Recognition of the A Chain Carboxy-Terminal Heparin Binding Region of Fibronectin Involves Multiple Sites: Two Contiguous Sequences Act Independently to Promote Neural Cell Adhesion

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Abstract. Cellular interactions with fibronectin-treated substrata have a complex molecular basis involving multiple domains. A carboxy-terminal cell and heparin binding region of fibronectin (FN) is particularly interesting because it is a strong promoter of neurite outgrowth (Rogers, S. L., J. B. McCarthy, S. L. Palm, L. T. Furcht, and P. C. Letourneau, 1985. J. Neurosci. 5:369-378) and cell attachment (McCarthy, J. B., S. T. Hagen, and L. T. Furcht. 1986. J. Cell Biol. 102:179-188.). To further understand the molecular mechanisms of neuronal interactions with this region of FN, we screened two peptides from the 33-kD heparin binding fragment of the FN A chain, FN-C/H II (KNNQKSEPLIGRKKT) and CS1 (Humphries, M. J., A. Komoriya, S. K. Akiyama, K. Olden, and K. M. Yamada. 1987. J. Biol. Chem. 262:6886-6892), for their ability to promote B104 neuroblastoma cell-substratum adhesion and neurite outgrowth.

Both FN-C/H II and CS1 promoted B104 cell attachment in a concentration-dependent and saturable manner, with attachment to FN-C/H II exceeding attachment to CS1. In solution, both exogenous FN-C/H II or CS1 partially inhibited cell adhesion to the 33-kD fragment. Similar results were obtained with anti-FN-C/H II antibodies. In contrast, soluble GRGDSP did not affect B104 cell adhesion to FN-C/H II. These results indicate that both FN-C/H II and CS1 represent distinct, RGD-independent, cell adhesion-promoting sites active within the 33-kD fragment, and further define FN-C/H II as a novel neural recognition sequence in FN. B104 adhesion to FN-C/H II and CS1 differs in sensitivity to heparin, yet each peptide inhibited adhesion to the other peptide, suggesting cell adhesion is somehow related at the cellular level.

Within the A chain 33-kD fragment, FN-C/H II and CS1 are contiguous, and might represent components of a larger domain with greater neurite-promoting activity since only the 33-kD fragment, and neither individual peptide, was effective at promoting B104 neurite outgrowth. These data further support the hypothesis that cell responses to FN are mediated by multiple sites involving both heparin-sensitive and -insensitive mechanisms.

The extracellular matrix (ECM)1 is a complex mixture of components that plays an important role in regulating cell shape and behavior, thus affecting embryonic development and cellular differentiation. One ECM glycoprotein of much interest is fibronectin (FN), which promotes cell adhesion and motility (Couchman et al., 1982; McCarthy et al., 1988), and affects morphogenesis and embryonic cell migrations (Boucaut et al., 1984a,b; Dufour et al., 1988a; for review see Furcht, 1981; Hynes, 1985; Ruoslahti, 1988; Yamada, 1989). FN may function in nervous system development by promoting the outgrowth of axons from peripheral nervous system (PNS) neurons (Carbonetto et al., 1983; Rogers et al., 1983, 1989), and is present along pathways of neural crest cell migration in a spatial and temporal expression that correlates with the localization of neural crest cells (Duband and Thiery, 1982a,b; Bronner-Fraser, 1986; Dufour et al., 1988b; for review see Sanes, 1989). In the central nervous system (CNS), FN immunoreactivity has been noted as well, restricted to particular regions and stages of development that correlate to specific neuronal migrations and neurite outgrowth (Stewart and Pearse, 1987; Chum and Shatz, 1988). For example, FN promotes retinal neurite outgrowth (Akers et al., 1981; Hall et al., 1987). It is clear that FN may have diverse roles in developmental interactions with a variety of cells.

Cell adhesion to FN has a complex molecular basis involving multiple cell-attachment domains. The arginyl-glycyl-aspartyl-
serine (RGDS) tetrapeptide has been well characterized as a sequence that promotes cell attachment (Pierschbacher and Ruoslahti, 1984a, b; McCarthy et al., 1986), and plays a role in neurite outgrowth (Rogers et al., 1987; Waite et al., 1987; Letourneau et al., 1988; Mugnai et al., 1988b). Cells interact with the RGDGCS domain attachment sequence in FN through an α5β1 integrin-dependent mechanism (Pytel et al., 1985; Horwitz et al., 1985; Ruoslahti and Pierschbacher, 1987; for review see Buck and Horwitz, 1987; Hynes, 1987). RGDGCS is unable to account for all of the cell adhesion- and neurite-promoting activity of FN (Rogers et al., 1985, 1987; Izzard et al., 1986; McCarthy et al., 1986; Waite et al., 1987), indicating that molecules other than the RGD-specific integrins also mediate cell recognition of FN.

The carboxy-terminal cell and heparin binding region of FN promotes RGD-independent cell attachment (McCarthy et al., 1986; Izzard et al., 1986; Liao et al., 1989) and is a strong promoter of neurite outgrowth (Rogers et al., 1985, 1987; Waite et al., 1987). Rogers et al. (1985, 1987) demonstrated that neurons from the PNS and CNS attach to and extend neurites on substrata treated with a 33-kD proteolytic fragment of fibronectin from the carboxy-terminal heparin binding region of the A chain. High concentrations of soluble RGD-containing peptide do not inhibit neuronal adhesion nor decrease neurite length on surfaces coated with the 33-kD heparin binding fragment (Rogers et al., 1987). CNS neurons actually prefer the heparin binding fragment as a substratum over the intact FN molecule, and are able to form stable neurites on the isolated heparin binding domain of FN even though they do not form stable neurites on surfaces coated with intact FN (Rogers et al., 1985). In contrast, PNS neurons form stable neurites on either substratum (Rogers et al., 1985), demonstrating an example of different neuronal responses to specific FN domains.

