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Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple adhesion sites on fibronectin molecules

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Cellular adhesion to fibronectin (FN) can be mediated by several sequences located in different portions of the molecule. In human FN, these are: (i) the bipartite RGDS domain containing the RGDS cell-binding sequence functioning in synergy for full cellular adhesion with a second site (termed here the synergistic adhesion site) and (ii) the recently characterized CS1 and REDV adhesion sites within the alternatively-spliced type III homology-connecting segment. Using specific adhesive ligands and inhibitory probes, we have examined the role of each of these domains in the adhesion, spreading, and motility of avian neural crest cells *in vitro*. Both the RGDS domain and the CS1 adhesion site were found to promote attachment of neural crest cells, but only the RGDS domain supported their spreading. However, the RGDS sequence could mediate both attachment and spreading efficiently only when it was associated with the synergistic adhesion site. In migratory assays, it was found that both the RGDS domain and the CS1 site are required in association, each with functional specificity, to permit effective locomotion of neural crest cells. The REDV adhesion site was apparently not recognized by avian neural crest cells, presumably because this sequence is absent from chicken FN. Finally, it was found that recognition of both the RGDS domain and CS1 binding site by neural crest cells involved receptors belonging to the integrin family. From these results, we conclude that neural crest cells can interact with several binding sites of FN molecules, and use them for distinct functions. Our results also suggest the possibility of an instructive role for FN in the control of adhesive and migratory events during embryonic development.

Key words: cell-binding sites/cell motility/fibronectin/neural crest

Introduction

During migration to their final differentiation sites, neural crest cells utilize defined, transient pathways that are composed of extracellular matrix molecules, including collagen type I, laminin, hyaluronate, cytotactin (also called tenascin), vitronectin and fibronectin (for reviews, see Thiery and Duband, 1986; Thiery *et al.*, 1988). Among these components, the glycoprotein fibronectin (FN) plays a crucial role in neural crest-cell motility. *In vivo*, migration of these cells is correlated with the presence of FN (for reviews, see Thiery *et al.*, 1985; Thiery and Duband, 1986). In addition, cessation of movement of neural crest cells in some cases coincides with the local disappearance of FN (Thiery *et al.*, 1982). *In vitro*, FN constitutes the most suitable substrate for displacement of neural crest cells (Newgreen *et al.*, 1982; Rovasio *et al.*, 1983; Tucker and Erickson, 1984). Finally, direct interaction between FN and its 140-kd receptor complex is required for neural crest-cell movement both *in vitro* and *in vivo* (Rovasio *et al.*, 1983; Boucaut *et al.*, 1984; Bronner-Fraser, 1986; Duband *et al.*, 1986).

FN interactions with its receptor require a short sequence located in the central region of the molecule identified as the tetrapeptide Arg–Gly–Asp–Ser (RGDS; for a review, see Ruoslahti and Pierschbacher, 1987). Synthetic peptides containing this sequence are able to mediate attachment of a variety of cells when immobilized on a suitable substrate (Pierschbacher and Ruoslahti, 1984a,b), and can also inhibit adhesion of fibroblastic cells to FN (Yamada and Kennedy, 1984, 1985). In addition, RGDS-containing decapeptides used as competitors can perturb the migration of neural crest cells both *in vivo* and *in vitro*, indicating that, beside its role in cell attachment and spreading, this sequence is also involved in cell motility (Boucaut *et al.*, 1984). However, there are indications that even though the RGDS sequence is necessary, it does not account solely for the FN-mediated adhesion. The affinity of synthetic peptides or of an 11.5-kd cell-binding fragment containing for fibroblasts is ~100-fold lower than larger fragments or intact molecules (Akiyama *et al.*, 1985). In addition, the same 11.5-kd fragment cannot support haptotactic motility of B16-F10 melanoma cells in contrast to a larger fragment of 75-kd (McCarthy *et al.*, 1986).

Recently, several additional cell-adhesion sites have been mapped along FN molecules. Firstly, a binding site located upstream of the RGDS binding-site has been identified by deletion and site-directed mutagenesis (Obara *et al.*, 1987, 1988). This site functions independently from the RGDS site, but synergizes with it to provide full cellular adhesion of fibroblasts (Obara *et al.*, 1988). In the present paper, we shall refer to this site as the synergistic adhesion site. Secondly, two other adhesion sites have been mapped in a distant region of the FN molecule, the type III homology-connecting segment (IIICS). One of these sites is located in the C-terminal portion of the IIICS region (peptide CS5) and was identified in human FN as the Arg–Glu–Asp–Val tetrapeptide (REDV; Humphries *et al.*, 1986). However, the adhesive activity of this site is much lower than that of intact FN. The other site termed CS1 is located the N terminus of the IIICS region and may function in an additive manner with the REDV binding site (Humphries *et al.*, 1987, 1988). Interestingly, the various cell-adhesion sites on FN possess

Table I. List of the different cell-adhesion sites on FN and their corresponding inhibitory or adhesive substrate probes used in the present study

Adhesion sites	Inhibitory probes	Adhesion probes
RGDS site	GRGDS (1) RGDS (1) GRGD (1) SDGR (1) GRDGS[a] (1) KGES[a] (1) 333 mAb (3)	λCBD20 (2)
RGDS site + Synergistic adhesion site	same probes as for the RGDS site	λCBD1 (2)
CS1 site	CS1 (1) GRGES (1) GRGDS (1) CS1-C[a] (1)	CS1–IgG (4) CS2–IgG[a] (4) FN421 (2)
CS5 site	REDG (1) GRGDS (1) CS5 (1)	CS5–IgG (4) FN421 (2)

As inhibitory probes, we used soluble peptides and monoclonal antibodies indicated respectively by the numbers (1) and (3). β-Galactosidae fusion proteins (2) and IgG-conjugated peptides (4) composed the adhesive substrate probes.
[a]Control probes, the specificities of these probes have been established previously (Humphries *et al.*, 1986, 1987, 1988; CS1-C is the CS1 sequence lacking the six N-terminal amino acid residues and the five C-terminal residues).

cellular specificity. While fibroblastic BHK cells only interact with the RGDS and synergistic adhesion sites, B16-F10 melanoma cells recognize these sites poorly, and primarily use the two IIICS sites for their spreading (Humphries *et al.*, 1986, 1987). Moreover, the sequence encoding the IIICS domain can be partly or entirely spliced from FN precursor mRNAs (Tamkun *et al.*, 1984; Kornblihtt *et al.*, 1985), resulting in many FN variants that contain both, one, or none of the cell-adhesion sites of the IIICS region. Finally, a proteolytic 33-kd fragment of FN that includes the C-terminal heparin-binding site promotes attachment and spreading of B16-F10 melanoma cells but not the haptotactic motility of these cells; it is possible that this region contains a fifth adhesion site (McCarthy *et al.*, 1986). The existence of these multiple cell-adhesion sites along FN molecules provides the potential for flexibility in the control of cell behavior, which might have important implications during development, e.g. in neural crest-cell migration.

In the present study, we have investigated the role of each of the cell-binding domains of FN in attachment, spreading and motility of neural crest cells, using specific inhibitory and adhesion probes. Our results show that neural crest cells can interact *in vitro* with both the RGDS and the CS1 adhesion domains through integrin receptors. In addition, these sites possess distinct functional specificities, possibly reflecting differences in the association between their corresponding receptors and cytoskeletal elements. These results indicate the complexity and specificity of different cell-surface interactions, and suggest that spatio-temporal changes in the forms of FN molecules throughout the embryo may be important in regulating neural crest cell migration and consequently their differentiation.

