

# Amphibian Neural Crest Cell Migration on Purified Extracellular Matrix Components: A Chondroitin Sulfate Proteoglycan Inhibits Locomotion on Fibronectin Substrates

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**Abstract.** The ability of purified extracellular matrix components to promote the initial migration of amphibian neural crest (NC) cells was quantitatively investigated *in vitro*. NC cells migrated avidly on fibronectin (FN), displaying progressively more extensive dispersion at increasing amounts of material incorporated in the substrate. In contrast, dispersion on laminin substrates was optimal at low protein concentrations but strongly reduced at high concentrations. NC cells were unable to migrate on substrates containing a high molecular mass chondroitin sulfate proteoglycan (ChSP).

When proteolytic peptides, representing isolated functional domains of the FN molecule, were tested as potential migration substrates, the cell binding region of the molecule (105 kD) was found to be as active as the intact FN. A 31-kD heparin-binding fragment also stimulated NC cell migration, whereas NC cells dispersed to a markedly lower extent on the isolated collagen-binding domain (40 kD), or the latter domain linked to the NH<sub>2</sub>-terminal part of the FN molecule.

Migration on the intact FN was partially inhibited by antibodies directed against the 105- and 31-kD fragments, respectively; dispersion was further decreased when the antibodies were used in combination.

Addition of the ChSP to the culture medium dramatically perturbed NC cell migration on substrates of FN, as well as of 105- or 31-kD fragments. However, preincubation of isolated cells or substrates with ChSP followed by washing did not affect NC cell movement. The use of substrates consisting of different relative amounts of ChSP and the 105-kD peptide revealed that ChSP counteracted the motility-promoting activity of the 105-kD FN fragment in a concentration-dependent manner also when bound to the substrate.

Our results indicate that NC cell migration on FN involves two separate domains of the molecule, and that ChSP can modulate the migratory behavior of NC cells moving along FN-rich pathways and may therefore influence directionally and subsequent localization of NC cells in the embryo.

**D**IRECTIONAL cell migration is a fundamental embryonic process which involves dynamic interactions between the migrating cells and their environment. The neural crest (NC)<sup>1</sup> is a suitable system for studies of the complex process of embryonic cell movement (7, 15, 24, 38, 64, 69), since NC cells migrate extensively in the embryo in an apparently regulated manner. Proceeding along delimited pathways, NC cells eventually reach distinct locations where they differentiate into numerous cell types that will compose several of the organs of the body (39, 72).

The extracellular matrix (ECM) that migrating NC cells encounter during their migratory phase (4, 40, 48, 61, 65) has been ascribed guiding functions during migration, and has been thought to influence the final homing of NC cells (4, 29, 40, 48, 49, 53, 61, 71). Recent studies by Löfberg and

co-workers (41 and unpublished results) demonstrate that ECM, transplanted on membrane microcarriers from older to younger regions of the axolotl embryo, are able to elicit a precocious local initiation of NC cell migration. These findings demonstrate that the onset of NC cell migration is dependent upon the maturation of the surrounding ECM.

Numerous attempts have been made to define the composition of the ECM permissive for NC cell movement (4, 48, 49). Fibronectin (FN) and laminin (LN) have been shown to be ubiquitous components of the ECM found along the migratory pathways of the NC (13, 14, 34, 47, 47, Perris, R., C. Fällström, Y. von Boxberg, D. F. Newgreen, and J. Löfberg, unpublished data); these cell adhesion proteins also strongly stimulate NC cell migration *in vitro* (16, 19, 44, 45, 58, 67). Sulfated proteoglycans have also been identified along the NC migratory routes and their differential distribution has been thought to contribute to the modeling of suitable paths for the migrating NC cells (2, 11, 49, 53, 66; Perris, R., C. Fällström, Y. von Boxberg, D. F. Newgreen,

1. *Abbreviations used in this paper:* ChSP, chondroitin sulfate proteoglycan; D, directionality; ECM, extracellular matrix; FN, fibronectin; LN, laminin; NC, neural crest; NT, neural tube; VN, vitronectin.

and J. Löfberg, unpublished data). However, when tested in vitro, exogenously administered glycosaminoglycans and proteoglycans have yielded inconsistent effects on NC cell locomotion (44, 48, 49, 67). These differences probably reflect variations in experimental conditions such as: the species studied, the developmental stage of cells, the types of substrate on which NC cells were seeded, and the structure of the glycosaminoglycans/proteoglycans used.