One way to further elucidate the molecular basis for cell adhesion to the carboxy-terminal heparin binding domain of FN has been to generate synthetic peptides representing potential cell adhesion sequences within this region. Previous studies have identified three such peptides from within the heparin binding region that have RGD-independent cell attachment activity. C1 (DELPQLVTLPHNPNLHGPEDL-VPST) is found within the alternately spliced IIIcs region of the A isoform of FN (Kornbluth et al., 1985) and promotes cell-specific adhesion and spreading of melanoma cells (Humphries et al., 1987; McCarthy et al., 1990), as well as PNS neurite outgrowth (Humphries et al., 1988). Recently, it has been demonstrated that α5β1 integrin mediates hematopoietic cell adhesion to the C1 sequence (Wayner et al., 1989; Guan and Hynes, 1990; Mould et al., 1990). Two heparin-binding peptides from within the 33-kD fragment that promote melanoma cell adhesion have also been isolated: FN-C/H I (YEKPSPREVPRPVPGRV) and FN-C/H II (KNNQKRGLIRKKT) (McCarthy et al., 1988). These peptides differ from each other in that they are able to bind heparin (McCarthy et al., 1988), and FN-C/H II differs from FN-C/H I in that it is contiguous with C1.

In this study, we focused on the response of neural cells to defined sequences from the carboxy-terminal heparin binding domain of FN by contrasting FN-C/H II and C1 for their ability to promote neuroblastoma adhesion. The response of neural cells to FN-C/H II has not previously been described.

Our data support the hypothesis that FN-C/H II represents a specific RGD-independent neuronal cell attachment sequence within the carboxy-terminal cell and heparin binding domain of FN. Neuroblastoma adhesion to the C-terminal heparin binding region of the FN A chain involves multiple sites, since C1 is also active within this domain. Further, the A chain-derived 33-kD fragment, but neither peptide alone, promotes neurite outgrowth, suggesting that FN-C/H II and C1, which are contiguous within this fragment, are contained in a larger domain. Cell adhesion to FN-C/H II differs from cell adhesion to C1 in sensitivity to heparin, yet each peptide inhibits cell adhesion to the other peptide, implying cell adhesion to these two distinct peptides is somehow coordinated at the cellular level.

Materials and Methods

Cell Lines

The neuroblastoma cell line, B104, isolated by in vivo mutagenesis and generously donated by Dr. D. Schubert (Salk Institute), expresses several neuronal characteristics, including electrical excitability, neurotransmitter synthesis, and neurite-like process formation (Schubert et al., 1974). Cells were maintained free of mycoplasma contamination, in DME (Gibco Laboratories, Grand Island, NY), buffered with bicarbonate, and supplemented with 100 ng/ml penicillin-streptomycin, 0.25 μg/ml fungizone, 2 mM L-glutamine (Gibco Laboratories), 7.5% calf serum, and 2.5% FBS (Hyclone Laboratory, Logan, UT).

Protein, Fragment, and Peptide Preparation

Human plasma FN was purified as a byproduct of Factor VIII production by sequential ion-exchange and gelatin affinity chromatography as described (McCarthy et al., 1986). A 2-min trypsin digest followed by a cathepsin D digest produced the 75-kD and 33-kD fragments as described previously (McCarthy et al., 1986; see Fig. 1 for fragment location).

Peptides were synthesized at the University of Minnesota Microchemical Facility by Dr. Robert Wohlheuter using a peptide synthesizer (System 990; Beckman Instruments, Fullerton, CA). The procedures used are based on the Merrifield solid-phase system as described previously (Stewart and Young, 1984). Lyophilized crude peptides were purified by preparative reverse-phase HPLC on a C-18 column, using an elution gradient of 0-60% acetonitrile with 0.1% trifluoroacetic acid in water. The purity and composition of the peptides were routinely verified by HPLC analysis of hydrolysates prepared by treating the peptides under nitrogen in 6 N HCl overnight at 110°C, and the sequence was verified on the sequenator (McCarthy et al., 1986; Chellberg et al., 1989). The FN peptides specifically used for this study are: FN-C/H II (formally termed "Peptide II"; McCarthy et al., 1988), representing residues 1,946-1,960 (KNNQKRELIRKKT); C1 with a primary sequence of DELPQLVTLPHNPNLHGPEDLVPST (Humphries et al., 1987) and GRDQSP, representing residues 1,492-1,497 (based on sequence information from Kornbluth et al., 1985). FN-C/H II was synthesized with a tyrosine residue on the carboxy terminal end to allow for iodination (see below). The sequences shown use the single letter amino acid code (K = lysine, R = arginine, H = histidine, E = glutamic acid, D = aspartic acid, Q = glutamine, N = asparagine, P = proline, G = glycine, S = serine, T = threonine, V = valine, I = isoleucine, L = leucine, Y = tyrosine).