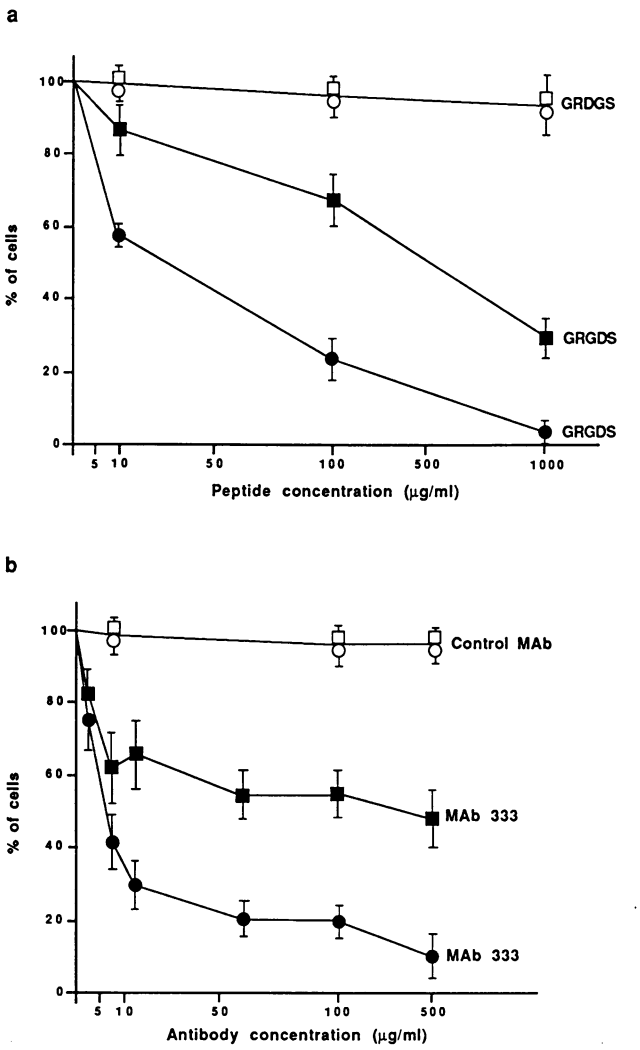


Fig. 1. Effect of GRGDS and GRDGS peptides (**a**) and of 333 and control mAbs (**b**) on the attachment (squares) and spreading (circles) of neural crest cells onto FN substrates. Results are expressed as percentage of the values obtained in the absence of peptides and antibodies.

Results

Attachment, spreading and motility of neural crest cells were quantitated using several different types of assays: (i) peptides and monoclonal antibodies (mAbs) were used as competitors for crest cell attachment, spreading or motility on FN substrates; (ii) fusion proteins and IgG-conjugated peptides containing each of the cell-adhesion sites were used as substrata for neural crest cells. The inhibitory and adhesion probes used in this study and their specificities are listed in Table I.

Role of adhesion sites in attachment and spreading of neural crest cells

RGDS binding site and its associated synergistic adhesion site. In inhibition assays, GRGDS peptides interfered strongly with both attachment and spreading of neural crest cells in a dose-dependent manner, whereas control GRDGS peptides were inactive (Figure 1a). Attachment of neural crest cells to FN was also perturbed by the 333 mAb but, in contrast to the complete inhibition by GRGDS peptides, it was not totally abolished at even high concentrations of the antibody; maximal inhibition was ~55% decreased

Table II. Attachment and spreading of neural crest cells on FN at 10 μg/ml in the presence of soluble peptides at varying concentrations

Peptide	Concentration (mg/ml)	Cells attached (%)	Cells spread (%)
GRGDS	1	29 ± 2	3 ± 1
GRGDS	0.1	67 ± 6	22 ± 4
RGDS	1	45 ± 1	10 ± 1
RGDS	0.1	66 ± 3	22 ± 8
GRGD	1	80 ± 2	35 ± 3
SDGR	1	93 ± 4	73 ± 9
GRDGS	1	93 ± 6	92 ± 6

Results are expressed as percentage of the values obtained in the absence of peptides.

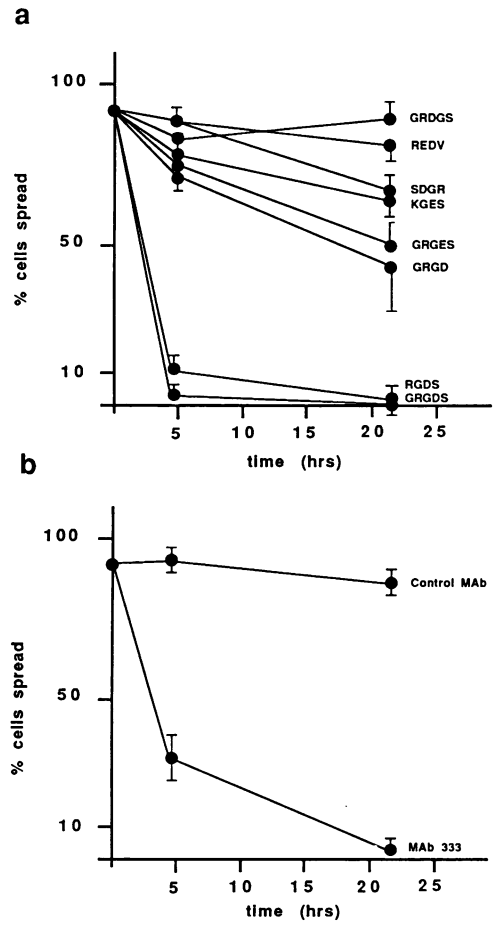


Fig. 2. Rounding-up effects of various peptides (**a**) and of the 333 mAb (**b**) on spread neural crest cells. Peptides were used at 1 mg/ml and the antibodies at 0.1 mg/ml. Cells were incubated in the presence of the inhibitory probes and the number of cells spread counted at varying periods of time. The results are expressed as the percentage of cells that remained spread at the time indicated.

attachment (Figure 1b). In contrast to attachment, the spreading of neural crest cells was strongly inhibited by the 333 mAb, with a nearly 90% decrease in spreading at 500 μg/ml and a 50% decrease at concentrations as low as 5 μg/ml (Figure 1b). Other RGD-derived peptides known to interfere with fibroblast interaction with FN (Yamada and Kennedy, 1985, 1987) were also tested for their inhibitory activity on neural crest attachment and spreading. All of them, except SDGR, were found to inhibit cell spreading

