Modulation of Matrix Adhesive Responses of Human Neuroblastoma Cells by Neighboring Sequences in the Fibronectins

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Abstract. Attachment and neurite extension have been measured when Platt or La-N1 human neuroblastoma cells respond to tissue culture substrata coated with a panel of complementary fragments from the individual chains of human plasma (pFN) or cellular fibronectins (cFN) purified from thermolysin digests. A 110-kD fragment (fl10), which contains the Arg-Gly-Asp-Ser sequence (RGDS)-dependent cell-binding domain but no heparin-binding domains and whose sequences are shared in common by both the α- and β-subunits of pFN, facilitated attachment of cells that approached the level observed with either intact pFN or the heparan sulfate–binding platelet factor-4 (PF4). This attachment on fl10 was resistant to RGDS-containing peptide in the medium. Neurite outgrowth was also maximal on fl10, and half of these neurites were also resistant to soluble RGDS peptide. Treatment of cells with glycosaminoglycan lyases failed to alter these responses on fl10. Therefore, there is a second "cell-binding" domain in the sequences represented by fl10 that is not RGDS- or heparan sulfate–dependent and that facilitates stable attachment and some neurite outgrowth; this domain appears to be conformation-dependent.

Comparisons were also made between two larger fragments generated from the two subunits of pFN–fl45 from the α-subunit and fl55 from the β-subunit–both of which contain the RGDS-dependent cell-binding domain and the COOH-terminal heparin-binding domain but which differ in the former's containing some IIICS sequence at its COOH terminus and the latter's having an additional type III homology unit. Heparin-binding fragments (with no RGDS activity) of f29 and f38, derived from fl45 or fl55 of pFN, respectively, and having the same differences in sequence, were also compared with f44+47 having the "extra domain$_a$" characteristic of cFN. Attachment on fl45 was slightly sensitive to soluble RGDS peptide; attachment on fl55 was much more sensitive. There were also differences in the percentage of cells with neurites on fl45 vs. fl55 but neurites on either fragment were completely sensitive to RGDS peptide. Mixing of f29, f38, or PF4 with fl10 could not reconstitute the activities demonstrated in fl45 or fl55, demonstrating that covalently linked sequences are critical in modulating these responses. However, mixing of f44+47 from cFN with fl10 from pFN increased the sensitivity to RGDS peptide. Attachment on f29 was poor, on f44+47 intermediate, and on f38 or PF4 very good, demonstrating the modulation of responses by the additional type III homology unit (in f38) and by alternately spliced sequences represented in the "extra domain$_a$" (in f44+47). The COOH-terminal heparin-binding domain appears to modulate RGDS-dependent cell-binding activity as well (by comparing fl10 with either fl45 or fl55): (*a*) better attachment on fl10; (*b*) resistance of attachment on fl10 to RGDS peptide; (*c*) higher percentage of cells with neurites on fl10 and their resistance to RGDS peptide; and (*d*) increased resistance of attachment on fl55 when cells were treated with heparitanase. These data suggest that the responses of human neuroblastoma cells are mediated by two different cell-binding domains, as well as by the heparan sulfate–binding domain. Furthermore, the cell-binding responses can be modulated in four different ways: by neighboring sequences found in the IIICS region of pFN or cFN, by "extradomain$_a$," by the additional type III homology unit in the β-subunit of pFN, and by the COOH-terminal heparan sulfate–binding domain.

THE mechanisms by which specific neuronal subpopulations in animals adhere and react to fibronectin-containing extracellular matrices are being resolved with a variety of experimental approaches in tissue culture model systems. Chick embryonic retinal neurons adhere and extend neurites on fibronectin-coated tissue culture substrata in

chemically defined medium (1). The same applies to chick dorsal root and sympathetic neurons (39), as well as to neurons from rat superior cervical ganglia (41). In the case of chick sympathetic neurons that also respond effectively to laminin-coated substrata, neurite outgrowth is promoted by the heparan sulfate-binding domain of this molecule and not by its cell-binding domain (16). In similar experiments, chick dorsal root ganglia have been shown to extend neurites effectively on substrata coated with the tetravalent heparan sulfate-binding protein, platelet factor-4 (PF4)[1] (Chernoff, E. A., S. L. Tobey, and L. A. Culp, unpublished data; 15). Cell surface heparan sulfate proteoglycan interacts with a 170K glycoprotein to mediate some responses of chick neural retina cells (13), and heparan sulfate proteoglycan has been localized at the basal lamina interface with peripherai neurons of various sorts in vivo (5, 17).

Neuroblastoma tumor cells from rodents or humans share a common origin with peripheral neurons from the neural crest of the developing embryo and also effective adherence and neurite outgrowth on fibronectin-coated substrata (4, 12, 14, 38, 40). Therefore, these tumor cells because of ease of clonal growth and metabolic/biochemical analyses can be used as a partial model for the molecular mechanisms of interaction of neurons with extracellular matrices, with the caveat that these cells have been exposed to some unique selection pressures during tumor progression. The same applies to a peripheral neural tumor, PC12, which also requires nerve growth factor for neurite development (2). However, the mechanisms of neurite outgrowth of neuroblastoma cells on human plasma fibronectin (pFN) have recently been shown to have different molecular bases (15, 46). Human and rat neuroblastoma cells adhere and spread in a pseudopodial-like fashion on PF4-coated substrata, consistent with the high concentrations of heparan sulfate proteoglycans in their substratum adhesion sites (14, 29, 47*a*), but fail to extend neurites on PF4 under the very same conditions that chick dorsal root neurons do (46). In contrast, several cell-binding fragments of pFN lacking heparin-binding activity display neurite-promoting activity with primary peripheral neurons from the chick (40), and neuroblastoma cells extend neurites on a chymotrypsin-generated 120K cell-binding fragment of pFN (46). The neurite-generating activity of this fragment has been shown to depend only on the Arg-Gly-Asp-Ser sequence (RGDS) contained within this fragment (48), a sequence recognized by a 140K glycoprotein complex on the surface of many cell types (3, 9, 11, 19, 36, 37). There is a different glycoprotein complex (115K/150K) on the surface of some cells which acts as a receptor for the vitronectin molecule and which also recognizes an RGDS sequence (44). Furthermore, testing of neurite outgrowth of neuroblastoma cells on substrata coated with intact pFN molecules with soluble RGDS peptides has revealed a second "cell-binding" activity in the intact pFN, which is completely insensitive to inhibition and independent of heparan sulfate binding (48). A third mechanism of neurite outgrowth with these cells is mediated by a cell surface ganglioside (45), which acts cooperatively with an unidentified cell surface protein when these cells adhere to an appropriate ganglioside-binding substratum (31). Therefore, these neural crest-derived tumor cells have evolved multiple and alternative mechanisms by which they adhere and extend differentiated processes on a fibronectin-coated matrix. This system becomes particularly valuable for studying the molecular bases for this "differentiation" process and for resolving the multiple binding activities in the fibronectins responsible.

To pursue the latter objective, fragments of human plasma or cellular fibronectins (cFN) that have overlapping permutations of the known binding domains of these molecules (30, 40, 49) were tested. Chick embryonic peripheral neurons were observed to generate neurites effectively on fibronectin fragments containing either the COOH-terminal heparin-binding domain or a cell-binding domain (40). Since both pFN and cFN are composed of more than one polypeptide generated by alternative splicing mechanisms of a common pre-messenger RNA, as discovered in both protein biochemical analyses (7, 34, 43) and in gene structure/expression studies (21, 25), it would be optimal to examine specific regions of individual fibronectin polypeptides. This possibility became feasible with the scheme developed by Zardi and his colleagues (8, 10, 33) for isolating sequences from the two different pFN α- and β-subunits, which differ in the amounts of the alternately spliced IIICS region of the molecule (21), and sequences from the alternately spliced "extra domain" (ED_a) (21) region characteristic of the cellular fibronectins. In the studies reported here, the adherence and neurite outgrowth properties of two human neuroblastoma cells have been measured on substrata coated with these various fragments for comparison with the intact fibronectins and with heparan sulfate-binding PF4. Sensitivity to inhibition with an RGDS-containing peptide and/or with pretreatment of the cell with specific glycosaminoglycan (GAG) lyases, in order to test the contribution of a recently discovered cryptic GAG-binding domain (28), has been used to discriminate and further characterize the multiple mechanisms of adherence and neurite outgrowth on the fibronectins.