Coupling of Peptides to Ovalbumin (OA)

Peptides FN-C/H II and C1 were coupled to ovalbumin as suggested by Humphries et al. (1987), based on a procedure by Baumberger and Wilchek (1980). Briefly, equal amounts (by weight) of peptide and ovalbumin were solubilized and mixed with a 10-fold excess (by weight) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Sigma Chemical Co., St. Louis, MO) dissolved in water. After mixing overnight at 4°C, the sample was extensively dialyzed in PBS to remove excess carbodiimide and uncoupled peptide (10000-D exclusion; Spectrum Medical Industries, Los Angeles, CA).
Generation and Purification of Anti-FN-C/H II Antibodies

Polyclonal antibodies were generated against FN peptides coupled to keyhole limpet hemocyanin (KLH; Sigma Chemical Co.) using carbodiimide as the coupling reagent, as described above, however for the dialysis procedure, dialysis tubing with a 1,000-D exclusion (Spectrapore 6; Spectrum Medical Industries) was used to remove only the excess carbodiimide and not the uncoupled peptide. This mixture was then concentrated in Aquacide II (Calbiochem-Behring Corp., La Jolla, CA) to a final concentration of 10 mg/ml, and aliquots were stored at -70°C. The coupling of the peptide to KLH was verified by the use of radiodinated FN-C/H II in a parallel reaction mixture. The coupled mixture was then used to immunize New Zealand White rabbits. Immunization was performed by mixing an equal volume of peptide/KLH conjugate with CFA, and injecting this mixture into multiple sites on the shaved backs of the rabbits (>1 mg of conjugate/rabbit). Subsequent biweekly boosts in incomplete Freund's adjuvant were given in one shot intramuscularly. Sera was collected 14 d after the fourth immunization, and tested by ELISA (see below) for reactivity against proteins and uncoupled synthetic peptides. ELISA plates (either Immulon I or C; Dynatech Laboratories, Chantilly, VA) were prepared by adsorbing 1 µg of the synthetic peptides, FN, 33-kD fragment, control proteins, and control peptides into each well, using Voller's carbonate buffer to dilute the ligands (Smith and Furcht, 1982). The plates were then blocked by incubating the wells for 2 h at room temperature with PBS containing 2 mg/ml of ovalbumin (PBS/BSA). Various concentrations of antisera or purified IgG were diluted in PBS containing 0.05%Tween 20 and 0.36 M NaCl (PBS/Tween) and incubated in the wells for 1 h at room temperature on a rotating platform. The plates were washed three times with PBS/Tween and incubated for 1 h at room temperature on a rotating platform. After the third rinse in PBS/Tween, the plates were developed extensively with PBS/Tween and developed with the subsequent addition of 0.4 mg/ml of orthophenylene diamine containing 0.02% H2O2 in 0.1 M sodium phosphate and 24 mM citric acid. The reaction was terminated by the addition of 2.5 M H2SO4 and the intensity at 490 nm was determined using an automatic microplate reader (EL311; Bio-Tek Instruments, Burlington, VT).

Cell Adhesion and Spreading Assays

Substrates were prepared using two different procedures. FN, proteolytic fragments of FN, and synthetic peptides were diluted to the appropriate concentrations in Voller's carbonate buffer. If peptides had not been conjugated to OA, they were adsorbed onto 96-well tissue culture plastic (Costar Corp., Cambridge, MA) overnight in a dry oven at 29°C. If peptides were conjugated to OA, they were adsorbed to Immulon 1 plates in a humidified oven. A second method of quantitating adherent cells was also used that allowed for the estimation of cell attachment equals the number of cells counted divided by the number of cells adherent to the substrata. Additionally, duplicate plates that had been fixed and counted using phase microscopy verified the accuracy and sensitivity of this method. To quantitate adherent cells more quickly, the following assay based on the procedure of Landegren (1984) was used. After the third rinse in DPBS, 60 µl of hexosaminidase substrate (3.75 mM-p-nitrophenol-N-acetyl-b-D-glucosaminide, 50 mM citrate, 0.25% Triton X-100) was added to each well, and incubated for 21-24 h at 37°C. The assay was stopped and the color development by the addition of 90 µl of 50 mM glycine buffer, 5 mM EDTA, pH 10.4. Absorances were measured using a plate reader (EL311; Bio-Tek Instruments) at 405 nm. For each assay, a standard curve was constructed using known numbers of cells to determine the number of cells adherent to the substrata. Additionally, duplicate wells were then blocked with BSA in DPBS; the results were analyzed for significant binding. Polyclonal antibodies were generated against FN peptides coupled to keyhole limpet hemocyanin (KLH; Sigma Chemical Co.) using carbodiimide as the coupling reagent, as described above, however for the dialysis procedure, dialysis tubing with a 1,000-D exclusion (Spectrapore 6; Spectrum Medical Industries) was used to remove only the excess carbodiimide and not the uncoupled peptide. This mixture was then concentrated in Aquacide II (Calbiochem-Behring Corp., La Jolla, CA) to a final concentration of 10 mg/ml, and aliquots were stored at -70°C. The coupling of the peptide to KLH was verified by the use of radiodinated FN-C/H II in a parallel reaction mixture. Adherent Cells

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Peptide and Antibody Inhibition Assays

Substrata and cells were prepared as described above for the adhesion assays. Whenever an assay involved a soluble inhibitor, the substratum coating concentration used was one near the concentration that promoted half-maximal adhesion for that particular fragment or peptide. This concentration was different for peptides that had been conjugated to OA compared with those that had not. For peptide inhibition assays, cells at 50,000 cells/ml were incubated at 37°C for 25-30 min in F12H-BSA containing various concentrations of soluble peptides. After this incubation, the mixture of cells and peptide was plated directly into wells and cells were allowed to attach for 40 min, until they were observed to begin attaching and spreading on FN-coated surfaces. For antibody inhibition assays, various concentrations of purified anti-FN-C/H II IgG or normal rabbit IgG were incubated in the wells for 30 min. Cells were then added to the wells and allowed to attach for 40-50 min. For both types of assays, nonadherent cells were removed by rinsing, and adherent cells were fixed and counted.