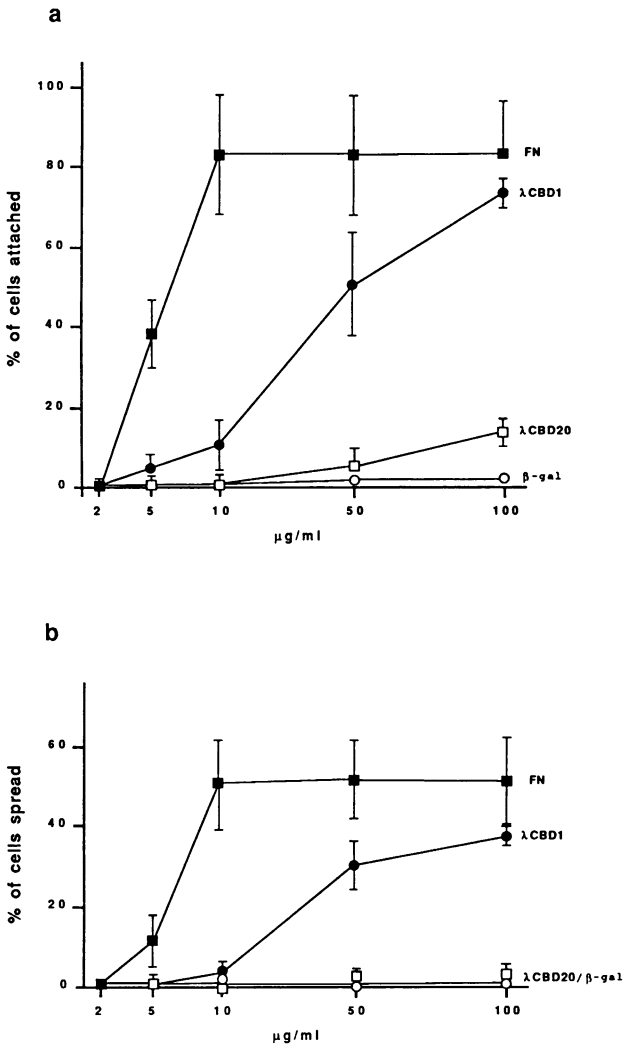


Fig. 3. Attachment (**a**) and spreading (**b**) of neural crest cells on FN, β-galactosidase (β-gal) and fusion proteins covering either the RGDS binding site plus the synergistic adhesion site (λCBD1) or the RGDS site alone (λCBD20). The results are expressed as the percentage of attached and spread cells in relation to the total number of cells deposited on the substratum.

and, to a lesser extent, cell attachment; among these peptides, GRGDS had the highest activity (Table II).

In assays of perturbation of the morphology of previously spread neural crest cells, only GRGDS and RGDS peptides and the 333 antibody could induce rapid rounding-up of cells; after 5 h in the presence of these competitors, only a small proportion of neural crest cells remained spread (Figure 2; see also Figure 8). The rounding-up of cells was frequently followed by the formation of compact aggregates of cells. The other peptides tested either exhibited much weaker activity (e.g. GRGD) or were totally ineffective (i.e. SDGR, GRDGS and KGES; Figure 2).

In adhesion assays, the λCBD1 fusion protein displayed lower specific activities for attachment or spreading than FN, but it was able to mediate nearly complete adhesion at high concentrations (50–100 μg/ml; Figure 3). The λCBD20 fusion protein (which lacks the synergistic site) appeared much less active at all concentrations than either FN or λCBD1 fusion protein (Figure 3). In a complementary experiment, we allowed neural crest cells to adhere onto λCBD1 fusion proteins in the presence of GRGDS, GRGES,

Table III. Attachment and spreading of neural crest cells on substrates precoated with 10 μg/ml FN in the presence of peptides inhibiting the binding sites located in the IIICS region

Peptide	Cells attached (%)	Cells spread (%)
GRGDS	29 ± 2	3 ± 1
GRDGS	93 ± 6	92 ± 6
CS1	91 ± 4	82 ± 6
GRGES	89 ± 10	77 ± 10
CS1-C	85 ± 11	78 ± 13
REDV	89 ± 11	74 ± 8
CS5	88 ± 10	91 ± 7

Peptide concentrations were 1 mg/ml. Results are expressed as percentage of the values obtained in the absence of peptides.

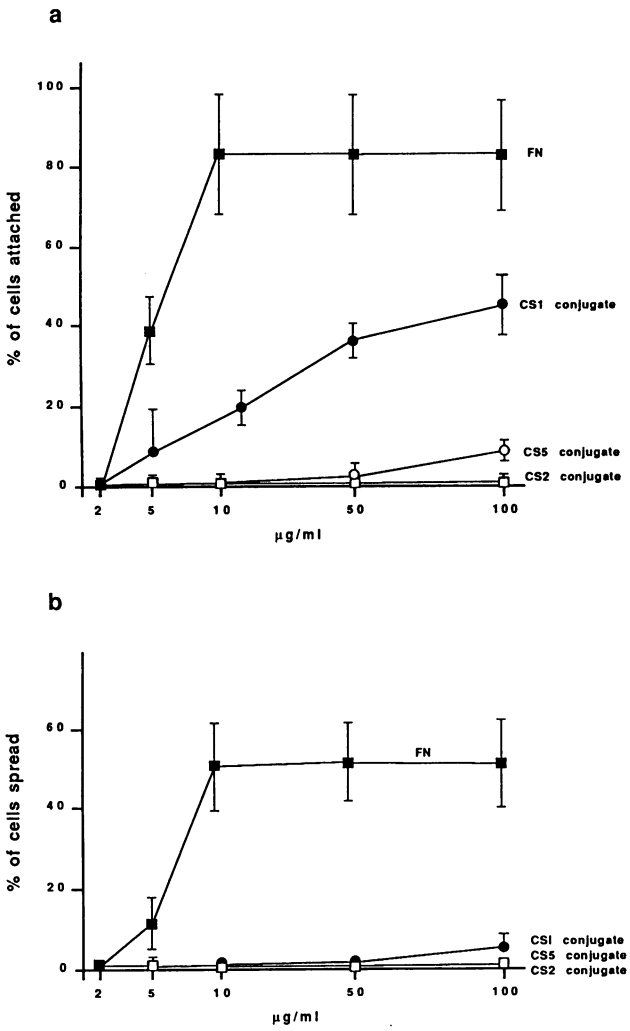


Fig. 4. Attachment (**a**) and spreading (**b**) of neural crest cells on FN and CS1, CS2 and CS5 peptides conjugated to IgGs (CS1, CS2 and CS5 conjugates, respectively). The results are expressed as the percentage of attached and spread cells in relation to the number of cells deposited on the substratum. Note that only the CS1 conjugate is able to promote cell attachment. In contrast, none of the conjugates can mediate spreading of neural crest cells.

CS1 peptides or 333 mAbs. Both GRGDS peptide and 333 antibody strongly inhibited attachment and spreading of neural crest cells, whereas CS1 and GRGES peptides were ineffective (data not shown).

Table IV. Attachment of neural crest cells on IgG-conjugated CS1 at 100 μg/ml in the presence of peptides at 1 mg/ml or of mAbs at 500 μg/ml

Inhibitory probe	Cells attached (%)
CS1	45 ± 6
GRGES	79 ± 15
CS1-C	88 ± 12
mAb 333	92 ± 8
Control mAb	97 ± 3

Results are expressed as a percentage of the values obtained in the absence of peptides.