Materials and Methods

Cell Culture

Two human neuroblastoma cell lines, Platt (12) and La-N1 (46), were grown *Mycoplasma*-free as stock cultures in DME, supplemented with 10% neonatal calf serum, 250 U/ml penicillin, and 250 μg/ml streptomycin sulfate at 37°C in a 10% CO_2/humidified air mixture. Both cell lines were from advanced tumors whose cells contained a 50–100-fold amplification of the *N-myc* oncogene (48). In select cases, cells were supplemented with highly purified gangliosides by established procedures (23, 31). Briefly, subconfluent cultures after 48 h of growth were exposed to 50 μM purified ganglioside in DME containing 1% neonatal calf serum for 24 h; cells grew continuously in this medium and biochemical analyses of the ganglioside fractions of cells using thin-layer chromatography methods demonstrated the incorporation of the particular ganglioside into the membrane fraction (data not shown).

Binding Proteins and Their Fragments

pFN was purified from blood bank plasma by gelatin-Sepharose affinity chromatography (18) and stored at −85°C in CAPS buffer (0.15 M NaCl, 1.0 mM $CaCl_2$, 10 mM 3-cyclohexylamino-1-propane sulfonic acid; pH 11.0). Human PF4 was purified from outdated platelet packs obtained from

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1. *Abbreviations used in this paper*: cFN, cellular fibronectin; ED_a, extra domain; GAG, glycosaminoglycan; PF4, platelet factor-4; pFN, plasma fibronectin; REDV, Arg-Glu-Asp-Val; RGDS, Arg-Gly-Asp-Ser.

the Cleveland Red Cross by heparin-Sepharose affinity chromatography (26), was completely free of contamination with pFN, and was stored in PBS supplemented with 0.2 M NaCl at −85°C.

A variety of purified fragments of human plasma or cellular fibronectins (cFN) were generated as described by Zardi and his collaborators (8, 10, 28, 33). Fibronectins were treated with thermolysin and then specific fragments purified by sequential affinity chromatography on columns of gelatin-Sepharose or heparin-Sepharose, or supplementally with a column derivatized with one of a variety of monoclonal antibodies whose epitopes had been mapped. The following fragments are used as described more completely in references 8 and 10 and in Fig. 1: 110K fragment (f110) from pFN contains the RGDS-dependent cell-binding domain (9, 11, 19, 37) and an RGDS-independent cell-binding activity (48) but no COOH-terminal heparin-binding domain; 145K fragment (f145) from the α-subunit of pFN contains all of the sequence of f110 plus a region that includes the COOH-terminal heparin-binding domain and some IIICS sequence at its C-terminus; 155K fragment (f155) from the β-subunit of pFN contains all of the sequence of f110 plus a larger region containing the COOH-terminal heparin-binding domain and an additional type III homology unit; 29K fragment (f29) from the α-subunit of pFN contains the COOH-terminal heparin-binding domain and some IIICS sequence but no RGDS-dependent or independent cell-binding domains; 38K fragment (f38) from the β-subunit of pFN contains the COOH-terminal heparin-binding domain but no cell-binding domains and an additional type III homology unit (this fragment is derived from cleavage of f155); a mixture of 44K and 47K fragments from cFN(f44+47) contains the COOH-terminal heparin-binding domain and one of the "extra domains" (ED_a) attributed to cellular fibronectins, but no cell-binding domains (Fig. 1).

Adhesion Assays

For all experiments, the stock cultures were rinsed twice with PBS and then fed 0.5 mM EGTA in PBS for shaking at 37°C for 30 min (31, 46, 48). The detached cells were pelleted by centrifugation, rinsed twice in PBS by re-centrifugation, and suspended in adhesion medium (serum-free DME plus 250 μg/ml heat-treated BSA). Meanwhile, tissue culture plastic cluster wells (48-well dishes) or glass coverslips in select cases were coated with 20 μg/ml of the proteins being tested in protein-free DME for 1 h at 37°C; the wells were rinsed twice with PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and then re-fed with 200 μl of adhesion medium.

To quantitate the attachment of cells on substrata coated with various proteins, stock cultures were radiolabeled during 48 h of exponential growth in medium containing 0.5 μCi/ml of [methyl-^{3}H]thymidine (sp. act. 10–20 Ci/mmol); these radiolabeling conditions did not inhibit the growth or neurite extension of cells. Unlabeled medium was added for an additional 24 h before subculturing into experimental wells (as described above). Aliquots of cells (2×10^4) were inoculated into wells, which were incubated for 1 h at 37°C to evaluate attachment as follows. The medium was aspirated off and the adherent cells rinsed twice with PBS containing divalent cations; the cells were then solubilized with 50 μl (for 96-well plates) of 1% SDS in 0.1 N NaOH for 1 h. Radioactive solubilized cells were quantitated by scintillation counting with correction for quenching and compared on percent bases with values of the original cell suspension.

For phase-contrast microscopy of cell adherence and neurite outgrowth, stock cultures were not radiolabeled. In most cases, plastic tissue culture cluster wells were used and, where noted, glass coverslips were also used with identical results. The cells were allowed to attach and spread over an 18-h period before glutaraldehyde fixation and photography on a Nikon Diaphot phase-contrast microscope. Photographs of these fields were used to quantitate neurite outgrowth as described previously for cell processes that were longer than one cell body diameter (12, 31, 46); statistical significance was determined with a Student's *t*-test.

Peptides A and B

To evaluate the contribution of the RGDS sequence in the fibronectins as a cell-binding activity (36) known to promote attachment and neurite outgrowth of these cells (46, 48), two peptides were tested as soluble inhibitors of cell responses. Peptide A (Gly-Arg-Gly-Asp-Ser-Pro-Cys) and peptide B as the control (Gly-Arg-Gly-Glu-Ser-Pro) were synthesized to specifications by Peninsula Laboratories (Belmont, CA) and purified by high pressure liquid chromatography. None of the effects of peptide A reported here could be attributed to the COOH-terminal cysteine residue (data not shown). The effectiveness of peptide A was further verified by demonstrating complete inhibition of attachment of BALB/c 3T3 cells at concentrations >100 μg/ml.

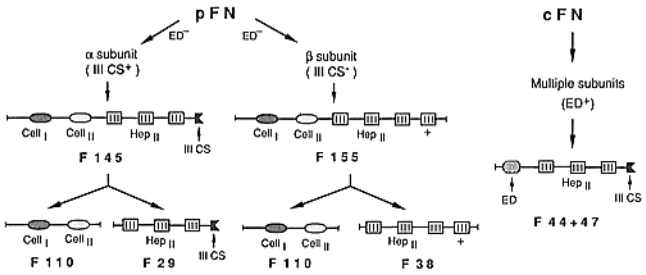

Figure 1. Origin of fragments from the two chains of human plasma fibronectins or the multiple chains of cellular fibronectins. Products of thermolysin digestion of pFN are shown with the larger f145 and f155 chains liberated initially from the α- and β-subunits, respectively. There are two regions of sequence difference in these fragments: some IIICS-derived amino acids at the COOH terminus of f145 (IIICS) but not in f155, which lacks a IIICS region, and an additional type III homology unit (identified with a +) at the COOH terminus of f155. Otherwise, these two fragments both contain the RGDS-dependent cell-binding domain ($Cell_I$) and the COOH-terminal heparin-binding domain (Hep_{II}). (They also may contain a second cell-binding domain [$Cell_{II}$] as explained in the text which is RGDS independent, although its location is still undefined; it is likely that this domain results from the conformational folding of the protein to bring two distant sequences together.) F145 is subsequently cleaved to give rise to f110 and f29, which separates the RGDS-dependent cell-binding domain from the COOH-terminal heparin-binding domain into the respective fragments. F155 is cleaved to give rise to the same f110 fragment, as well as f38 with its additional type III homology unit. Cellular FNs go through a similar cleavage sequence to give rise to two sets of fragments (f44+47), which contain the "extra domain" (ED_a) characteristic of cFNs, the Hep_{II} domain, and some IIICS sequence at the COOH terminus.

GAG Lyase Treatment of Cells

These treatments were as described by Laterra et al. (27). Briefly, EGTA-detached cells ([^{3}H]thymidine-radiolabeled to quantitate attachment) were rinsed twice in PBS plus divalent cations and treated in suspension with either 0.1 U/ml chondroitinase ABC/1 × 10^6 cells or 0.02 U/ml Flavobacterium heparitanase in PBS supplemented with divalent cations and 2 mg/ml BSA for 1 h with constant agitation at 37°C; then a second dose of enzyme was added for an additional 0.5–1 h. Viability of cells after the enzyme treatments was routinely >93% when compared with untreated control cells. Control or enzyme-treated cells were then inoculated into wells containing adhesion medium and in some cases peptide A or B.