Heparin (and Sulfated Polyanion) Inhibition

Substrata and cells were prepared as described above for the adhesion assays. Substrata were incubated at 37°C for 25 min with F12H-BSA containing various concentrations of soluble heparin (Sigma Chemical Co.). After three rinses in PBS, 5000 cells in 0.1 ml were added to each well and incubated at 37°C until cells were observed to begin attaching and spreading on substrata. Nonadherent cells were removed by rinsing, and adherent cells were quantitated by visually counting or assaying hexosaminidase. The same procedure was used for other sulfated polyanions. Dextran sulfate and bovine kidney heparan sulfate were obtained from Sigma Chemical Co. Chondroitin-4-sulfate from rat chondrosarcoma was a generous gift of Dr. Theodore Oegema (University of Minnesota). The charge characteristics of
Figure 1. Location of the 33- and 66-kD heparin binding fragments and the synthetic peptide sequences within the intact FN molecule. Also depicted is the location of the IIIcs region found only on the A isoform of human plasma FN. The amino and carboxy ends of the 33-kD fragment are based on previous sequence data (McCarthy et al., 1988). Selected biological domains, indicated by roman numerals at the top, are based on the nomenclature of Furcht (1981). I, weak heparin binding; II, collagen binding; III, free sulfhydryl; IV, RGD-mediated cell adhesion; V, carboxy-terminal strong heparin binding and cell adhesion; VI, free sulfhydryl. Location of tryptic (T) and cathepsin D (C) sites on fibronectin are shown.

Results

Neuroblastoma Cells Adhere to Multiple Sites Within the Carboxy-Terminal Heparin Binding Region of FN A Chains

The rat neuroblastoma cell line used for this study, B104, is a useful model to study neural responses to FN domains because one can isolate large quantities of cells for future characterization of molecules involved in adhesion. B104 cells are electrically excitable, synthesize neurotransmitter enzymes, and are able to extend long, thin neurite-like processes (Schubert et al., 1974, 1986; Tobey et al., 1985). B104 cells are able to attach and spread on intact FN (not shown).

Digestion of FN with trypsin and cathepsin-D yields a 33-kD heparin binding fragment from the A chain, containing a portion of the differentially spliced IIIcs region, and a 66-kD heparin binding fragment from the B chain, lacking the IIIcs region (Fig. 1; McCarthy et al., 1986). We determined the response of B104 cells to these fragments using three parameters: adhesion, spreading, and neurite outgrowth. B104 cells adhered in a concentration dependent manner on substrata coated with either heparin binding binding fragment, but showed a greater adhesive response to the A chain (33-kD) compared to the B chain (66-kD) derived fragment. After a 1-h incubation, more than twice as many B104 cells adhered to substrata coated with the 33-kD fragment than to substrata coated with equivalent or greater amounts of the 66-kD fragment (Fig. 2 A). The 33-kD fragment was also more effective at promoting B104 cell spreading than the 66-kD fragment. As seen with adhesion, cells cultured on 33-kD-treated substrata encompassed almost twice the surface area (in square microns) as cells cultured on 66-kD-treated substrata (Fig. 2 B). In fact, spreading on the 33-kD fragment slightly exceeded spreading on intact FN (not shown). At higher coating concentrations (100 μg/ml), B104 cells showed some spreading on the 66-kD-coated substratum.

Using radiolabeled FN fragments, we determined whether differences in B104 cell response to the 33- and 66-kD fragments were due to differences in binding of each fragment to the plastic plates. Differences in binding and molecular...
weights between the two fragments do not account for the greater adhesion and spreading activity of the 33-kD fragment, since similar molar amounts of both the 33- and 66-kD fragments were bound at each coating concentration tested (Table I).

In addition to effectively promoting B104 cell adhesion and spreading, the 33-kD heparin binding fragment promoted neurite outgrowth. Neurite-like process formation was scored after cells were cultured for 24 h, to allow B104 cells sufficient time to extend neurites, on substrata coated with the 33- or 66-kD fragments. B104 cells extended long, thin processes on a substratum coated with 20 µg/ml of the 33-kD fragment (Fig. 3 A). B104 cells spread, but extended only short processes on a substratum coated with 100 µg/ml of the 66-kD fragment (Fig. 3 B). From these observations, it appears the 33-kD fragment may contain a neurite-promoting activity absent in the 66-kD fragment.

Two previously described cell adhesion promoting synthetic peptides from within the 33-kD fragment were examined for their ability to promote B104 cell adhesion. Peptide CSI, which is present only in plasma FN A-chains (Humphries et al., 1987) has been shown to promote cell adhesion by an α4β1 integrin (Wayner et al., 1989; Mould et al., 1990). A second synthetic peptide, termed FN-C/H II (fibronectin cell and heparin binding peptide II), has been previously shown to promote melanoma cell adhesion and spreading (McCarthy et al., 1988) and is present within all isoforms of FN. FN-C/H II has been shown to bind [3H]heparin, whereas CSI is inactive in this regard (McCarthy et al., 1988, 1990). These two peptides represent contiguous sequences on plasma FN A-chains (Fig. I).

The synthetic peptides were coupled to a carrier protein, OA, since this had been shown to improve the ability of CSI to promote melanoma cell adhesion and spreading (Humphries et al., 1987). We quantitated the OA-coupling efficiencies and binding of peptide-OA to the wells using radiolabeled peptides (Table II). CSI exhibited a two-fold higher coupling efficiency to OA than FN-C/H II. The amount of peptide-OA bound to the wells was similar whether FN-C/H II or CSI was coupled to the OA (~40-50% binding at 1 µg/ml OA). However, due to the different coupling stoichiometries, more CSI bound than did FN-C/H II at each coating concentration.