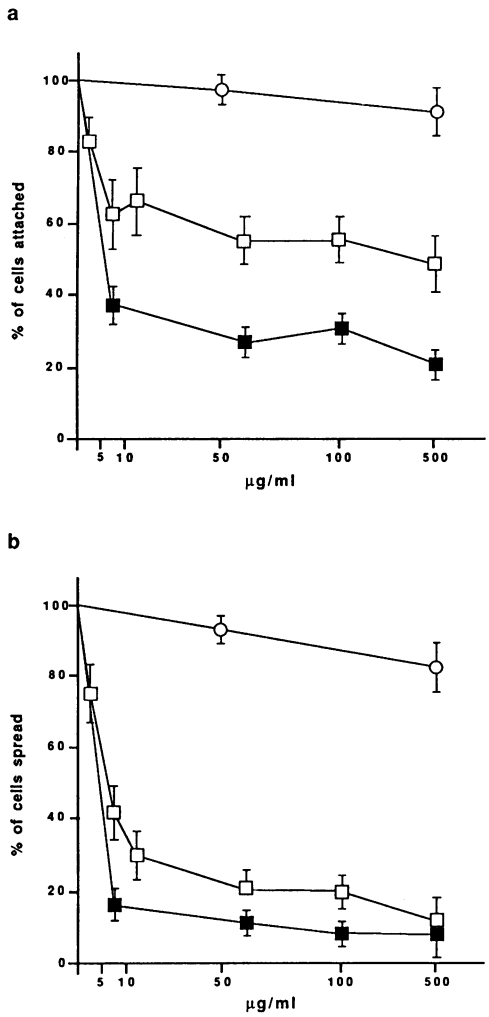


Fig. 5. Attachment (**a**) and spreading (**b**) of neural crest cells in the presence of CS1 peptide (open circles), of 333 mAb (open squares) at varying concentrations, or of CS1 peptide at 1 mg/ml with 333 antibody at varying concentrations (filled squares). Results are expressed as percentage of the values obtained in the absence of peptides and antibodies.

Adhesion sites in the IIICS region. As shown in Table III, all peptides for probing the IIICS sites, except GRGDS, did not interfere significantly with either attachment or spreading of neural crest cells onto FN substrates. When assayed for perturbation of the previously spread morphology of cells (Figure 2a; see also Figure 8), REDV or CS1 also did not

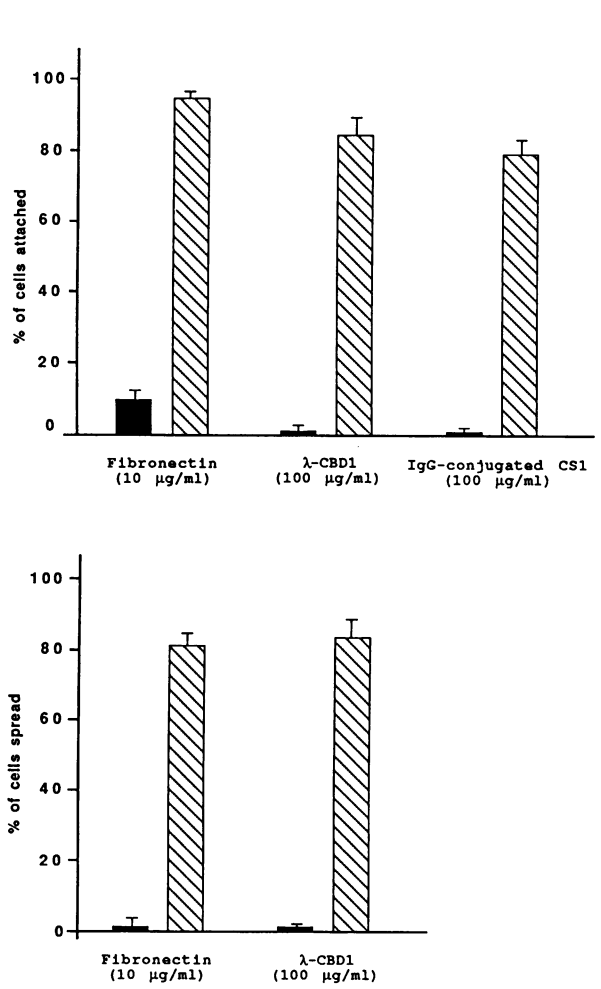


Fig. 6. Attachment (**a**) and spreading (**b**) of neural crest cells on FN, λCBD1 fusion protein and IgG-conjugated CS1 peptide in the presence of 1 mg/ml monovalent antibodies directed against the β subunit of the avian fibronectin receptor. Results are expressed as percentage of the values obtained in the absence of antibodies. The antibodies show a strong inhibitory effect on adhesion of all substrates. The effect of the antibodies on the spreading on IgG-conjugated CS1 was not evaluated because no spreading occurred on this substrate in the absence of any competitor (see Figure 5). Black and crosshatched bars correspond to antibodies to the receptor and to control antibodies, respectively.

induce rounding-up of neural crest cells. GRGES was slightly more active, but its perturbing effect could only be detected after 20 h (Figure 2a). The interaction of neural crest cells with binding sites located in the IIICS region was further investigated by measuring the extent of cell adhesion to substrates composed of IgG-conjugated peptides or of fusion proteins. As shown in Figure 4, the CS1 conjugate could promote in a dose-dependent manner the attachment of neural crest cells, but not their spreading. However, the activity of the CS1 conjugate was always weaker than that of intact FN. In contrast to the CS1 conjugate, CS2 and CS5 conjugates showed no attachment-promoting activity even at high concentrations (Figure 4). Attachment of neural crest cells to CS1 conjugate could be inhibited by CS1 peptides in solution and, to a lesser extent, by GRGES, while CS1-C peptide (i.e. a peptide covering only part of CS1 and lacking adhesion-promoting activity when conjugated to IgGs) or 333 mAbs had no effect (Table IV). Finally, the fusion protein FN421 covering the IIICS domain was able to support attachment but not spreading of neural crest cells at a rate similar to that of the CS1 conjugate (not shown).

The extent of attachment and spreading of neural crest cells on FN was measured in the presence both of 333 mAbs at varying concentrations and of CS1 peptides at 1 mg/ml. As shown in Figure 5a, a combination of the 333 antibody and of CS1 peptide significantly increased the inhibition of attachment of neural crest cells as compared to either the antibody or the peptide alone. The level of inhibition obtained with the two mixed probes was comparable to that with the GRGDS peptide, which is known to affect both RGDS and CS1 sites. In contrast, when REDV or CS1-C were used in combination with the 333 antibody, no detectable increase in inhibition of attachment was observed as compared to the antibody alone (not shown). Spreading of neural crest cells was also decreased in the presence of both 333 antibody and CS1 peptide, but less significantly than attachment, since the 333 antibody alone showed strong inhibitory activity (Figure 5b).

Attachment and spreading of neural crest cells were also measured on substrata composed of λCBD1 fusion protein in association with FN421 fusion protein or with CS1 conjugate at various concentrations. In all combinations of proteins, we did not observe any additive effect on cell attachment and spreading; the proportions of attached and spread cells in all cases were comparable to those with λCBD1 protein alone (data not shown).

Receptors for RGDS and CS1 adhesion sites

In order to determine whether neural crest cells interact with the RGDS and the CS1 adhesion sites through receptors belonging to the 140-kd complex family (for reviews, see Ruoslahti and Pierschbacher, 1987; Hynes, 1987; Buck and Horwitz, 1987; Yamada, 1988), we measured the extent of crest cell attachment and spreading on FN, λCBD1 fusion protein or IgG-conjugated CS1 peptide in the presence of monovalent antibodies to the β subunits of the avian 140-kd complex. As shown in Figure 6, these antibodies were able to prevent totally both attachment and spreading of neural crest cells to FN, λCBD1 protein or CS1 conjugate.