Materials

BSA, EGTA, heparin, and gelatin were purchased from Sigma Chemical Co., St. Louis, MO; Sepharose 4B from Pharmacia Fine Chemicals, Piscataway, NJ; 48-well cluster dishes from Costar, Cambridge, MA; neonatal calf serum from Biologos, Naperville, IL; [methyl-^{3}H]thymidine from Amersham Corp., Arlington Heights, IL; chondroitinase ABC from Miles Biochemicals, Elkhart, IN; and glutaraldehyde (50% wt/wt) and technical pan films from Eastman Kodak Co., Rochester, NY. Flavobacterium heparinum heparitanase was kindly provided by Dr. Jeremiah Silbert of Boston's Veterans Administration Outpatient Clinic. The highly purified ganglioside GT1b was generously provided by Fidia Research Laboratories, Abano Terme, Italy. Dr. Michael Pierschbacher of the La Jolla Cancer Foundation kindly provided the 11.5K fragment of human pFN containing the RGDS sequence and generated by chymotrypsin/pepsin treatments (35).

Results

Purity of Fibronectin Fragments

The fragments of human plasma or cellular fibronectins to

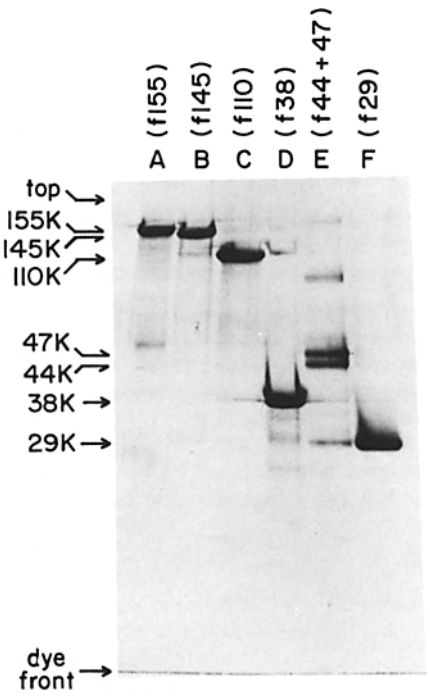

Figure 2. Purity of fibronectin fragments. Human plasma or cellular fibronectins were purified and then proteolytically digested with thermolysin for affinity fractionation as described previously (8, 10) and in Materials and Methods. 10 μg of each fragment class were applied to a gradient polyacrylamide gel containing SDS and a reducing agent for electrophoresis. The gel was then stained with Coomassie Blue for evaluation of patterns. Molecular weights are provided on the left side.

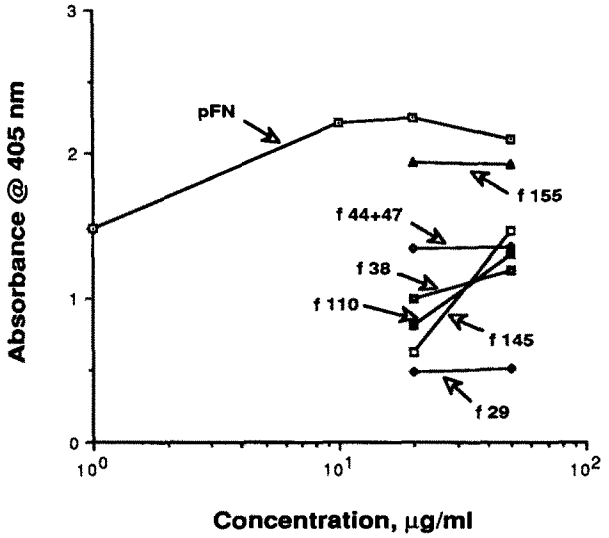

Figure 3. Adsorption of fibronectin fragments to the substratum evaluated by ELISA. 96-well tissue culture dishes were coated with 20 or 50 μg/ml of the indicated fibronectin fragments or with 1, 10, 20, or 50 μg/ml intact pFN for 1 h as described in Materials and Methods. Wells were then rinsed several times with PBS-II and postadsorbed with 250 μg/ml BSA in attachment medium for another hour. ELISA was then conducted using an antibody-excess amount of goat anti-pFN antiserum as described previously (28), followed by rabbit anti-goat alkaline phosphatase conjugate and *p*-nitrophenyl phosphate solution, with extensive rinsing between all steps. Optical density measurements were taken at 405 nm at 1 h and standard errors routinely fell within ±7% of the values shown.

be used in these studies after thermolysin digestion and subsequent multiple affinity chromatographies are shown in Fig. 1. The purity of these fragments was assessed by PAGE in the presence of SDS and a reducing agent (Fig. 2). The two high molecular weight fragments (fl45 and fl55) could be more readily resolved from each other by longer periods of electrophoresis and are derived from the α- and β-subunits of human pFN, respectively, as described previously (8, 10, 33) and differ by the latter's containing an additional type III homology unit and the former containing some IIICS sequence at its COOH terminus (five amino acids of IIICS [33]) (Fig. 1). These fragments do not include the Arg-Glu-Asp-Val (REDV) functional domain recently discovered by Humphries et al. (20). Fl10 contains sequences shared in common by both subunits of human pFN around the RGDS-dependent cell-binding domain and lacks any detectable heparin-binding activity when assayed as a soluble fragment. Also shown is a second cell-binding domain in these larger fragments that is RGDS-independent (48 and evidence below) and whose location has not been mapped precisely (its location in Fig. 1 is arbitrary). In contrast, f38 and f29 contain the COOH-terminal heparin-binding domains of the two human pFN subunits but lack the RGDS-dependent and -independent cell-binding domains; they also differ with the former containing the additional type III homology unit and the latter possibly containing some IIICS sequence at its COOH terminus. These sequences are also shared with f44+47 from human cFN, which also contain the so-called "extra domain$_a$" of the cellular fibronectins (Fig. 1). The purity of these fragments is evident from these gel patterns with only minor contaminants evident in f44+47.

The effectiveness of binding of these fragments to the substratum was then evaluated quantitatively by enzyme-linked immunosorbent assay (ELISA) using methods described previously (28). As shown in Fig. 3, intact pFN saturates the substratum at a concentration well below 10 μg/ml using polyclonal goat antibody to human pFN; the multiplicity of epitopes available for antibody binding is reflected in the higher maximum optical densities for pFN than those observed with any of the fragments (which reflect only subsets of the epitopes available in the whole molecule). Fragments fl55, f44+47, f38, and f29 saturate substratum sites below 20 μg/ml (i.e., 50 μg/ml fails to elicit higher antibody binding and reactivity than 20 μg/ml). However, fl10 elicits somewhat more effective antibody binding at 50 μg/ml, and fl45 at this concentration doubles the amount of antibody-reactive material. These results demonstrate that, even though fl45 and fl55 are very similar in size and sequence, the extra type III homology unit in fl55 leads to much more stable binding of this fragment on the substratum at a lower concentration.

Attachment Activity with Fibronectin Fragments

Adherence was tested on each fragment for comparison with similar data for intact human pFN and for human PF4 (27). As shown in Fig. 4 using a concentration of 20 μg/ml for adsorption of all proteins, both Platt and La-N1 neuroblastoma cells attached poorly to the f29 univalent heparin-binding

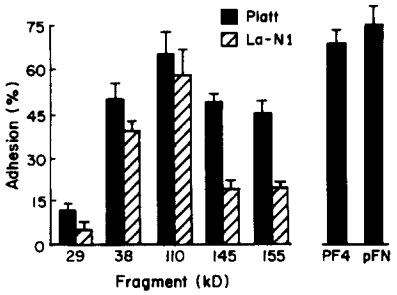

Figure 4. Attachment of Platt and LaN-1 cells on intact human pFN, pFN fragments, or PF4. Platt (*solid bars*) or La-N1 (*hatched bars*) cells were radiolabeled with [^{3}H]thymidine as described in Materials and Methods. They were then detached by EGTA treatment, rinsed twice with PBS, and inoculated into plastic cluster wells coated with 20 μg/ml intact human pFN, various pFN fragments (f29, f38, fl10, fl45, or fl55 as indicated), or PF4. The cells were allowed to adhere for 1 h at 37°C. The wells were then rinsed twice with PBS containing divalent cations, and the attached cells were solubilized for quantitation of adherence as described in Materials and Methods. The values are given as a percentage of the same number of radiolabeled cells in the original cell suspension. Bars represent standard errors of multiple determinations. La-N1 cell attachments on pFN or PF4 (not shown) were virtually identical to the values observed for Platt cells (see reference 48).