B104 cells adhered to substrata treated with either FN-C/H II-OA or CSI-OA in a concentration dependent and saturable manner (Fig. 4). OA conjugated to itself (OA-OA) did not promote cell adhesion. As a substratum, FN-C/H II-OA was more active at promoting B104 adhesion than CSI-OA. FN-C/H II-OA maximally promoted adhesion of ~70% of the cells, while CSI-OA maximally promoted ~50% cell adhesion. To reach half of this maximum adhesion required treatment of the substrata with a greater concentration of CSI-OA than FN-C/H II-OA. When the concentration of OA was used as a basis for determining a coating concentration of 1 µg/ml, 4.3 pmoles of FN-C/H II promotes adhesion of 50% of the cells, while 6.1 pmol of CSI promotes adhesion of only 20% of the cells. Similar results were obtained for unconjugated FN-C/H II and CSI (not shown).

B104 cells also spread on substrata coated with either peptide conjugate (not shown). Within 1 h, both peptides promoted spreading of ~80-90% of adherent cells. In contrast to what was previously observed on the intact 33-kD fragment (Fig. 3 A), cells cultured overnight on either of the two peptide-OA conjugates did not exhibit long, neuronal processes (Fig. 3, C and D). Instead, the cell morphology on the two synthetic peptides resembled that observed on the 66-kD heparin binding fragment, with short, stubby processes being formed by these cells (compare Fig. 3 B with C and D).

**Peptides FN-C/H II and CSI Both Represent RGD-independent Cell Adhesion Promoting Sites within the 33-kD Heparin Binding Fragment**

Cell adhesion to CSI has previously been shown to be RGD-independent (Humphries et al., 1988; McCarthy et al., 1990). Attachment of B104 cells to FN-C/H II was also RGD independent (Fig. 5). B104 cells were incubated in various concentrations of soluble GRGDSP before allowing them to attach to surfaces treated with FN, the 75-kD FN fragment that contains an RGD sequence, or FN-C/H II. Soluble peptide was present throughout the assay. Soluble GRGDSP inhibited B104 adhesion to substrata coated with the 75-kD fragment or FN in a concentration-dependent manner, with 600 µg/ml of GRGDSP causing 57 and 19% inhibition, respectively (Fig. 5). This concentration of soluble GRGDSP had no effect on the adhesion of B104 cells to a FN-C/H II-coated substratum (Fig. 5), demonstrating that FN-C/H II is an RGD-independent site of cell adhesion.

Additional experiments were performed to establish that FN-C/H II represents an active site within the 33-kD fragment. As one approach, cell attachment assays were performed in the presence of increasing concentrations of purified IgG generated against FN-C/H II. An ELISA confirmed that this IgG reacted with FN-C/H II and intact FN (Table III). The antibody did not react with laminin, FN-C/H I (a different FN peptide that binds heparin and promotes cell adhesion), or OA.

In functional assays, anti-FN-C/H II IgG inhibited the attachment of B104 cells to a FN-C/H II substratum (Fig. 6).

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**Table I. Amount of FN Heparin-binding Fragments Bound to Plastic Wells**

| Coating concentration | Input fragment | Bound fragment |
|------------------------|---------------|---------------|
| µg/ml                  | pmol/well     | pmol/well     |
| 33-kD fragment         |               |               |
| 100                    | 246           | 7.0           |
| 30                     | 69            | 7.2           |
| 10                     | 22            | 4.2           |
| 3                      | 4.5           | 1.6           |
| 66-kD fragment         |               |               |
| 100                    | 143           | 12.0          |
| 30                     | 38            | 4.9           |
| 10                     | 15            | 3.4           |
| 3                      | 3.4           | 1.5           |

The 33- and 66-kD COOH-terminal heparin binding fragments of FN were radiolabeled with 3H by reductive methylation (Materials and Methods). Labeled fragments were then serially diluted and adsorbed to plastic wells exactly as was done for the adhesion assay in Fig. 2. Using the input disintegrations per minute, the specific activity, and the molecular weight of each fragment, the amount of input FN fragment for each well (0.1 ml/well) was calculated. Based on disintegrations per minute recovered, the amount of FN fragment bound to each well was then calculated.
Figure 3. B104 process formation in response to the FN heparin binding region. B104 neural cells were cultured for 24 h on substrata treated with: (A) 20 μg/ml of the 33-kD (A chain-derived) fragment, (B) 100 μg/ml of the 66-kD (B chain-derived) fragment, (C) 1 μg/ml of FN-C/H II-OA, (D) or 2 μg/ml of CS1-OA. Except for the 33-kD fragment, these concentrations all induce cell spreading to the maximum cell surface area observed for each given peptide in 1-h assays. Arrows in A point to long, neurite-like processes. Bar, 30 μm.

At the highest concentration of IgG tested (500 μg/ml), inhibition of cell adhesion to a surface coated with FN-C/H II was virtually complete (~80% inhibition). This concentration of anti-FN-C/H II IgG also significantly decreased attachment of B104 cells to surfaces coated with the 33-kD heparin binding FN fragment or intact FN by ~35% (Fig. 6). As expected, the anti-FN-C/H II IgG had no effect on attachment of B104 cells to the 75-kD FN fragment. Normal rabbit IgG (500 μg/ml) caused no significant inhibition of cell attachment (<8% for any of the substrata tested). Similar experiments were conducted with anti-CS1 antibodies. However, these antibodies were of extremely low affinity in
Table II. Amount of Peptide-OA Bound to Immulon-1 Wells

| Coating (OA) | Amount Input Bound (OA) peptide/well pmol | Bound peptide/well pmol |
|--------------|----------------------------------------|------------------------|
| FN-C/H II    | 30                                     | 162                    | 13                     |
|              | 10                                     | 68                     | 9.1                    |
|              | 3                                      | 16                     | 6.3                    |
|              | 1                                      | 9.1                    | 4.5                    |
| CSI          | 30                                     | 362                    | 35                     |
|              | 10                                     | 123                    | 19                     |
|              | 3                                      | 30                     | 11                     |
|              | 1                                      | 16                     | 6.1                    |

FN-C/H II and CSI, with radiolabeled 125-I, were conjugated to OA as described in Materials and Methods. Coupling efficiencies of around 11% for FN-C/H II and 39% for CSI were obtained. FN-C/H II coupled to OA with a stoichiometry of 2.9 mol of FN-C/H II to 1 mol of OA, while CSI coupled with a stoichiometry of 6.3 mol of CSI to 1 mol of OA. OA-conjugated peptides were then serially diluted and adsorbed to wells based on the known concentration of OA (micrograms/milliliter), as is done for adhesion assays. Using the input counts per minute and the coupling efficiency, the amount of input peptide for each well (0.1 ml/well) was calculated. Based on counts per minute recovered, the amount of peptide bound to each well was then calculated.