The focus of this in vitro study was to determine the effects of a well-characterized high molecular mass hyaluronate-binding chondroitin sulfate proteoglycan (ChSP) on initial NC cell migration on FN. To gain a better understanding of the mechanisms by which proteoglycans may regulate NC cell migration on FN, we also localized the regions of the FN molecule responsible for the motility-promoting effects exerted by the intact protein and examined how ChSP affected locomotion on isolated portions of the FN molecule. The degree of NC cell dispersion away from the co-explanted neural tube was adopted here to assess the migratory response induced by the different types of substrates examined.

## Materials and Methods

### ECM Components

The following purified ECM molecules were kind gifts: heparin (Dr. Ulf Lindahl, Department of Veterinary Medical Chemistry, Swedish University of Agricultural Science, Uppsala, Sweden); a high molecular weight, hyaluronate-binding chondroitin sulfate proteoglycan, purified from bovine nasal cartilage under dissociative conditions (Dr. Bruce Caterson, Department of Biochemistry, West Virginia University, Morgantown, WV); vitronectin (VN) (Dr. Erkki Rouslahti, La Jolla Cancer Research Foundation, La Jolla, CA); LN (Dr. Rupert Timpl, Max-Planck Institute for Biochemistry, Martinsried, Munich, Federal Republic of Germany). LN was also purchased from Bethesda Research Laboratories, Gaithersburg, MD. FN was purified from human plasma as previously described (70).

### Purification of FN Fragments

FN fragments containing the collagen-binding domain (70 and 40 kD) were generated by digestion of FN (3 mg/ml in PBS) at 22°C with 10 µg/ml chymotrypsin (type II; Sigma Chemical Co., St. Louis, MO) for 7 min. The digestion was terminated by the addition of phenylmethylsulfonyl fluoride (1 mM) to the incubation mixture. The digest was subjected to chromatography on a column of gelatin-Sepharose as described earlier (25). The gelatin-binding peptides eluted from the gelatin-Sepharose (20 mg) were finally purified by chromatography on a DEAE-Sepharose column (10 ml bed volume). The sample was applied to the DEAE column in 20 mM Tris-HCl buffer, pH 7.4, and eluted with a linear gradient (100 ml) of NaCl from 0 to 200 mM in this buffer. Under these conditions the 70-kD fragment eluted well ahead of the 40-kD fragment.

Purification of a cell-binding fragment (105 kD) and a heparin-binding fragment (31 kD) from chymotrypsin-digested FN has previously been described (74). The precise localization of the various fragments within the FN molecule has recently been described in detail elsewhere (43, 60, 74).

### Antibodies against FN Fragments

Antibodies against the 31-kD fragment and the NH<sub>2</sub>-terminal 29-kD fragment of FN (17), respectively, were raised in hens and purified from egg yolk as previously described (74). Potential antibodies in the IgG preparations possessing cross-reactivity with other parts of the FN molecule were removed by chromatography on columns of Sepharose conjugated with the appropriate FN fragments. Thus, anti-31-kD IgG was absorbed against the 29- and 105-kD fragments, while anti-29-kD IgG was absorbed versus the 31- and 105-kD fragments.

Antibodies specific for the cell-binding FN fragment were obtained by affinity chromatography of rabbit anti-FN IgG (26) on a column of Sepharose conjugated with 105-kD fragment. The preparations of IgG were

found to react specifically with 31-, 29-, and 105-kD fragments, respectively, in enzyme-linked and Western blot analysis.