fragment of the α-chains of pFN, particularly in contrast to their effective adherence to PF4 whose activity is comparable to intact pFNs (46). However, both cells attached more effectively to f38, which contains the same heparin-binding domain from the β-chain of pFN as well as an additional type III homology unit of the molecule. When f44+47 was tested, its activity was intermediate between those of f29 and f38 (data not shown). Attachment to fl10 was most effective with either cell type and approached the activity of intact pFN while the activities of fl45 or fl55 were somewhat reduced, particularly for the La-N1 cells. These results were evaluated by the Student's *t*-test and the following comparisons were statistically significant with a $P < 0.005$ for each specific cell (f29 vs. f38; f29 or f38 vs. PF4; fl10 vs. fl45 or fl55; and fl45 or fl55 vs. pFN). Furthermore, both Platt and La-N1 cells displayed the same relative patterns of attachment. The dose dependence of attachment of Platt cells on these various fragments is shown in Fig. 5. The concentration of fragments to achieve a maximal response was ~20 μg/ml (compared with 3–5 μg/ml for intact pFN) with the single exception of fl45, consistent with the weaker binding of this particular fragment to the substratum (see Fig. 3). Also, the same data were obtained for either tissue culture plastic or glass substrata. These results indicate that there are differences in the attachment efficiency of cells on these fragments and that the sequences represented in the ED_a (e.g., by comparing f44+47 with either f29 or f38) and the extra type III homology repeat (e.g., by comparing f38 with f29) of the molecule can affect responses mediated by either the COOH-terminal heparin-binding domain or the RGDS-dependent cell-binding domain (by comparing fl10 with fl45 or fl55).

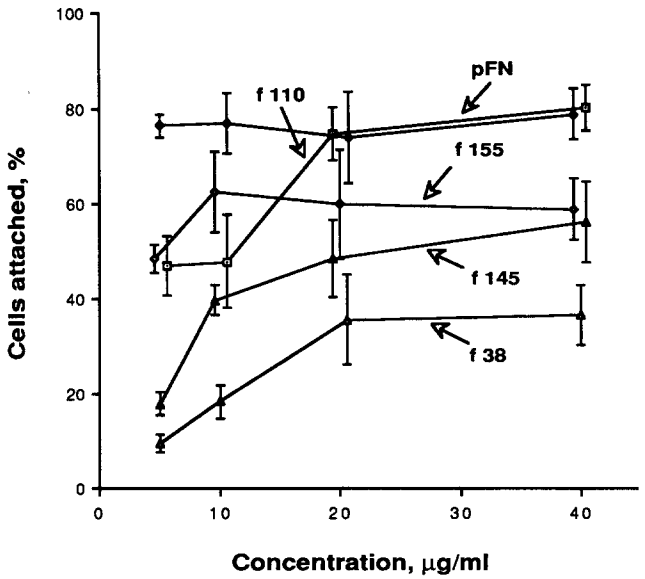

Figure 5. Dose dependence of Platt cell attachment on pFN or its fragments. 96-well tissue culture dishes were coated with pFN, f38, fl10, fl45, fl55 at concentrations varying from 5 to 40 μg/ml for 1 h; they were then rinsed twice with PBS-II and postadsorbed with attachment medium. EGTA-detached and [^{3}H]thymidine radiolabeled Platt cells were inoculated into wells in fresh attachment medium. After 1 h attachment, attached cells were assayed as described in Materials and Methods. Standard deviations are calculated for quadruplicate samples.

Effects upon Attachment by Medium-containing RGDS Peptide

It has been shown previously that some adhesion responses of cells are mediated by the RGDS-containing cell-binding domain of the fibronectin molecule which reacts with a 140K glycoprotein integrin complex (3, 9, 11, 19, 36, 37). However, these human neuroblastoma cells (both Platt and La-N1) recognize at least two and possibly three different "cell-binding" activities on human pFN, only one of which is RGDS-dependent (31, 46, 48). Therefore, it was important to assay these various fragments of pFN for the sensitivity of attachment of cells to RGDS-containing peptide (i.e., peptide A) when added in medium at the same time as cell inoculation. In all cases, the control peptide B (with a Glu substitution for the critical Asp residue) was without effect in these experiments (data not shown).

As shown in Fig. 6, attachment to f38 was completely resistant to peptide A, which is expected since f38 lacks the RGDS-dependent cell-binding domain but does contain the heparin-binding domain. Of significance is the resistance of attachment of Platt cells to peptide A on fl10, which does contain the RGDS cell-binding domain but no heparin-binding activity when assayed in solution; these same preparations of peptide A inhibited attachment of BALB/c 3T3 cells at concentrations between 30 and 100 μg/ml. However, this fragment (fl10) has been shown to display a cryptic GAG-binding domain that becomes expressed when it binds to the substratum (28) and this may provide the basis for resistance to peptide A (see below for further analysis of this issue). In contrast, fl45 demonstrated some sensitivity to peptide A and fl55 was much more sensitive (Fig. 6); these fragments do contain the COOH-terminal heparin domain. The insensitivity on fl10 and the sensitivity on fl55 to peptide A are identical for several preparations of the fragments; the same applies if fl10 and fl55 are purified separately from the original thermolysin digest or if purified fl55 is used as the sub-

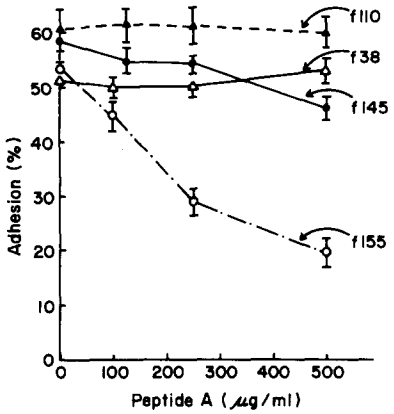

Figure 6. Sensitivity of Platt cell attachment on pFN fragments to peptide A. Attachment of radiolabeled cells was measured as described in Materials and Methods and the legend to Fig. 4 in plastic wells precoated with human pFN fragments. Different amounts of peptide A were added to the attachment medium along with the cell suspension. After a 1-h incubation, attached cells were washed, solubilized, and quantitated by scintillation counting. Percentage of attachment was calculated in reference to the original cell suspension as described in Materials and Methods; standard errors are shown. It should be noted that attachment on pFN was unaffected by all concentrations of peptide A used (not shown [48]).

strate for protease digestion to obtain fl10. Therefore, a contaminant that co-purifies with one of the fragments is not a likely contributor to this differential sensitivity to peptide A. It would appear that inclusion of the heparin-binding domain in either of these fragments increases the dependence of attachment upon the RGDS cell-binding domain. Since both of these fragments contain the same COOH-terminal heparin-binding domain, this differential response to fl45 vs. fl55 must be due to differences in sequence provided by the additional type III homology unit of fl55 or the partial IIICS sequences at the COOH terminus of fl45.

These results led to an experiment in which combinations of fragments on the substratum were tested for comparison with the individual fragments. As shown in the Untreated columns of Table I, substrata coated with an equal-weight combination of fl10 plus f38 (a mixture that represents virtually all the peptide sequence in fl55) continued to be resistant to peptide A while fl55 demonstrated significant sensitivity to peptide A (by Student's *t*-test, $P < 0.005$ for row 7, column 1 vs. column 2); raising or lowering the concentration of f38 did not change this result (data not shown). Also using a weight ratio of 3:1 of fl10/f38 which more approximates the equivalent molarities of the binding domains in the intact molecule, identical results were observed (not shown). Mixing of fl10 plus PF4 was also without effect (row 5, columns 1 and 2 in Table I). Therefore, the covalently linked sequences in fl55 contained in the additional type III homology unit are necessary to demonstrate this effect and mere addition of mixtures of fragments cannot reconstitute the effect. The same was observed when La-N1 cells were used in the same type of experiment.