ELISA assays, and did not inhibit B104 adhesion to CSI (not shown).

As a second approach, the ability of exogenous FN-C/H II to inhibit cell adhesion on various FN-derived substrata was tested (Fig. 7). B104 cells were preincubated with increasing concentrations of soluble FN-C/H II, then the mixture of cells and soluble peptide was added to substrata coated with various ligands. Soluble FN-C/H II inhibited cell adhesion in a concentration-dependent and saturable manner to surfaces coated with FN-C/H II or the 33-kD heparin binding fragment (Fig. 7). Maximal inhibition of cell adhesion of FN-C/H II was almost complete (84%), whereas inhibition of cell adhesion on the 33-kD fragment was partial (39%). Soluble FN-C/H II had no effect on cell attachment to a surface coated with intact FN, consistent with the hypothesis that multiple cell attachment sites mediate adhesion to the FN molecule. The specificity of inhibition was demonstrated by the inability of soluble FN-C/H II to inhibit cell attachment to the 75-kD (RGD-containing) FN fragment (Fig. 7).

Similarly, soluble peptide CSI also specifically inhibited B104 cell adhesion to various FN-derived cell adhesion-promoting ligands (Fig. 8). As has been shown previously for melanoma cells (McCarthy et al., 1990), soluble CSI virtually eliminated cell adhesion to CSI-coated substrata (Fig. 8). Cell adhesion to substrata coated with the 33-kD fragment or FN was also partially inhibited (~50% maximum inhibition). As expected, soluble CSI had no effect on adhesion to the 75-kD (RGD-containing) fragment or an RGD-containing peptide (Fig. 8).

Since either FN-C/H II or CSI only partially inhibited cell adhesion to the 33-kD fragment, both peptides were added together in solution to determine if they would have an additive effect on inhibiting cell adhesion to this fragment. A synergistic effect was not seen since the addition of both peptides at concentrations as high as 500 μg/ml had no greater effect on B104 cell adhesion to the 33-kD fragment than either peptide alone (not shown). These results suggest that CSI and FN-C/H II may be promoting B104 cell adhesion by a related mechanism, and that additional adhesion-promoting determinants exist within the 33-kD fragment.

Figure 5. Effect of soluble GRGDSP on adhesion to FN-derived substrata. B104 cells were incubated with increasing concentrations of soluble GRGDSP peptide before allowing the cells to attach to surfaces treated with 250 μg/ml of FN-C/H II (open squares), 20 μg/ml of the 75-kD fragment (solid squares), or 10 μg/ml of FN (open diamonds). Cell adhesion was quantitated by visual counting and is relative to the 100% control cell adhesion observed for each substratum in the absence of inhibitors. Values represent the mean of triplicate wells ± the SD.
### Table III. Reactivity of Purified IgG Raised against FN-C/H II-KLH

| Anti-FN-C/H II antibodies (µg/ml) | Adsorbed antigen        |     |     |     |     |
|----------------------------------|-------------------------|-----|-----|-----|-----|
|                                  | FN-C/H II               | FN  | LMN | FN-C/H I | OA   |
| 1                                | 0.144                   | 0.000 | 0.000 | 0.028 |
| 4                                | 0.382                   | 0.265 | 0.020 | 0.007 |
| 20                               | 0.644                   | 0.179 | 0.000 | 0.066 |
| 100                              | 0.746                   | 0.600 | 0.000 | 0.093 |

Plates were coated with 1 µg/well of FN-C/H II or FN-C/H I, and 3 µg/well of FN, laminin (LMN), or OA. Purified IgG from rabbits immunized with FN-C/H II coupled to KLH was then added to the wells at the indicated concentrations. The ELISA was performed as described in Materials and Methods, and absorbance readings at 490 nm were taken. The values shown are an average of duplicate wells with background readings using normal rabbit IgG subtracted. Where the background reading exceeded the absorbance using anti-FN-C/H II IgG, zero has been used.

II, the ability of each peptide in solution to inhibit B104 cell adhesion to the other peptide was determined. As expected, preincubation of B104 cells with soluble FN-C/H II-OA significantly inhibited cell adhesion to FN-C/H II-OA (Fig. 9 A). Of interest, the addition of soluble FN-C/H II-OA inhibited cell adhesion to CSI-OA by 58% (Fig. 9 A). Conversely, the addition of soluble CSI-OA significantly inhibited cell adhesion to both CSI-OA and FN-C/H II-OA (Fig. 9 B). OA-OA had no effect on cell adhesion to either FN-C/H II-OA or CSI-OA (not shown), indicating that the cross inhibition observed for the two peptides is not due to nonspecific effects of cross-linked OA. FN-C/H II-OA and CSI-OA did not inhibit adhesion to each other by directly binding to each other, since radiolabeled FN-C/H II-OA did not bind to a substratum treated with CSI-OA, and radiolabeled CSI-OA did not bind to a substratum treated with FN-C/H II-OA (not shown). These results suggest that B104 cell adhesion to CSI and FN-C/H II may somehow be related at the cellular level.