### Preparation of Culture Substrates

Culture substrates composed of the selected ECM molecules were prepared in tissue culture dishes (35 mm; Nunc, Roskilde, Denmark) by coating the middle area (1 cm<sup>2</sup>) of the dishes with 100 µl of 0.01–1,000 µg/ml of the proteins dissolved in 0.05 M CO<sub>2</sub>-buffer, pH 9.6. After surface coating, dishes were extensively washed with PBS and uncovered areas were blocked with 2% BSA (type IV; Sigma Chemical Co.) in the CO<sub>2</sub> buffer for 60 min at 37°C. In addition, three different types of dual substrates, composed of both ChSP and the 105-kD FN peptide, were produced. One type of substrate, having different relative amounts of the two ECM components, was generated by incubating dishes first with various concentrations of ChSP (0.1–100 µg/ml) and subsequently with 10 µg/ml of the 105-kD fragment. Alternatively, half of the surface of the dish was coated with ChSP (40–80 µg/ml) and the other half with 105-kD peptide (10 µg/ml). A third type of substrate was prepared by scraping narrow parallel tracks on a ChSP-coated dish and subsequently incubating the exposed plastic surface with 105-kD FN fragment (10 µg/ml). In all cases of double-coating possible noncovered areas were blocked with BSA.

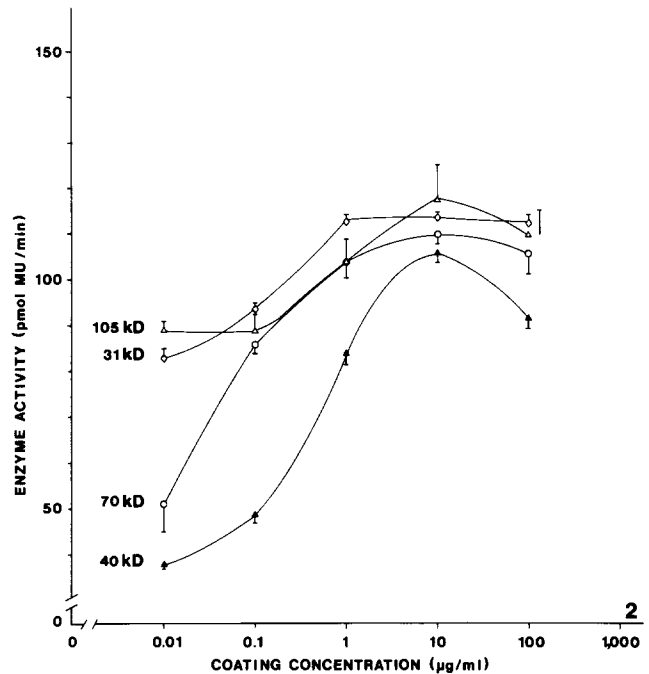
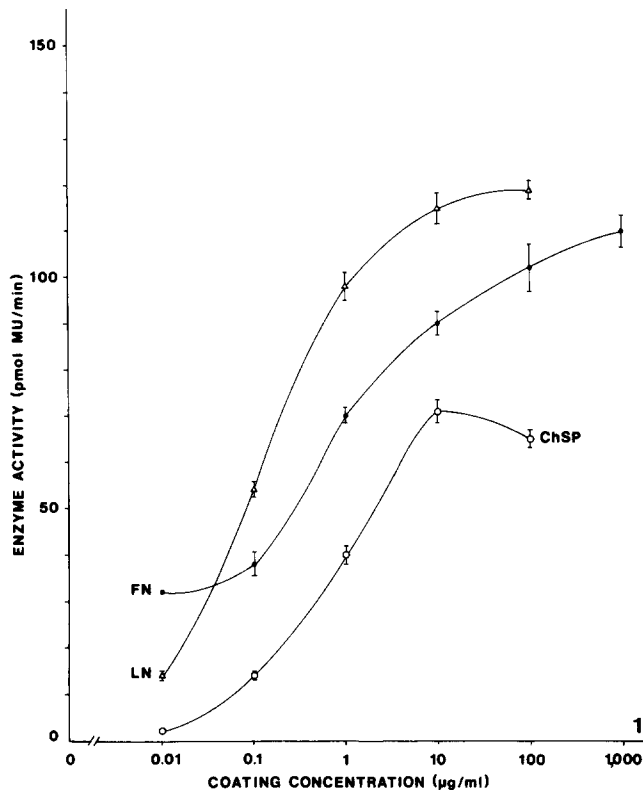
Antibodies used to block FN substrates were added at various concentrations to the FN-coated dishes, which were then incubated for 2 h at 37°C or at 4°C overnight. The binding efficiency of various anti-FN fragment antibodies to FN adsorbed onto the plastic was controlled by a two-site sandwich ELISA (35).