FN adhesion sites and neural crest cell motility

In the initial series of experiments, neural tubes were deposited on various substrates and neural crest cells were photographed periodically. When neural tubes were deposited on FN, neural crest cells emigrated from them within 2–4 h and actively moved about on the substrate. After 15 h in culture, neural crest cells organized into a large halo around the neural tube and exhibited a well-spread morphology with several long processes per cell (Figure 7a). On λCBD1 fusion protein, neural crest cells were able to leave the neural tube, but the timing of their emigration was delayed. The diameter of the halo was similar to that obtained on FN, but the population was less dense and cells were generally less flattened (Figure 7c). In contrast, substrates composed of λCBD20 fusion protein did not permit extensive migration of neural crest cells. Cells started to leave the neural tube only after 8–10 h and organized into a small halo where most cells were round, sparse, and often aggregated with their neighbors (Figure 7d). On substrates composed of IgG-conjugated CS1 (Figure 7e) or of FN421 fusion protein (Figure 7f), cells were sparse, rarely spread, and often assembled into loose aggregates; however, the area

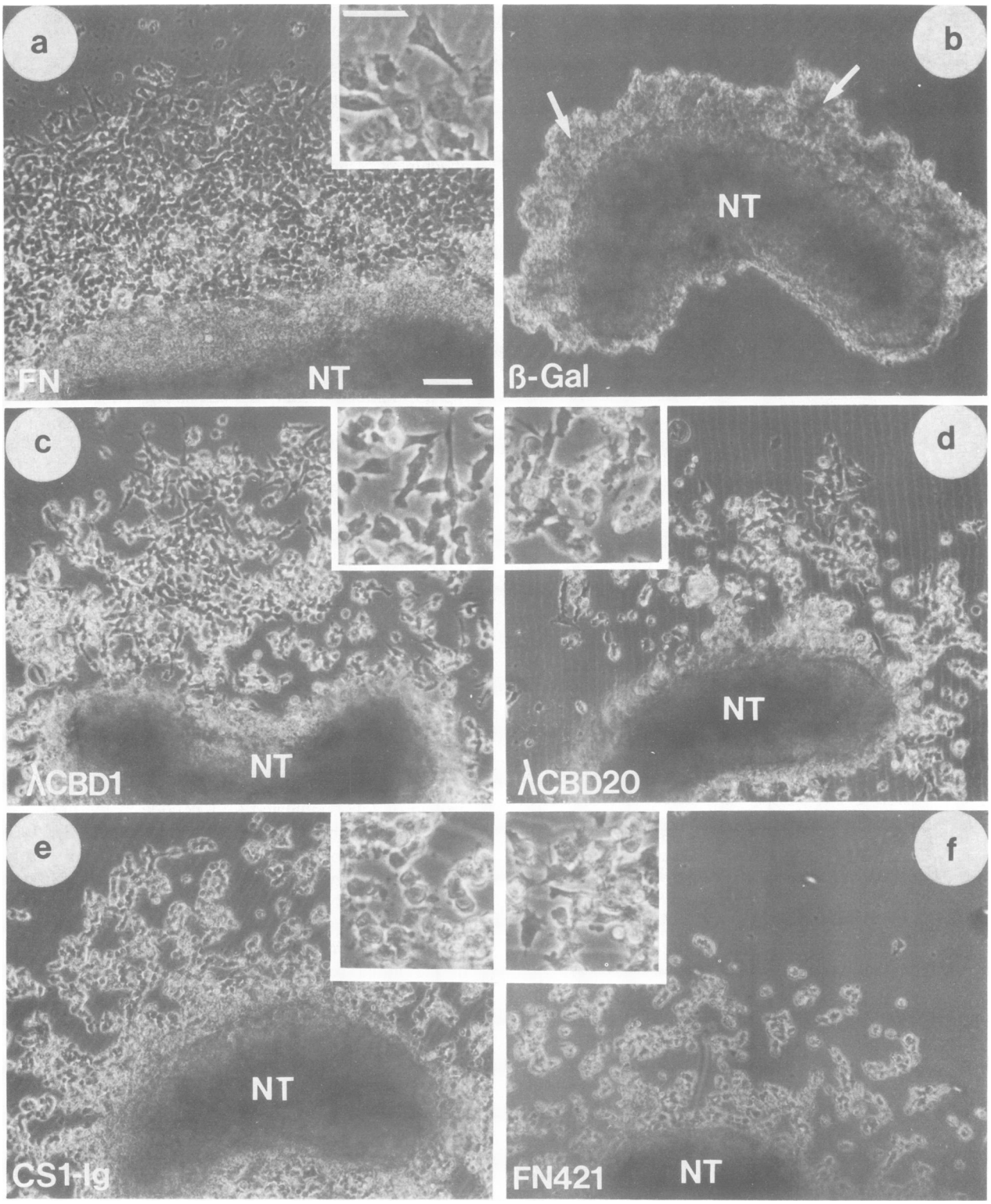


Fig. 7. Migration of neural crest cells on FN at 10 μg/ml (**a**), β-galactosidase at 50 μg/ml (**b**), λCBD1 fusion protein at 50 μg/ml (**c**), λCBD20 fusion protein at 50 μg/ml (**d**), peptide CS1 conjugated to IgGs at 50 μg/ml (**e**) and FN421 fusion protein at 20 μg/ml (**f**). Neural tubes were deposited on Petri dishes coated with the substrates to be tested, and halos of neural crest cells were photographed regularly. The micrographs show general views of the halos after 15 h of culture and insets show the morphology of the cells. NT, neural tube. Arrows in (b) point at neural crest cells aggregated along the neural tube. (General views: bar = 100 μm; insets: bar = 25 μm.)

of the explant was comparable to that on FN or λCBD1 fusion protein. In the presence of CS2 conjugates, bovine serum albumin or β-galactosidase, neural crest cells remained as compact aggregates attached to the neural tube (Figure 7b). No cells were seen migrating away on the substratum, and in many cases the neural tube could not even adhere to the substratum. When the substratum was coated with λCBD1 fusion protein plus CS1 conjugate or with λCBD1 plus FN421 fusion proteins, migration of neural crest cells was not improved as compared with the proteins used