**Heparin Inhibits Cell Adhesion to FN-C/H II, But Not CSI**

Although FN-C/H II and CSI are able to cross-inhibit B104 cell adhesion to each other, these peptides differ in that FN-C/H II binds [3H]heparin, whereas CSI does not. Because of this difference, we compared the effect of exogenous heparin on cell adhesion to substrata treated with CSI or FN-C/H II. Cell adhesion to FN-C/H II-OA is virtually eliminated when wells are pretreated with 100 ng/ml of soluble heparin, while cell adhesion to CSI-OA is unaffected (Fig. 10). These results suggest a distinction between the mechanism of cell adhesion to FN-C/H II and CSI. These results also indicate that the ability of neural cells to attach to FN-C/H II is directly related to the heparin binding activity of FN-C/H II, consistent with a role for a cell-surface glycosaminoglycan or proteoglycan mediating cell adhesion to FN-C/H II.

We next evaluated the specificity of heparin-mediated inhibition of cell adhesion on FN-C/H II-OA coated substrata by comparing the relative ability of other glycosaminoglycans...
Soluble CS1 (μg/ml)

Figure 8. Inhibition of B104 attachment by soluble CS1. B104 neural cells were incubated with increasing concentrations of soluble CS1 before allowing the cells to attach to surfaces treated with 1 μg/ml CS1-OA (open squares), 5 μg/ml of the 33-kD heparin binding fragment (solid diamonds), 20 μg/ml of the 75-kD fragment (solid squares), 1 μg/ml of GRGDSP-OA (open triangles) or 10 μg/ml of FN (open diamonds). Adherent cells were quantitated by means of the hexosaminidase enzyme assay (Materials and Methods). Values are relative to the adhesion observed in the absence of inhibitors (as in Fig. 5) and represent the mean of three wells ± the SD.

to inhibit cell adhesion on this peptide (Fig. 11 A). Dextran sulfate was also included in these studies as a control for nonspecific charge effects of heparin. While both heparin and dextran sulfate were effective at competing for cell adhesion to FN-C/H II-OA, dextran sulfate was at least an order of magnitude less effective in this regard, suggesting that the heparin induced inhibition of B104 neural cell adhesion was not just due to nonspecific charge effects. Heparan sulfate glycosaminoglycans were also effective at inhibiting cell adhesion to FN-C/H II-OA in a concentration-dependent manner; however, the amount required to produce a 50% inhibition was two orders of magnitude greater than the amount of heparin which produced a similar degree of inhibition. Chondroitin 4-sulfate was not effective at competing for cell adhesion to FN-C/H II-OA at any concentration tested. The ability of these sulfated glycoconjugates to inhibit cell spreading on FN-C/H II-OA followed essentially an identical pattern (Fig. 11 B). These results are virtually identical to those observed using these sulfated glycoconjugates to inhibit the binding of [3H]heparin to FN-C/H II (not shown).

Discussion

The carboxy-terminal heparin binding domain of plasma FN A-chains is a potent RGD-independent promoter of CNS and PNS neurite outgrowth (Rogers et al., 1987; Waite et al., 1987). Furthermore, many other cell types have been shown to adhere to this fragment (Izzard et al., 1986; McCarthy et al., 1986, 1990; Liao et al., 1989; Wayner et al., 1989; Visser et al., 1989; Mould et al., 1990), indicating a more general biological role for this domain in promoting cell fragment are necessary for neuroblastoma spreading and neurite outgrowth.
adhesion. The current studies were designed to further elucidate the molecular basis of neuronal cell adhesion on this domain of plasma FN A-chains. These results demonstrate that two structurally distinct peptides derived from this fragment can independently support neuroblastoma cell adhesion and spreading, further supporting the hypothesis (McCarthy et al., 1988, 1990) that cell adhesion to this domain has a complex molecular basis involving both heparin-sensitive and -insensitive mechanisms.

Several lines of evidence indicate that both FN-C/H II and CSI represent independent active sites within the 33-kD A chain-derived fragment. Both peptides are able to effectively compete for cell adhesion to this fragment, although the effect is only partial in either case. Specific anti-FN-C/H II IgG also partially inhibits cell adhesion to this fragment, further supporting the contention that FN-C/H II is exposed and active when in the context of the larger fragment. While the neurite-promoting activity of CSI has previously been described (Humphries et al., 1988), these results indicate FN-C/H II also represents a site effective at promoting neural cell adhesion.

Although both FN-C/H II and CSI promote RGD-independent neuronal cell adhesion, each peptide has distinct structural and functional properties. CSI is relatively hydrophobic, has a net negative charge, and fails to bind heparin. In contrast, FN-C/H II is hydrophilic, has a net positive charge, and specifically binds $[^3$H]heparin in solid phase binding assays (McCarthy et al., 1988). As expected, exogenous heparin was extremely effective at inhibiting neuronal cell adhesion to FN-C/H II, but was ineffective at inhibiting neuroblastoma cell adhesion to peptide CSI. Despite these differences, each of the two peptides could effectively cross-inhibit cell adhesion to the other peptide in our assays, suggesting that recognition of these two sequences within FN A chains is somehow linked at the cellular level. The ability of these peptides to cross-inhibit cell adhesion to each other was not due to nonspecific effects created by the opposite charges of these two peptides, since the peptides did not directly bind each other. Considering the location of FN-C/H II immediately adjacent to CSI, one possibility is that FN-C/H II and CSI bind two sites on the same molecule or two distinct molecules that are closely associated on the cell surface, although we have no evidence yet that indicates the exact relationship between these two sequences.