### Protein-binding Assay

The relative amount of material bound to the plastic dish was determined for each ECM protein according to the following procedure: 1 mg of the purified ECM proteins, apart from VN, was dissolved in 1 ml PBS, mixed with an equal volume of biotin-β-aminocaproic acid *N*-hydroxysuccinimide ester (1 mg/ml in dimethylsulphoxide; Calbiochem-Behring Corp., La Jolla, CA) and incubated for 3 h at room temperature. Biotinylated proteins were dialyzed against 0.05 M bicarbonate buffer, pH 9.6, at 4°C for 48 h in order to remove uncoupled biotin-X-NHS. 96-well Microfluor plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 100 µl of the various biotinylated proteins (0.01–100 µg/ml) in 0.05 M bicarbonate, pH 9.6, by the same procedure used for coating of culture substrates. The plates were washed with 0.02 M Tris, 0.5 M NaCl, 2 mM MgCl<sub>2</sub>, pH 7.4 (buffer A), and incubated for 15 min at 20°C with streptavidin-conjugated β-galactosidase (Bethesda Research Laboratories) diluted 1:5000 in buffer A supplemented with 0.05% Tween 20 and 0.1% BSA. The conjugate contained approximately one molecule of enzyme for each molecule of streptavidin (1,028 U of active β-galactosidase per milligram of active streptavidin). The dishes were washed with buffer A containing 0.5% Tween 20, and after 0, 12, 18, or 24 h the fluorometrical enzyme reaction was started by the addition of 0.2 mM 4-methylumbelliferyl-β-galactoside (Sigma Chemical Co.) in 0.1 M phosphate buffer, pH 7.2, containing 2 mM MgSO<sub>4</sub>, 0.4 mM MnSO<sub>4</sub>, 4 mM EDTA, 0.1% NaN<sub>3</sub> (60 µl per well). The accumulation of 4-methylumbelliferone was monitored after 0, 15, 30, and 45 min in a Microfluor reader (excitation wavelength, 365 nm; emission, 450 nm; Dynatech Laboratories, Inc.). All values obtained were expressed as picomoles 4-methylumbelliferone generated per minute. The background values (microwells coated with BSA only) were subtracted from the results shown (Figs. 1 and 2).

### Cell Culture and Migration Assay

Premigratory NC cells were co-isolated together with the underlying neural tube (NT) from 3 1/2-d-old embryos of the Mexican axolotl (*Ambystoma mexicanum*). Preparation of the embryos for microsurgery and the procedure of explantation of the neural primordia (NC-NT) were performed as previously described (51). To obtain comparable NC cell populations, the neural primordia were excised at axial levels between the 3rd and 12th trunk segments. Isolated neural primordia were rinsed for 30–45 min in the culture medium (see below) supplemented with 0.1% BSA to allow maximal recovery of the extirpated NT tissue and thereby minimize release of cell debris. NC-NT explants were then deposited onto the various ECM substrates and cultured for 18 h at 20°C in a serum-free culture solution (PL-85) composed as follows (g/liter): NaCl, 4.755; CaCl<sub>2</sub>, 0.14; Ca(NO<sub>3</sub>)<sub>2</sub> × 4 H<sub>2</sub>O, 0.080; MgSO<sub>4</sub> × 7 H<sub>2</sub>O, 0.189; Hepes, 3.570; KCl, 0.1275; Na<sub>2</sub>HPO<sub>4</sub> × 2 H<sub>2</sub>O, 0.06; NaHCO<sub>3</sub>, 0.200; and L-glutamine 50 µg/ml (pH 7.8–8.0).