In a similar experiment, cells were also pretreated with either chondroitinase ABC or *Flavobacterium* heparitanase under the same conditions used by Laterra et al. (27) to demonstrate that cell surface heparan sulfate proteoglycan does mediate certain adhesion responses of fibroblasts. This was done to test the relative contributions to these responses of the well-characterized COOH-terminal heparin-binding domain and a recently discovered cryptic GAG-binding domain, which maps close to the RGDS domain (28). Table I illustrates that f38 and PF4 attachment processes are resistant to chondroitinase ABC but highly sensitive to heparitanase (by Student's *t*-test, $P < 0.0005$ for rows 1 or 2, columns 1 vs. 5), responses that are expected from these heparan sulfate-binding proteins. These results also demonstrate the effectiveness of enzymatic removal of cell surface heparan sulfates as shown previously (27). However, under the same treatment conditions, attachment to fl10 remains resistant to either enzyme treatment (row 3, columns 1, 3, and 5) and to peptide A after the enzyme treatment (row 3, columns 2, 4, and 6). Therefore, the resistance of attachment to fl10 cannot be due to the cryptic GAG-binding domain in this fragment but must be attributed to a second "cell-binding" activity in the molecule located in these sequences (Fig. 1) or to much greater affinity of the RGDS sequence to its cell surface receptor. The same applies to substrata coated with a mixture of fl10 plus f38 (row 4). In contrast,

*Table I. Effects of Enzyme Pretreatment upon Peptide a Sensitivity of Attachment on Fragments**

		Untreated		Chondroitinase ABC		Heparitanase	
		−Pep A	+PepA	−Pep A	+Pep A	−Pep A	+Pep A
Fragment		1	2	3	4	5	6
1	PF4	65.6 ± 3.1	ND	61.8 ± 2.2	ND	14.0 ± 1.0	ND
2	f38	55.7 ± 3.3	ND	56.7 ± 4.0	ND	16.6 ± 1.8	ND
3	fl10	62.8 ± 5.1	63.3 ± 2.9	59.1 ± 2.3	62.3 ± 1.6	55.0 ± 2.2	59.5 ± 1.4
4	fl10+f38	63.1 ± 2.2	63.2 ± 1.8	61.3 ± 2.1	64.6 ± 2.0	56.8 ± 2.2	53.9 ± 2.0
5	fl10+PF4	62.6 ± 3.5	69.8 ± 2.8	61.8 ± 1.3	57.3 ± 2.5	33.0 ± 1.8	28.8 ± 3.7
6	fl45	58.0 ± 3.0	58.1 ± 4.6	51.9 ± 3.8	56.8 ± 2.4	42.4 ± 2.3	45.9 ± 4.2
7	fl55	52.6 ± 4.8	29.8 ± 2.6	37.6 ± 1.3	38.8 ± 2.4	38.9 ± 1.1	40.4 ± 2.6

* EGTA-detached and [^{3}H]thymidine-radiolabeled Platt cells were rinsed twice in PBS with divalent cations and resuspended in the same solution supplemented with 2 mg/ml BSA. The suspended cells were treated with chondroitinase ABC or *Flavobacterium* heparitanase as described in Laterra et al. (27) and in Materials and Methods; control cells were handled identically without the addition of any enzyme. Cells were then inoculated into plastic cluster dishes precoated with the indicated array of fibronectin fragments and/or PF4 at a total protein concentration of 20 μg/ml; one set of wells contained 250 μg/ml of peptide A (+ Pep A) while a second set of wells did not contain peptide A (−Pep A). After 1 h to allow cell attachment, unattached cells were rinsed out of the wells with PBS-containing divalent cations and the adherent cells assayed as described in Materials and Methods. The values presented are the percentages of the total number of cells inoculated into wells divided into the number of cells adherent (× 100). Errors for multiple samples of each fragment are standard errors.

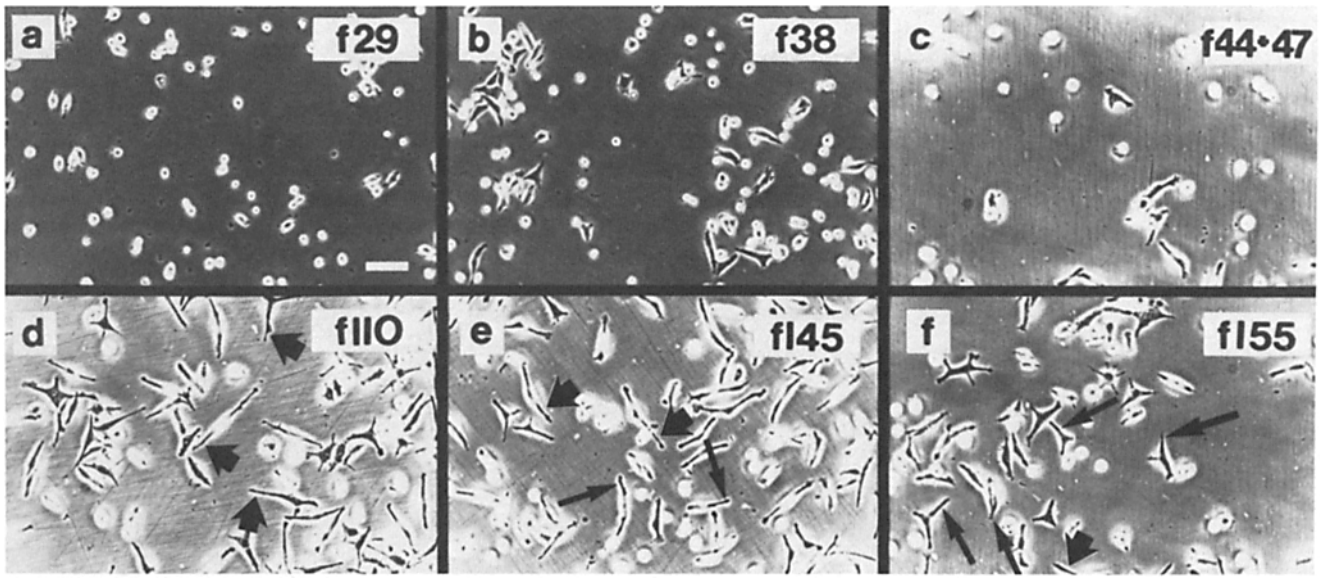

Figure 7. Morphology and neurite outgrowth of Platt cells on various pFN or cFN fragments. Cluster dishes were coated with the following fibronectin fragments described in detail in Materials and Methods: (a) f29; (b) f38; (c) f44+47; (d) fI10; (e) fI45; (f) fI55. EGTA-detached Platt cells were inoculated into these cluster dishes and allowed to spread and generate neurites for 18 h. The wells were then fixed with glutaraldehyde and photographed under phase-contrast microscopy. Long narrow neurites are formed on fI10 and to a lesser extent on fI45 and fI55 (*broad arrowheads* in *d–f*). Many short and broader extensions were more common on fI45 and fI55 (*arrows* in *e* and *f*). Under these same experimental conditions, intact pFN generated cells that were indistinguishable from those on fI55 in f (data not shown). Bar, 20 μm.

the mixture of fI10 plus PF4 becomes sensitive to heparitanase (by Student's *t*-test, $P < 0.0005$ for row 5, columns 1 vs. 5) but not to peptide A, demonstrating the dominance in this situation of the tetravalent heparan sulfate–binding activity of PF4 when acting as a supplement to the RGDS-dependent cell-binding activity. While fI45 displays little sensitivity to prior chondroitinase treatment of cells (row 6, columns 3 and 4), fI55 does display some (row 7, columns 3 and 4); in contrast, both fI45 and fI55 display sensitivity to prior heparitanase treatment (row 5, columns 1 vs. 5, $P < 0.005$; row 7, columns 1 vs. 5, $P < 0.025$). Moreover, the enzyme treatment generates a resistance to peptide A not observed in the untreated control. This result suggests that cell surface heparan sulfate proteoglycan binding to the substratum ligand (specifically fI55) increases the specific activity of the RGDS-dependent cell-binding domain.

Neurite Outgrowth on Fibronectin Fragments

On an appropriate substratum provided by more than one "cell-binding" activity of human pFN but not by its heparan sulfate–binding activity, Platt and La-N1 cells will generate neurites that have been characterized and quantitated previously using the intact pFN molecule (12, 31, 46, 48). Therefore, fragments were tested for their ability to permit this "differentiation" response. As shown in Fig. 7, *a–c*, the heparin-binding domain alone, as represented in the f29 or f38 fragments of pFN or the f44+47 fragments of cFN, were incompetent for neurite generation as expected from previous studies (46); cells only extended pseudopodial-like processes on these substrata without any evidence of a neurite fiber. In contrast, Platt cells on fI10 were able to generate neurites (*broad arrowheads* in Fig. 7 *d*) and only 40% of this activity was sensitive to peptide A but not to peptide B (Table II, row 1, columns 1 vs. 2 with a Student's *t*-test, $P < 0.005$; also, see Fig. 8, *a* and *b*). Varying the concentration of peptide A in this assay demonstrated that neurites on fI10 were resistant as high as 1,250 μg/ml (data not shown). Although fI45 and fI55 could also generate two morphologically different neurites (*broad arrowheads* and *arrows* in Fig. 7, *e* and *f*; rows 2 and 3 in Table II) in a reduced percentage of the cells, all of these processes were completely sensitive to peptide A (Fig. 8, *c* and *d*) with Student's *t*-test values of $P < 0.0005$; this stands in contrast to the complete inhibition of BALB/c 3T3 attachment on these fragments at concentrations of peptide A below 100 μg/ml. Varying the concentration of peptide A in this assay revealed their sensitivity as low as 50 μg/ml (data not shown). These results indicate that addition of the COOH-terminal heparin-binding domain to the molecule (*a*) reduces the percentage of cells competent for making neurites and (*b*) makes those cells that do generate them completely sensitive to the RGDS peptide. Identical results were obtained with La-N1 cells.