This result is in sharp contrast to previous results examining the ability of peptide CSI to compete for melanoma cell adhesion to FN-C/H I, which represents an additional cationic heparin binding/cell adhesion promoting sequence within the 33-kD fragment (McCarthy et al., 1990). Those
studies demonstrated that peptide CS1 was not effective at competing for cell adhesion to FN-C/H I coated substrata, and further argue that the ability of peptide CS1 to inhibit cell adhesion to FN-C/H II is not due to nonspecific charge effects. Unlike FN-C/H II, FN-C/H I is not contiguous with CS1, providing further evidence that the ability of CS1 and FN-C/H II to cross inhibit adhesion to each other may relate to their adjacent location.

Several recent studies examining the molecular basis of cell adhesion to FN have suggested that multiple domains on the intact molecule mediate cell adhesion and modulate cell phenotype (Rogers et al., 1985, 1987; Humphries et al., 1987, 1988; McCarthy et al., 1986, 1990; Dufour et al., 1988b; Mugnai et al., 1988c; Obara et al., 1988; Wayner et al., 1989; Lewandowska et al., 1990). Our results support the view that multiple sites mediate cell responses to FN. CS1 and FN-C/H II are two such sites, and additional sites within the FN heparin binding fragment likely exist. While either FN-C/H II or CS1 alone is partially effective at inhibiting cell adhesion to the 33-kD fragment, the simultaneous addition of both synthetic peptides in solution is not additive at inhibiting neuronal cell adhesion to the larger proteolytic fragment. This result suggests that interference of either site is sufficient to block cell adhesion to this domain, and that additional site(s) not blocked by soluble FN-C/H II and CS1 promote neuronal cell adhesion to the 33-kD fragment. Our preliminary results demonstrate that FN-C/H I, which can promote melanoma cell adhesion (McCarthy et al., 1988, 1990) is also effective at promoting neuronal cell adhesion and may represent at least one additional site.

The data presented here are consistent with the hypothesis that both FN-C/H II and CS1 contribute to a larger domain within the 33-kD fragment that has high apparent affinity for cell surfaces. Both FN-C/H II and CS1 are present as a contiguous sequence on the 33-kD fragment. In contrast, the IIIcs region containing the CS1 sequence is not present within FN B chains (Kornblith et al., 1985), with the result that the 66-kD fragment contains FN-C/H II but lacks CS1. The 33-kD fragment is much more active at promoting neuroblastoma adhesion, spreading, and neurite outgrowth than the 66-kD fragment, confirming previous results (McCarthy et al., 1986; Wayner et al., 1989), and suggesting that the presence of peptide CS1 is essential for the neurite-like process formation observed on the 33-kD fragment. However, it is likely a heparin-sensitive interaction, such as binding to FN-C/H II, is also necessary for neuroblastoma cell spreading and neurite growth on the 33-kD fragment since soluble heparin inhibited B104 cell spreading on the 33-kD fragment. Neither FN-C/H II or CS1 alone promoted neurite outgrowth, implying that both sequences must be within this larger domain to promote the formation of neurite-like processes by B104 cells. These results are in contrast to previous studies that demonstrate that CS1 can support neurite outgrowth of PNS neurons (Humphries et al., 1988). This difference could relate to the fact that the neuroblastoma cells used in the current studies are CNS cell lines and could differ from primary PNS neurons in the number and/or affinity of cell surface receptors for peptide CS1. Our results thus imply that CS1 acts in concert with heparin binding sites within FN A chains to contribute to a larger cell adhesion–promoting domain with higher affinity than that expressed on FN B-chains.

It will be extremely important to isolate and identify receptor(s) involved in neuronal cell adhesion to this domain of FN A-chains in order to understand the molecular basis for cell adhesion to the 33-kD fragment. An overwhelming literature exists which implicates cell surface proteoglycans in mediating cell adhesion to FN as well as other ECM pro-
teins (Rapraeger and Bernfield, 1983; Woods et al., 1985; Perris and Johannson, 1987; Saunders and Bernfield, 1988; Mignani et al., 1988b; Culp, 1989; for review see Hock et al., 1984; Gallagher et al., 1986; Margolis and Margolis, 1989). In this study, we show that neural cell adhesion and spreading in response to FN-C/H II is extremely sensitive to exogenous heparin, indicating that cell adhesion to FN-C/H II may involve a heparin-like molecule on the cell surface. Additionally, it has recently been demonstrated (Wayner et al., 1989; Guan and Hynes, 1990; Mould et al., 1990) that α6β1 integrin plays an important role in mediating the adhesion of several cell types to peptide CS1. One possible explanation for the high adhesivity of the 33-kD fragment is that the contiguous sites within this fragment act in a coordinated manner to drive the lateral association of cell surface proteoglycans with other cell surface receptors (e.g., α6β1 integrins). A complete understanding, at both the cellular and molecular level, of the mechanism of action of this fragment will help explain the mechanism by which ECM proteins such as FN modulate cell adhesion and phenotype.

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American Paralysis Association, Leukemia Task Force (J. B. McCarthy, II may involve a heparin-like molecule on the cell surface. Additionally, it has recently been demonstrated (Wayner et al., 1989; Guan and Hynes, 1990; Mould et al., 1990) that α6β1 integrin plays an important role in mediating the adhesion of several cell types to peptide CS1. One possible explanation for the high adhesivity of the 33-kD fragment is that the contiguous sites within this fragment act in a coordinated manner to drive the lateral association of cell surface proteoglycans with other cell surface receptors (e.g., α6β1 integrins). A complete understanding, at both the cellular and molecular level, of the mechanism of action of this fragment will help explain the mechanism by which ECM proteins such as FN modulate cell adhesion and phenotype.

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