**Figures 1 and 2.** Adsorption of various proteins onto tissue culture plastic. The results shown have been subtracted from the background values (equals close to 0; wells coated with only BSA). The results represent mean  $\pm$  SD of three independent analyses. The SD are not indicated unless they exceeded the width of the symbols. Unlike other proteins FN was studied at coating concentrations up to 1 mg/ml in order to approach its saturation point. The values indicate the relative amount of each protein adsorbed onto the plastic at various coating concentrations as monitored directly after the coating procedure. Identical results were obtained at measurements after 6, 12, 18, and 24 h of incubation of the substrate in buffer. Note that the absolute amount of protein bound to the plastic cannot be determined from these data.

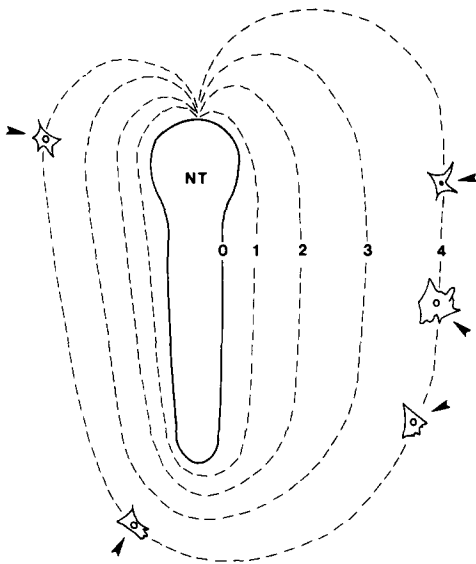
Gentamycin (50  $\mu$ g/ml) and benzylpenicillin (250 IU/ml) were used as antibiotics. After exactly 18 h of incubation (the time needed in vivo for an embryo at a premigratory NC stage to reach a slightly postmigratory developmental stage; references 40, 41, and 61) the cultures were evaluated and photographed under phase-contrast optics in an inverted microscope (Leitz, Diavert). The migratory response of NC cells to the different ECM substrates tested was estimated on the basis of two closely connected parameters: (a) maximal migration distance of discrete NC cells within the emigrated population and (b) total number of cells emigrated from the NT explant. Four (0–4) arbitrarily defined localization zones were chosen to denote the maximal distance NC cells had migrated after 18 h (Figs. 3 and 4). No mitotic activity was observed within this period of culture. The number of NC cells emigrated from the NT explant was estimated in each culture by counting individual cells in the outgrowth. To facilitate cell counting at the time of evaluation (see below), NC cultures were occasionally incubated for 30 min at 20°C with the fluorescent dye carboxyfluorescein diacetate succinimyl ester (Molecular Probes Inc., Junction City, OR) diluted 1:500 from a stock solution of 10 mM in DMSO (5). After careful rinsing in PBS, the vitally stained cells (Fig. 5) could be observed and photographed under epifluorescent illumination in a Leitz Ortholux microscope equipped with the fluorescein-specific filter set I<sub>2</sub> (Fig. 5). Cell behavior and overall rate of frontal advance of the emigrating NC cell population (46) was complementarily analyzed in several cultures by video time-lapse recording. Such recordings were performed using an inverted microscope equipped with a National system including a camera (WV-1350 AE/G), a time-lapse videotape recorder (VTR NV-8030), a time-date generator (WJ 800), and a tape (NV-P76H). Playback was on a monitor (AG3; Electrohome Inc., Stockholm, Sweden). The extent of randomness in directionality during movement was analyzed in the frontal individual cells after 12 h of culture on substrates of LN, FN, or of the 105-, 70-, 40-, and 30-kD FN fragments. The analysis of directionality was accomplished by tracing nuclear displace-

ment and migration paths of cells for a period of 1 1/2 h at 10-min intervals. The frequency of deviation from a reference migratory direction perpendicular to the NT explant was adopted to denote the degree of randomness during migration. Persistence in directionality was established by vector analysis according to a previously described procedure (16). A value of 1 represents maximal migratory directionality.