Dose responses of individual fragments for neurite formation in Platt cells are shown in Table III. Even at 2 μg/ml, intact pFN remains competent for inducing neurites while fI55 or fI10 do not ($P < 0.0005$). However, significant attachment activity still occurs on fI55 or fI10 at this concentration. Of note is the difference ($P < 0.005$) at 10 μg/ml between fI55 and fI10 in specific activities for neurite formation. This might reflect the differing contributions of the RGDS-dependent versus RGDS-independent cell-binding domains in the two fragments or, alternatively, different conformations of the two fragments on the substratum leading to very different affinities for the RGDS-dependent receptor.

Combinations of fragments were then tested for neurite

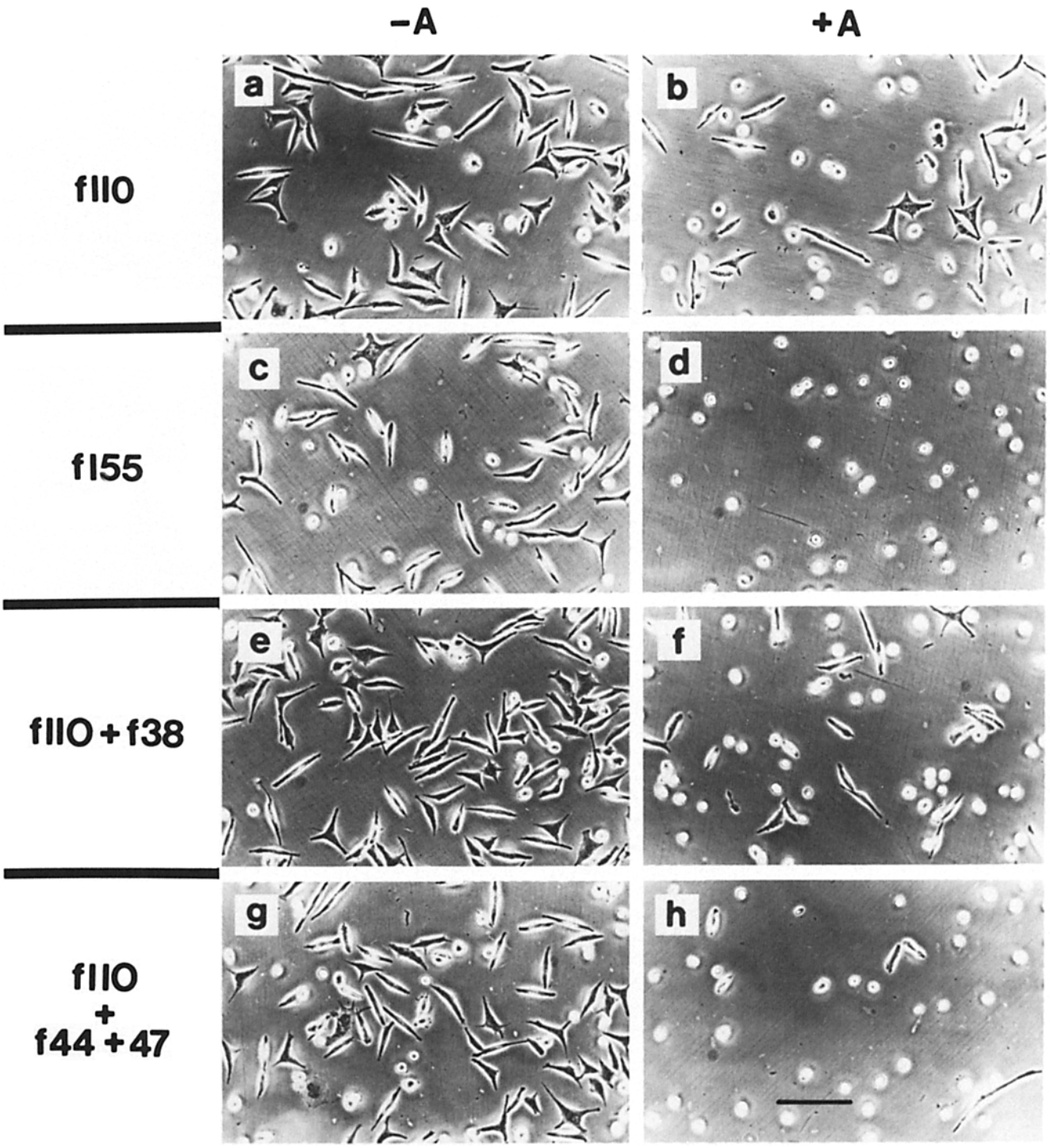

Figure 8. Sensitivity of neurite outgrowth on various fibronectin fragments to peptide A. EGTA-detached Platt cells were inoculated into plastic wells coated with the following fragments: (*a* and *b*) fI10; (*c* and *d*) fI55; (*e* and *f*) a mixture of 20 μg/ml each of fI10 plus f38; (*g* and *h*) a mixture of 20 μg/ml each of fI10 plus f44+47. Peptide A (250 μg/ml) was also added at the same time as cells in *b*, *d*, *f*, and *h*. The cells were allowed to attach and spread for 18 h at 37°C. At this peptide A concentration, neurites on intact pFN were completely resistant (data not shown). The attached cells were then fixed and photographed. Bar, 20 μm.

generation. When equal amounts of f29 or f38 were mixed with fI10 before adsorbing the mixture to the substratum, some diminution of neurite outgrowth was observed, as well as some increase in the sensitivity to peptide A (Fig. 8, *e* and *f*; Table II, rows 4 and 5). In contrast, addition of the ED_a sequences of cFN via f44+47 did result in complete sensitivity to peptide A (Fig. 8, *g* and *h* and row 6 of Table II). These results demonstrate that (*a*) the intact fI45 and fI55 fragments become modulated by these additional heparin-binding sequences far more effectively than mixtures of separate poly-

*Table II. Sensitivity of Neurite Outgrowth on Fragments to Peptide A**

Fragment		Soluble Peptide: None	A	B
		1	2	3
1	f110	20.0 ± 1.3 (8)	11.8 ± 0.5 (4)	16.6 ± 0.7 (4)
2	f145	9.9 ± 1.3 (8)	0	11.0 ± 0.6 (4)
3	f155	13.2 ± 1.3 (8)	0	10.8 ± 1.4 (4)
4	f110+f29	16.6 ± 1.7 (8)	4.5 ± 0.7 (4)	14.8 ± 0.8 (4)
5	f110+f38	14.8 ± 0.4 (8)	6.2 ± 2.1 (4)	13.4 ± 1.1 (4)
6	f110+f44+47	15.3 ± 1.6 (4)	0	13.1 ± 0.6 (4)

* EGTA-detached Platt cells were inoculated into plastic cluster dishes precoated with indicated fibronectin fragment (total protein concentration of 20 μg/ml). 250 μg/ml of peptide A or B were added to the medium and the cells were allowed to attach for 18 h. They were then fixed with glutaraldehyde and scored for neurites. The values presented are the percentages of neurite-bearing cells divided by the total number of cells in the microscope field. When intact pFN is tested for comparison (data not shown), results virtually identical with f110 were observed. Standard errors and the numbers of observations (in parentheses) are included.

peptides and (*b*) the ED_a sequences from cFN can modulate RGDS dependence more effectively than the pFN-derived sequences in f29 or f38. The same results were obtained for La-N1 cells.

These experiments led to one in which f110 was premixed with a varying concentration of f38 to determine the dose response (i.e., for f38) upon neurite generation mediated by f110 and peptide A sensitivity of this process. ELISA data using antibodies directed toward each of these fragments have shown that either one of the fragments does not competitively displace the other fragment in binding to the substratum (Lewandowska, K., and L. A. Culp, manuscript submitted for publication), thereby verifying a true mixture of the two fragments on the substratum available to cell surface receptors. As shown in Table IV, neurite formation decreased significantly ($P < 0.025$) at a 1:1 weight ratio of f110 and f38, and this was accompanied by increased peptide A sensitivity (ratio of +peptide/−peptide changing from 0.49 to 0.25). At a weight ratio of 1:3 (f110/f38), neurite formation was one-third the maximal level and there was complete sensitivity to peptide A. Therefore, only higher concentrations of f38 reduce the ability of f110 to induce neurites and associated with this reduction in the fraction of cells making neurites is increased sensitivity to peptide A inhibition. This result contrasts with the complete inhibition of neurite formation of f155 alone on the substratum by peptide A at all concentrations of f155.