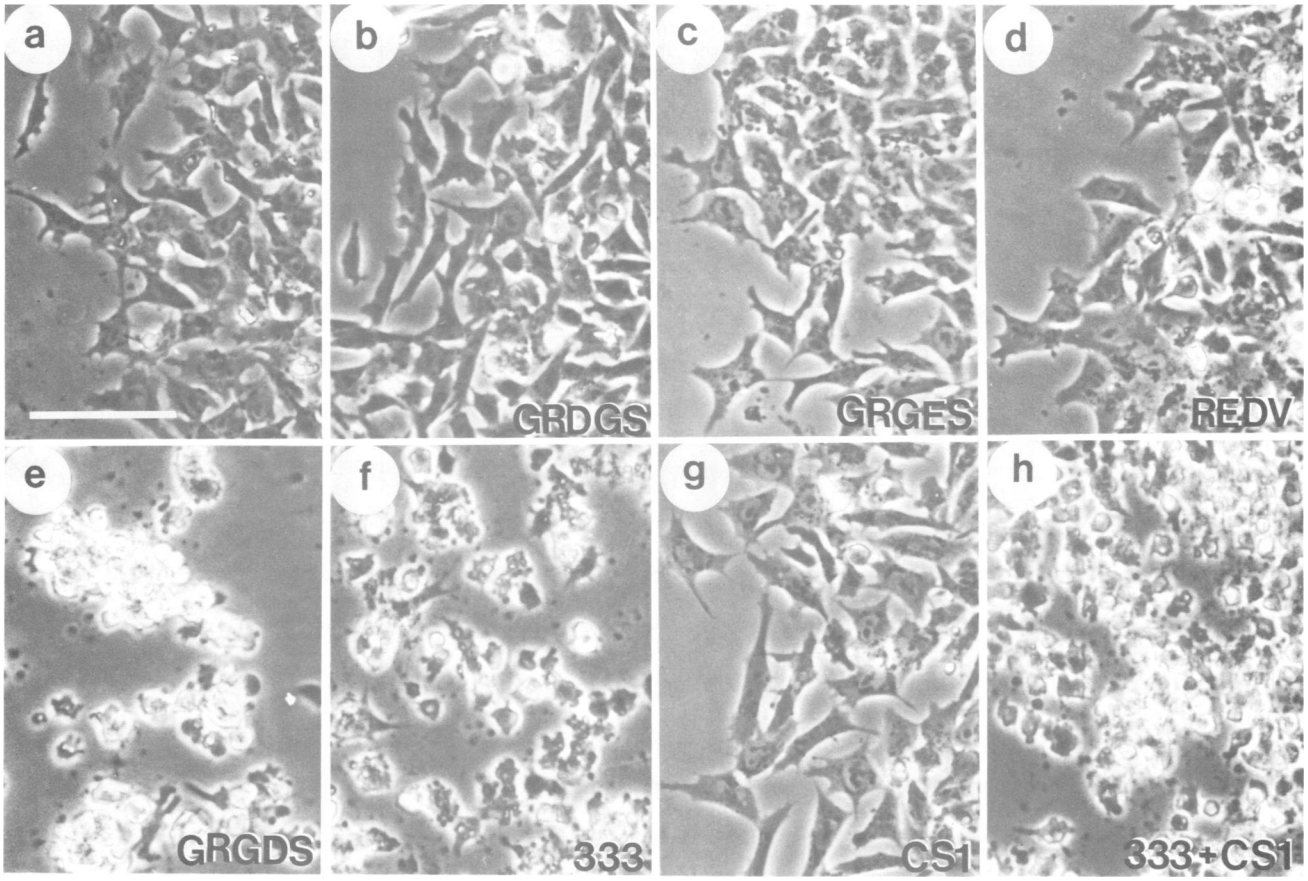


Fig. 8. Morphology of migrating neural crest cells in the absence of peptide or antibody (**a**), and in the presence of GRDGS (**b**), GRGES (**c**), REDV (**d**), GRGDS (**e**) or CS1 (**g**) at 1 mg/ml, compared with 333 mAb at 75 μg/ml (**f**) and CS1 at 1 mg/ml with 333 antibody at 75 μg/ml (**h**). Neural tubes were deposited in Terasaki wells previously coated with FN at 10 μg/ml in control culture medium, and neural crest cells were allowed to emigrate for ~6 h. Peptides and/or antibodies were added then in medium with 10% FN-depleted serum, and crest cells were photographed regularly. The micrographs show the morphologies of neural crest cells after 10 h in the presence of the competitors. Only GRGDS and the 333 antibody alone or associated with CS1 induced rounding-up of cells. (Bar = 50 μm.)

individually (not shown).

In another series of experiments, neural tubes were cultured on FN, and neural crest cells were allowed to emigrate for several hours. Peptides and/or antibodies were then added to the cells and the migratory behavior of cells was recorded using time-lapse microcinematography. Figure 8 shows the shapes of cells following treatment with peptides or antibodies and Figure 9 shows typical trajectories of cells located in or close to the front of migration, and the mean values of their speed of locomotion (S) and persistence of movement (P). On FN substrates in the absence of any competitor, neural crest cells formed a dense population, and exhibited a well-spread morphology (Figure 8a). Their migration speed was 60 ± 10 μm/h and the degree of persistence of movement was 0.70 ± 0.14. This resulted in a rapid and constant expansion of the halo. GRGDS peptides at high concentration induced complete rounding-up of cells, which soon became sparse and subsequently formed aggregates (Figure 8e). Isolated cells exhibited a rapid spinning movement and extended numerous cell processes which caused very rapid cell movement (e.g. S = 103 ± 19 μm/h) but without any constant directionality (P = 0.08 ± 0.04). Such rapid but random movement produced no outward expansion of the cell population. In the presence of 333 mAbs, neural crest cells also became round and sparse (Figure 3f), and the speed of locomotion was high (S = 84 ± 14 μm/h) and the persistence of movement low (P = 0.27 ± 0.11). In the presence of GRGES or CS1 peptides, neural crest cells retained their flattened morphology during the time course of the experiment (Figure 8c and g). However, while the persistence of movement of cells remained unchanged as compared to controls (P = 0.59 and 0.71, respectively), the speed of locomotion was significantly perturbed (S = 36 and 41 μm/h). This resulted in a substantial reduction in the expansion of the neural crest cell population. REDV and GRDGS peptides did not affect the morphology of neural crest cells (Figure 8b and d), and both the speed of locomotion and the persistence of movement were similar to those in the absence of competitors. When neural crest cells were confronted with both 333 antibodies and CS1 peptides, they rounded up rapidly (Figure 8h) and migrated randomly (P = 0.25 ± 0.06). However, their speed was reduced as compared to that obtained with the 333 antibody alone (S = 66 ± 6 versus 84 ± 14). Thus, combining the antibody and CS1 peptide resulted in a relative decrease in both speed and persistence of movement of neural crest cells.

Discussion

The aim of the present study was to investigate the role of the different adhesion sites of FN in the behavior of neural

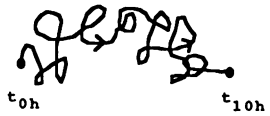
Competitor	Migratory tracks	Speed (μm/hr)	Persistence
No competitor		60 ± 10	0.70 ± 0.14
GRGDS (1mg/ml)		103 ± 19	0.08 ± 0.04
Mab333 (75 μg/ml)		84 ± 14	0.27 ± 0.11
GRGES (1mg/ml)		36 ± 8	0.59 ± 0.03
CS1 (1mg/ml)		41 ± 8	0.71 ± 0.13
REDV (1mg/ml)		56 ± 4	0.71 ± 0.13
GRDGS (1mg/ml)		61 ± 11	0.52 ± 0.11
Mab 333 (75μg/ml) + CS1 (1mg/ml)		66 ± 6	0.25 ± 0.06

Fig. 9. Migration tracks of neural crest cells on FN-coated substrates in the presence of various competitors at different concentrations. Neural tubes were deposited in Terasaki wells previously coated with FN at 10 μg/ml in control culture medium. Neural crest cells were allowed to emigrate from the neural tube for ~6 h, and their migration was recorded using time lapse videomicroscopy. The medium was replaced by fresh medium containing the competitor to be studied, and the behavior of neural crest cells was recorded for another 12 h. Migration tracks of several cells located at the migration front were plotted, and the total distance of migration was measured. A typical track, and the speed of locomotion and persistence of movement are indicated. Values represent the mean obtained for 15 cells in three different assays.

crest cells. The relative capacity of each site to permit attachment, spreading and motility was measured directly on substrates consisting of fusion proteins or of IgG-conjugated peptides containing these sites. Complementary experiments with inhibitory peptides and antibodies examined the consequences of inhibiting each site when cells were cultured on intact FN. Our major findings are: (i) neural crest cell attachment can be mediated by either the bipartite RGDS cell-binding domain or by the CS1 site, but not by the RGDS sequence alone; (ii) cell spreading is mediated only by the bipartite RGDS domain, and not by the CS1 or RGDS sites alone; (iii) cell motility requires both the bipartite RGDS domain and the CS1 site in association; and (iv) integrin receptors are involved in cell interactions with both of these major FN adhesive domains. Our results identify a hierarchy of requirements for cell interactions with different FN sites for different functions.

