University of Minnesota.

Received for publication 28 October 1986, and in revised form 27 April 1987.

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