*Table III. Dose Dependence of Neurite Outgrowth on Fragments**

Fragment	Concentration	Neurite-bearing cells
	μg/ml	% of total cell count ± SE
pFN	50	25.1 ± 3.5 (204)
	10	17.2 ± 2.1 (239)
	2	21.0 ± 1.1 (206)
f155	50	26.9 ± 3.9 (203)
	10	18.3 ± 2.0 (202)
	2	3.0 ± 0.9 (213)
f110	50	18.0 ± 3.3 (249)
	10	2.7 ± 0.3 (256)
	2	0 (126)

* 48 well tissue culture dishes were coated with intact pFN, f155, or f110 at 2, 10, and 50 μg/ml for 1 h. After postadsorption of all wells with 250 μg/ml BSA, Platt cells detached with EGTA and rinsed twice with attachment medium were inoculated at 25,000 cells per well. The cells were allowed to spread and make neurites for 18 h; they were then fixed with glutaraldehyde and scored for neurites. The numbers represent percent of neurite-bearing cells divided by total number of cells counted (given in parentheses). Six independent fields were examined in each case and standard errors are included.

*Table IV. Peptide A Sensitivity of Neurite Outgrowth on Fragment 110 Supplemented with Fragment 38**

Fragment Conc.		Neurite-bearing cells		
F110	F38	−Pep A	+Pep A	Ratio +/−
μg/ml		% ± SE		
40	0	17.6 ± 1.1	8.6 ± 3.6	0.49
30	10	16.6 ± 1.1	10.4 ± 1.0	0.63
20	20	10.7 ± 1.4	2.7 ± 0.5	0.25
10	30	5.3 ± 0.6	0.6 ± 0.4	0.11
0	40	0.9 ± 0.4	0	0

* 48 well tissue culture dishes were coated with f110 mixed at varying ratios with f38 with total protein concentration kept constant at 40 μg/ml. The wells were then rinsed and postadsorbed with attachment medium. EGTA-detached Platt cells, rinsed twice with attachment medium, were inoculated into wells with half of the wells supplemented with 250 μg/ml peptide A. After 18-h incubation cells were fixed with glutaraldehyde and scored for neurites as described in Materials and Methods. Percent of neurite-bearing cells per total number of cells is given along with standard errors based on six determinations in each case. A ratio of values obtained in the presence of peptide A divided by values without peptide A in the medium is also included. For comparison, f155 adsorbed at 20 or 40 μg/ml generated 19.0% neurite-bearing cells without peptide A and <1% with peptide A present in the medium.

Peptide A was shown previously to "reverse" neurite formation (48), i.e., addition of peptide A to Platt or La-N1 cells that had already formed neurites on a chymotrypsin-generated 120K cell-binding fragment of pFN resulted in their complete dissolution within hours. A similar experiment was used here to test for the dynamics of neurite outgrowth based on the premise that continuously migrating neurites, mediated only by the RGDS-dependent cell-binding domain, would be sensitive to inhibition with peptide A whereas static, nonmoving neurites (or ones formed by an RGDS-independent mechanism) would be resistant to peptide A. A 24-h exposure to peptide A after 29% of Platt cells had formed neurites on f110 resulted in dissolution of two-thirds of these processes (Table V) with a $P < 0.0005$. This occurred at a concentration of peptide A of 250 μg/ml, which was shown before to completely reverse formation on the RGDS-dependent 120K chymotrypsin cell-binding fragment (48); doubling the peptide A concentration resulted in

*Table V. Stability of Preformed Neurites on Fragments to Peptide A**

Fragment	Soluble peptide	
	None	A
fl10	28.6 ± 2.9	10.5 ± 0.7
fl45	8.4 ± 0.9	0
fl55	13.0 ± 2.2	0
fl10+f29	11.0 ± 0.6	6.8 ± 0.2
fl10+f38	13.5 ± 2.2	6.0 ± 0.4

* EGTA-detached Platt cells were inoculated into cluster dishes precoated with the indicated fibronectin fragment (20 μg/ml) and allowed to attach for 18 h. Peptide A at 250 μg/ml was then added to the medium and the incubation continued for an additional 24 h. The cells were then fixed and scored for neurites. When intact pFN was tested in the same protocol (data not shown), the same percentage of neurites were observed as on fl10 and >70% of these neurites were resistant to 250 μg/ml peptide A. Values presented are the percentage (mean ± SE of four observations) of neurite-bearing cells divided by total cell number in the field.

only a slightly smaller neurite-containing fraction of cells (data not shown). In contrast, neurite formation on fl45 and fl55 was completely sensitive to reversal and mixtures of fl10 and f29 or f38 continued to show the partial sensitivity of fl10 alone (Table V). Therefore, addition of a heparin-binding domain covalently linked, as represented in fl45 or fl55, generated much greater sensitivity to peptide A, presumably by increasing the "specific activity" of the RGDS-dependent cell-binding activity; simple addition of separate fragments f38 or f29 to fl10 could not reconstitute this activity.

Discussion

The human neuroblastoma cells used here provide an ideal biological system for evaluating the functional activities of the fibronectin fragments. They attach and spread by pseudopodial extension on heparan sulfate–binding substrata, including PF4 as observed previously (46) and fibronectin fragments f29, f38, and f44+47 as observed in these studies. In contrast, they extend neurites that can be readily quantitated in the population when reacting with one or more cell-binding regions of the fibronectin molecule (46, 48). It appears from the studies reported here that the second cell-binding activity of pFN (RGDS-independent) reported by Waite et al. (48) as contributing to neurite outgrowth can be found in the sequences of thermolysin-generated fragment fl10 that are not found in chymotrypsin-generated fragment fl20, the $Cell_{II}$ domain depicted in Fig. 1, although its precise location is still arbitrary and may be due to distant sequences brought together by conformational folding. Adherence on fl10 is completely resistant to peptide A and this resistance cannot be reduced by chondroitinase or heparitinase pretreatments of the cells, indicating that a cryptic GAG-binding domain close to the RGDS cell-binding domain (28) is not responsible. Similarly, neurite outgrowth on fl10 is only partially sensitive to peptide A. Waite et al. (48) observed that both attachment and neurite outgrowth on a chymotrypsin-generated cell-binding fragment of pFN (120K) were completely sensitive to peptide A. This second activity is not due to ganglioside binding since supplementation of cells with GT1b, known to bind to the fibronectins (24, 45, 50, 51) and to effect human neuroblastoma adhesion on the appropriate substratum as reported previously (31, 48), failed to alter responses of these cells (data not shown).

Moreover, these studies suggest that neighboring sequences in some of these fragments alter fragment conformation on the substratum, thereby generating very different affinities for the 140K integrin receptor on the cell surface as well as a second "receptor." This would be reflected in differing sensitivities to the soluble RGDS peptide in the former case, and there is clearly some evidence for this (e.g., the different sensitivities of neurite formation on fl45 or fl55 to peptide A). In fact, all of the data reported here suggest that the RGDS-independent cell-binding activity is very sensitive to the conformation of fragments on the substratum, suggesting that this activity is not simple primary sequence in the polypeptide chain but results from two distant regions of sequence folding into proximity. Therefore, further cleavage of fl10 into smaller fragments and assay of RGDS-independent neurite promotion may or may not permit refined mapping of this activity.

A second nonganglioside or nonproteoglycan cell-binding activity has also been implicated in fibroblast adhesion studies with fibronectin by indirect evidence (6, 32, 47). Also, Humphries et al. (20) have directly mapped a second RGDS-independent cell-binding domain to the alternately spliced IIICS region of the fibronectins. This activity is cell-type specific for melanoma, is not effective for baby hamster kidney cells, and is dependent upon an REDV sequence in the IIICS region. A second cell-binding activity reported here for human neuroblastoma cells maps differently from the REDV domain; it is contained within fl10 which has no IIICS sequence (none of the fragments used in these studies contains an entire IIICS region with the REDV sequence [33]). Therefore, there may be three cell-binding domains in the fibronectin molecule, only one of which is RGDS-specific and two of which may be cell-type specific. Further measurement of the dependence of adhesive responses of multiple cell types to these three binding domains should provide insight into the functional roles of three different "receptor" molecules in cells of developing embryos and in malignant cells during tumor progression.