These experiments indicate that the RGDS sequence alone is not sufficient to support attachment, spreading and motility of neural crest cells, but instead requires the presence of a

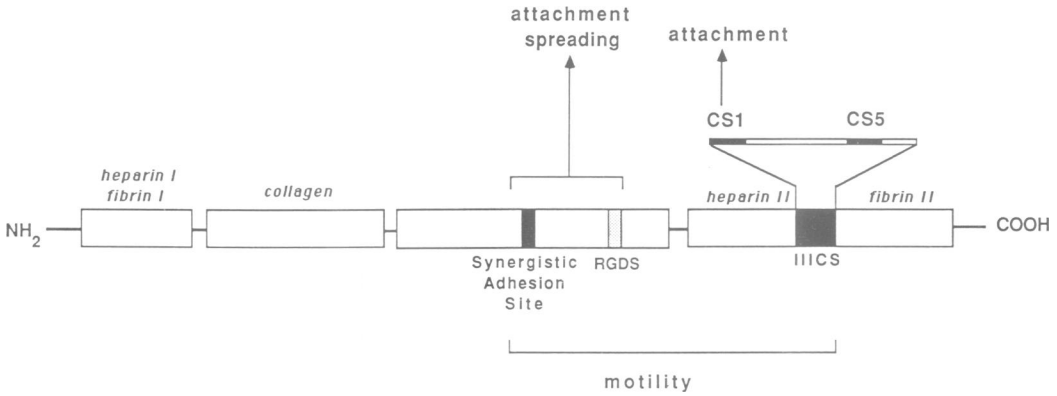


Fig. 10. Schematic representation of the localization of different cell-binding sites (synergistic adhesion site, RGDS site, CS1 and CS5 sites in the IIICS domain) along the FN molecule and their possible role in cell attachment, spreading and migration of neural crest cells. One of the two chains comprising FN is represented. The other major binding domains (i.e. collagen, fibrin I and II, and heparin I and II) are also indicated. The 'motility domain' is shown covering the various cell-binding sites and other regions of the molecule, because it cannot be excluded that areas of the molecule distinct from the cell-binding domains can contribute to the motility-promoting activity.

synergistic adhesion site initially identified in studies of fibroblast adhesion (Obara *et al.*, 1987, 1988), located >200 amino acids away towards the N terminus of the FN molecule. For conciseness, we term the combination of the RGDS sequence and the synergistic adhesion site the (bipartite) RGDS domain.

The spreading-promoting activity of FN is located primarily in the RGDS domain, since the adhesion sites of the IIICS region are unable to support spreading of neural crest cells. In contrast, the IIICS CS1 site as well as the RGDS domain can mediate simple attachment of neural crest cells. In adhesion assays, both fusion proteins containing the RGDS domain or the IgG-conjugated CS1 peptide at high concentrations produced a high percentage of neural crest cell attachment. In inhibition assays, only a 55% decrease in cell attachment was obtained with the 333 anti-RGDS domain antibody, but this decrease reached 90% when the antibody was used in conjunction with CS1 peptide. These and other results strongly suggest that the RGDS and CS1 sites are functionally independent but function in cooperation to provide maximal cellular attachment.

Both RGDS domain and CS1 substrates supported limited motility of neural crest cells, as shown by measurements of cell migration on fusion proteins and IgG-conjugated peptides. When neural crest cells were cultured on FN in the presence of 333 mAb known to perturb specifically the RGDS domain, they rounded up, their motility was either similar or even greater than on unperturbed FN, but the directionality of their movement was affected. Conversely, when peptides probing the CS1 site were used as competitors, the rate of neural crest cell locomotion was reduced, but both cell spreading and directionality of movement remained unchanged. Finally, GRGDS peptides or CS1 peptides in conjunction with the 333 antibody interfered with both speed of locomotion and persistence of movement. These experiments indicate that cell locomotion requires at least these two adhesion sites, each playing a specific distinctive role in the process. It is important to stress that cell spreading is not an absolute prerequisite for cell motility, as shown in the experiments using either CS1 conjugate as a substratum or 333 antibody as a competitor, where cells could still undergo some movement. However, it allows cohesion among the migrating population, thus maintaining the directionality of movement (see also Newgreen *et al.*, 1979; Rovasio *et al.*, 1983).

Therefore, sequence information for attachment, spreading and locomotion are localized in separate regions of FN molecules: (i) a 20−30-kd bipartite domain containing the RGDS sequence associated with the synergistic adhesion site and (ii) a short 25 amino acid sequence located within the IIICS domain (Figure 10). It is of interest that thrombospondin, another adhesive extracellular molecule, has sites for adhesion, haptotactic and chemotactic migrations that have been mapped to different regions of the molecule (Taraboletti *et al.*, 1987). On FN, one cannot exclude that other sequences may be necessary to obtain the full range of activities. This is particularly true for cell motility, which was never completely abolished even in the presence of probes competing with both RGDS and CS1 sites. Although one possible candidate would be the heparin-binding domain adjacent to the IIICS region (Laterra *et al.*, 1983; Rogers *et al.*, 1987; Mugnai *et al.*, 1988), our preliminary data showing inability of heparin to inhibit neural crest cell migration indicate that the heparin-binding site is apparently not involved in the locomotion of these cells.

Another cell-adhesion site located in the IIICS domain, termed the CS5 site and containing the REDV sequence, has been reported previously for B16-F10 melanoma cells (Humphries *et al.*, 1986, 1987). This site is apparently not recognized by migrating neural crest cells. The discrepancy between the activity of the CS5 sequence for mouse melanoma cells and for avian neural crest cells may result from species differences in the amino acid sequence of the IIICS region, since chicken FN lacks the REDV sequence (Norton and Hynes, 1987).

Since FN molecules contain multiple binding sites with functional specificities, the question arises of which receptors mediate the response of neural crest cells. Our data indicate that both RGDS and CS1 cell-binding domains interact with the surface of cells through 140-kd fibronectin receptors sharing close, if not identical, β subunits. Neurite outgrowth of peripheral nervous system cells derived from the neural crest on CS1−IgG conjugates is also blocked by anti-integrin antibodies (Humphries *et al.*, 1988). A common β subunit may be associated with distinct α subunits since, in avian embryos, two α chains, also termed band 1 and 2, have been described (Knudsen *et al.*, 1985; Hasegawa *et al.*, 1985). In mammalian cells, a family of receptor complexes share the same β subunit, but each possesses a unique α subunit (Wayner and Carter, 1987; Hemler, 1988). In our case, there

may be two receptors that recognize distinct cell-adhesion sites on FN; the receptor for the RGDS domain would mediate both cell attachment and spreading whereas the receptor for the CS1 sequence would only promote cell attachment. Since spreading but not attachment involves the cytoskeleton (see Burridge, 1986, for a review), these two receptors might therefore differ in their interactions with the cytoskeleton. During cell motility, the receptor for CS1 might participate in the formation of transient sites of cell–substratum attachment, while the receptor for the RGDS domain would also be involved in the organization of actin microfilaments.