The complexity of neuroblastoma interactions with the fibronectins reported here shares some findings with the results of McCarthy et al. (30) studying adherence and migration of melanoma cells on proteolytic fragments of human pFN. While a trypsin-generated 75K fragment mediated both attachment and motility of melanoma cells, a much smaller pepsin-generated 11.5K fragment facilitated RGDS-dependent attachment but not motility. This suggested a second cell-binding domain in sequences not contained within the 11.5K fragment but contained within the 75K fragment responsible for migration. The results of neurite outgrowth on fl10 reported here are remarkably consistent with the melanoma studies of McCarthy et al. (30). Furthermore, testing of the 11.5K pepsin fragment in the neuroblastoma system also failed to detect significant neurite outgrowth, a motility function on the substratum for a specialized region of the cell surface, while attachment on this fragment was completely RGDS sensitive (data not shown). Therefore, two tumor cell types of neuroectodermal origin, melanoma and neuroblastoma, may share multiple mechanisms of attachment and migration, with migration in the latter case being reflected in neurite outgrowth.

Table VI. Summary of Adhesion Response Comparisons on Various Fibronectin Fragments

Sequence differences	Adhesive response changes
(A) Additional type III homology unit in f155 and f38; partial IIICS sequences in f145 and f29	(A1) Much better attachment on f38 than f29 (A2) Attachment on f155 much more sensitive to peptide A (A3) Only covalently attached sequences effective (A4) Differences in neurite outgrowth on f145 or f155 (A5) Differential sensitivity to peptide A or enzymes on f145 or f155
(B) ED_a sequences in f44+47	(B1) Intermediate attachment responses between f29 and f38 (B2) Mixing of f44+47 with f110 increased peptide A sensitivity of neurite formation (f29 or f38 did not)
(C) COOH-terminal heparin-binding domain	(C1) Better attachment on f110 than f145 or f155 (C2) Greater resistance to peptide A of attachment on f110 (C3) More neurites formed on f110 and their partial resistance to peptide A (complete sensitivity of neurites to peptide A on f145 or f155) (C4) Heparitanase treatment leads to resistance of attachment on f155 to peptide A

These experiments have also revealed the significance of protein sequences neighboring known binding domains (particularly the extra type III homology unit) in the adhesive responses of human neuroblastoma cells. They also suggest, but certainly do not prove, that sequences generated by alternative splicing of pre-messenger RNAs may modulate neighboring binding domains (21, 25, 34, 41). The IIICS region can appear in multiple forms in cFN but in only two permutations in pFN (either with or without IIICS in the α- or β-chains, respectively). Zardi and his collaborators (8, 10) took advantage of the simplicity of the latter system by isolating two complementary sets of fragments that differ in an additional type III homology unit from the α- and β-chains of human pFN, i.e., f29 vs. f38 and fl45 vs. fl55 as displayed in Fig. 1. Fl45 and f29 contain five amino acids from the COOH terminus of the IIICS region as well (33). In all experiments reported here, there are statistically significant differences in responses to the complementary fragments as summarized in Table VI. First, cells adhered much more effectively to f38 than f29. Second, attachment to fl55 is much more sensitive to peptide A than attachment to fl45; since both fragments contain the COOH-terminal heparin-binding domain, this difference must be due to partial IIICS sequences of fl45 or the additional type III homology unit of fl55. Therefore, these sequences can modulate the biological "specific activity" of the RGDS-dependent cell-binding activity. Third, only covalently linked sequences can modulate this attachment activity, which cannot be reconstituted by mixing of fl10 with f38 or f29. And, fourth, there are significant differences in neurite extension and sensitivity to this differentiation process by peptide A addition or by pretreatment of cells with GAG lyase (particularly heparitanase). Since the specific activities of these fragments are comparable and differences persist with fragments in the medium, it is unlikely that these differences result from differential binding stability on the substratum, and this is supported by the ELISA data of Fig. 3.

One mechanism for the effects of the differing type III homology and IIICS sequences would be to affect the conformation of fragments bound to artificial glass or plastic substrata. This conclusion is supported by the requirement of covalently linked domains in modulation. That a conformational change can affect adhesion responses has been shown recently with human skin fibroblasts adhering and spreading more effectively on intact fibronectin that lacks asparagine-linked oligosaccharides when compared with fibronectin that is glycosylated (22). Substratum-induced conformation changes would be expected to influence the affinity between the binding domain on the substratum and the cell surface receptor (32), as well as to affect accessibility of more than one cell surface ligand which would have a "*cis*" orientation on the surface of the cell (i.e., the two "receptors" are either bound to each other or, alternatively, are sufficiently close to effect cooperative interactions [31]). Evidence for such cooperation is provided by two types of data in these studies. First, neurite formation on fl45 or fl55 can be reversed with peptide A while formation on fl10 can only be partially reversed, revealing differential dependence on a second cell-binding domain. Second, pretreatment of cells with heparitanase results in attachment responses that are resistant to peptide A on fl55, a result that contrasts with the marked sensitivity to peptide A of control cultures. There appears to be some form of cooperative interaction between the heparan sulfate proteoglycan on the surface of these cells (29, 47*a*) and the RGDS-dependent receptor for fibronectin. Whether such a modulation could be effected on a natural collagenous substratum by these differing sequences remains to be determined.

In a couple of instances, effects upon adhesive responses were noted by the fragments carrying the so-called ED_a region of the cellular fibronectins as summarized in Table VI (21, 25, 43). Attachment on f44+47 from cFN was intermediate between the very poor response to f29 and the good response to f38, both from pFN. Since all three fragment classes contain the same COOH-terminal heparin-binding domain, this difference of f44+47 with f29 or f38 must be due to the ED_a region of the fragments. Furthermore, mixing of f38 with fl10 only affected the peptide A sensitivity of neurite outgrowth at high ratios of f38/fl10 and only as a consequence of diminished proportion of cells able to form neurites. This contrasted with the complete sensitivity of fl45 and fl55 to peptide A and with the mixing of f44+47 with

fl10 resulting in neurites sensitive to peptide A. These results would infer that ED_a sequences can also affect the RGDS-dependent cell-binding activity of the molecule, at least as far as fragment-mediated responses are concerned. Other experimental approaches, for example, using molecular biologically cloned individual and intact fibronectin polypeptides, will be required to determine whether ED_a modulates binding reactions of the intact chains (42).

The third category of modulation of cell-binding activities in these fragments derives from the contribution of the COOH-terminal heparin-binding domain (Table VI). In virtually all cases the responses to fl10 were quite different from those on fl45 or fl55, the latter two sharing the same heparin-binding domain sequences. These include better attachment efficiency on fl10, greater resistance of attachment to peptide A on fl10, and many more neurite processes on fl10 and their greater resistance to peptide A (either by co-addition of peptide A with cells or by testing reversal of neurite formation). Heparitanase pretreatment of cells makes cells resistant to peptide A on fl55, implicating the binding of cell surface heparan sulfate proteoglycan to the substratum ligand as a regulator of the "specific activity" of the RGDS-dependent cell-binding activity. This conclusion is also supported by the complete sensitivity of neurite formation to peptide A on both fl45 and fl55 but not on fl10. The biochemistry of heparan sulfate proteoglycans has been investigated in a preliminary way for EGTA-detached Platt cells (29) and more recently for the adhesion sites left bound to the substratum by EGTA-detached Platt cells (47*a*). This latter study demonstrated that 90% of the glycosaminoglycan in adhesion sites is retained in high molecular weight heparan sulfate proteoglycans and that non-proteoglycan proteins in Platt adhesion sites can alter the binding of these proteoglycans selectively to fibronection but not to PF4 affinity matrices.

All of these results taken together reveal a complex and finely tuned set of regulators that influence the responses of cells on the multifaceted fibronectins. Even though the fibronectins retain their principal extracellular matrix binding domains, as well as the RGDS-dependent and -independent cell-binding domains, as a consequence of these multiple splicing events (21, 41), neighboring protein sequences and possibly alternately spliced sequences can affect the adhesive responses of these neural tumor cells in significant ways.

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Note Added in Proof: Rogers et al. (Rogers, S. L., P. C. Letourneau, B. A. Peterson, L. T. Furcht, and J. B. McCarthy. 1987. *J. Cell Biol.* 105:1435–1442) have recently reported that chick peripheral nervous system neurons can attach and extend neurites on either RGDS-containing or heparin-binding fragments of the FNs while central nervous system neurons preferentially interact with heparin-binding fragments. These data provide additional evidence for multiple and alternative mechanisms for neuron subpopulations to react with differing domains in the FNs.

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