Depending on the type of alternative splicing in the IIICS regin (Tamkun *et al.*, 1984; Kornblihtt *et al.*, 1985), FN molecules may or may not contain the CS1 binding domain. Since neural crest cell motility requires both the CS1 and RGDS domains, FN molecules that are missing the IIICS region cannot support effective migration. It is therefore conceivable that neural crest cell migration *in vivo* may be reduced or even prevented in certain regions of the embryo that are enriched in FN lacking the CS1 sequence. Such might be the case, for example, if the caudal half of the sclerotome which is never occupied by neural crest cells (Rickmann *et al.*, 1985; Teillet *et al.*, 1987). Conversely, as suggested by the high invasiveness of melanoma cells, the ability to recognize primarily the CS1 binding sequence may be correlated with invasive properties of cells. The presence of areas containing FN that are enriched in this sequence may result in specific behavior of some neural crest subpopulations. For instance, some migratory pathways are only taken by melanocyte and enteric neuron precursors, two populations that are thought to be more motile and invasive than other neural crest cells (Thiery *et al.*, 1982; Tucker *et al.*, 1986). One can speculate that these precursors respond to FN present in these pathways by invasive migration because they have receptors for the CS1 adhesion site and because FN molecules there are enriched in this sequence. Programmed fluctuations in the splicing of the IIICS region may therefore play a crucial regulatory role in path-finding mechanisms. If so, this would be the first example of an instructive role rather than permissive for extracellular matrix components.

In summary, this study demonstrates that neural crest cells interact differentially with several distinct binding domains on FN through integrin receptors. These receptors may differ in how they transduce signals to the cytoskeleton, which may help explain the functional specificities of these sites and allow them to operate in synergy. In addition, the migratory behavior of neural crest cells could be regulated by spatio-temporal changes in the expression of FN variants. It will therefore be instructive to examine the embryonic distribution of these variants and its implication for morphogenic events.

Materials and methods

Materials

FN was purified from human plasma by gelatin affinity chromatography (Rovasio *et al.*, 1983). The 333 mAb, which binds FN at a site close to the Arg–Gly–Asp–Ser adhesive recognition signal and inhibits fibroblastic cell adhesion to FN, was produced and characterized previously (Akyiama *et al.*, 1985; Humphries *et al.*, 1986). Custom synthesis of tetra- and pentapeptides was carried out by Peninsula Laboratories (Belmont, CA). Peptides were further purified by preparative reversed-phase HPLC as described (Yamada and Kennedy, 1987). The synthesis, purification and analysis of synthetic peptides spanning the entire IIICS region of human FN (CS peptides) were performed as described previously (Humphries *et al.*, 1986, 1987). These CS peptides were covalently conjugated to rabbit IgG to form CS–IgG conjugates, using the heterobifunctional cross-linker succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Humphries *et al.*, 1987). β-Galactosidase fusion proteins containing fragments of human FN and called λCBD1 and λCBD20 have been described elsewhere (Obara *et al.*, 1987). These fragments included a sequence of 80 kd promoting full adhesion of fibroblasts and a sequence of 33 kd containing the Arg–Gly–Asp–Ser site but lacking a second, synergistic adhesion site (Obara *et al.*, 1987, 1988). The production and characterization of a β-galactosidase fusion protein termed FN421 and containing the IIICS domain will be described elsewhere (M.J.Humphries and K.M.Yamada, in preparation). Rabbit polyclonal antibodies to the β subunit of the 140-kd receptor complex and their monovalent fragments were described previously (Chen *et al.*, 1985; Duband *et al.*, 1986).

Embryos and cell cultures

Japanese quail embryos (*Coturnix Coturnix japonica*) were used throughout this study. Eggs were incubated at 38 ± 2°C in an air chamber and staged according to the number of somite pairs and to the duration of incubation. Cultures of trunk neural crest cells were generated essentially as described previously (Duband *et al.*, 1986). Cells were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum previously depeleted in FN (see Rovasio *et al.*, 1983).

Assays for cellular attachment and spreading

Cellular attachment and spreading assays were performed in 10-cm bacteriological Petri dishes (CML; Consortium pour le Matériel de Laboratoire, Nemous, France). Small areas of the dishes were incubated at 37°C for 1 h with 20 μl of human plasma FN, CS–IgG conjugates or of fusion proteins at varying concentrations in phosphate-buffered saline (PBS), followed by incubation with heat-treated bovine serum albumin (3 min at 80°C) in PBS (3 mg/ml) for 30 min and extensive washes in PBS. Crest cells used for the assays were obtained from >50 neural tube explants cultured for 18 h on FN-coated dishes. Cells were harvested using treatment for 5 min at 37°C with 0.25% trypsin and 1 mM EDTA (Gibco Europe, UK) in PBS. The protease reaction was stopped by adding DMEM containing 10% serum. Cells were then collected and sedimented at 1000 r.p.m. for 10 min, then resuspended and incubated for at least 3 h at 37°C in DMEM with 10% serum to allow recovery from trypsin treatment. A 20 μl aliquot of cell suspension containing 2000 cells was deposited on the areas pre-coated with proteins to be tested. The dishes were then incubated at 37°C for 1 h, rinsed in PBS to remove the non-adherent cells, and fixed in a 3.7% formaldehyde solution in PBS. Attached and spread cells were counted with a Leitz inverted phase contrast microscope.

Assays for cellular migration

Cellular migration assays were performed in HLA-Terasaki plates (CML, France). It should be noted that plates designed for cell culture were not used because they gave high non-specific cell adhesion. Each well of the plates was coated with human plasma FN, CS–IgG conjugates or fusion proteins at varying concentration as for cell adhesion assays. Neural tubes were deposited in wells in the presence of DMEM with 10% FN-free serum and were allowed to attach to the substratum for 2 h. The wells were then covered with a glass coverslip sealed with modelling clay. For time-lapse video microcinematography, cultures were observed with a Leitz Laborlux phase-contrast microscope in a heated plexiglass chamber equipped with a video camera (× 3400; 0.5 Lux, Sanyo Electronic Co. Ltd, Osaka, Japan) connected to a TV monitor (WV 5400, National Matsushita Co., Osaka, Japan), a time generator, and a time-lapse recorder (NV 8030, National Matsushita Co.). Migration paths were plotted on the TV monitor and copied onto transparent paper. The total distance of migration of selected cells was measured and the speed of locomotion calculated. The degree of persistence of movement was defined as the ratio between the linear distance and the total distance covered by the cells.

Inhibition assays of cellular adhesion, spreading and migration

For inhibition of cell attachment and spreading, cells were deposited on areas previously coated with plasma FN, CS–IgG conjugates or fusion proteins in the presence of synthetic peptides or antibodies at varying concentrations and treated as described for the adhesion assay. Each adhesion and inhibition assay was performed six times in three different experiments. For assaying inhibition of cell migration, the procedure was identical to the migration assay, except that the culture medium was replaced by DMEM containing 10% FN-free serum and peptides or antibodies after 8 h of culture, corresponding to the time when a significant number of neural crest cells have started to leave the neural tube. This allowed the observation of

migratory behavior of cells prior to and after adding the peptides or antibodies. Experiments of perturbation of the spread morphology of cells by antibodies and peptides was performed as described previously (Duband *et al.*, 1986).

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