## Results

### General Observations on the Mode of Locomotion

Video time-lapse recordings showed that on all types of substrates NC cells migrated preferentially from the central portion of the NT explant after 4–6 h of culture. Cell motility was apparently initiated by blebs on the cell surface that gradually transformed into blunt projections later replaced by longer and thinner filopodial processes. Characteristically, the moving NC cells displayed multipolarity in their protrusive activity but consistently moved in the direction of a dominant projection. A transitory elongation of the cell body invariably preceded displacement. The initial migration was rapid and the leading cells that left the NT explant tended to disperse radially and form an even sheet on the dorsal side of the NT explant. Progressively, the cell mat increased in size and extended to both sides of the NT as well as to the area adjacent to the cut edges (Fig. 4). The overall



**Figure 3.** Schematic illustration of the migratory assay. From optimal representative cultures, four localization zones were arbitrarily designed. Each zone corresponds to a maximal distance of 1.2, 2.8, 4.8, and 7.6 mm, respectively, (as indicated to the right) from the NT explant. A culture was judged to show emigration corresponding to localization zones 1–4 when at least five discrete cells (arrows) could be observed at level with the boundary of the zones. Ordinarily, however, considerably more than five cells localized at the same distance from the NT explant, as emigrated NC cells formed uniformly spread sheets. NC cells were considered to be entirely impeded from emigration when <10 individual cells could be discerned beyond the edges of the NT explant (equals localization zone 0).

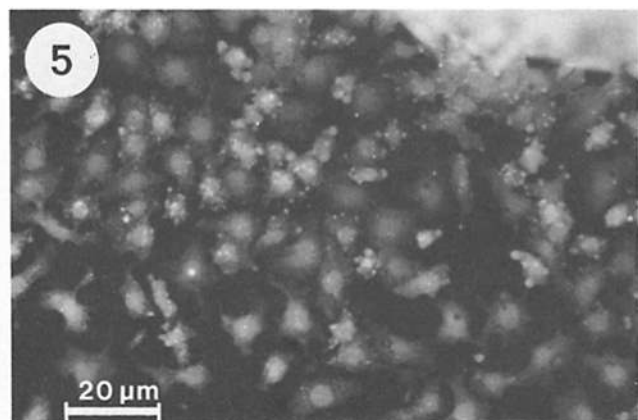
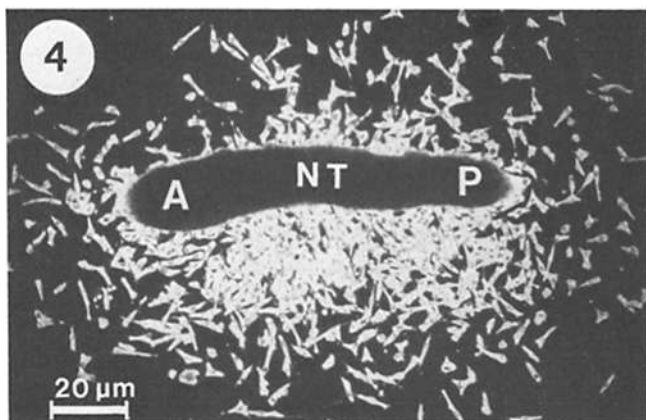
degree of cell–cell association in the established outgrowth and the shape of cells varied according to the type of substrate (see below). Although the overall direction during migration away from the NT explant was maintained throughout initial dispersion, the persistence in directionality of individual cells differed depending on the type of substrate. Directionality also seemed to be influenced by various types of cell–cell contacts resulting in momentary local paralysis

of pseudopodial activity, contact inhibition of movement, or contact-induced retraction. Thus, peripheral cells of the outgrowths were evidently polarized in their movement. Ordinarily, detachment of one cell from another was soon recompensed by association with a new neighboring cell.

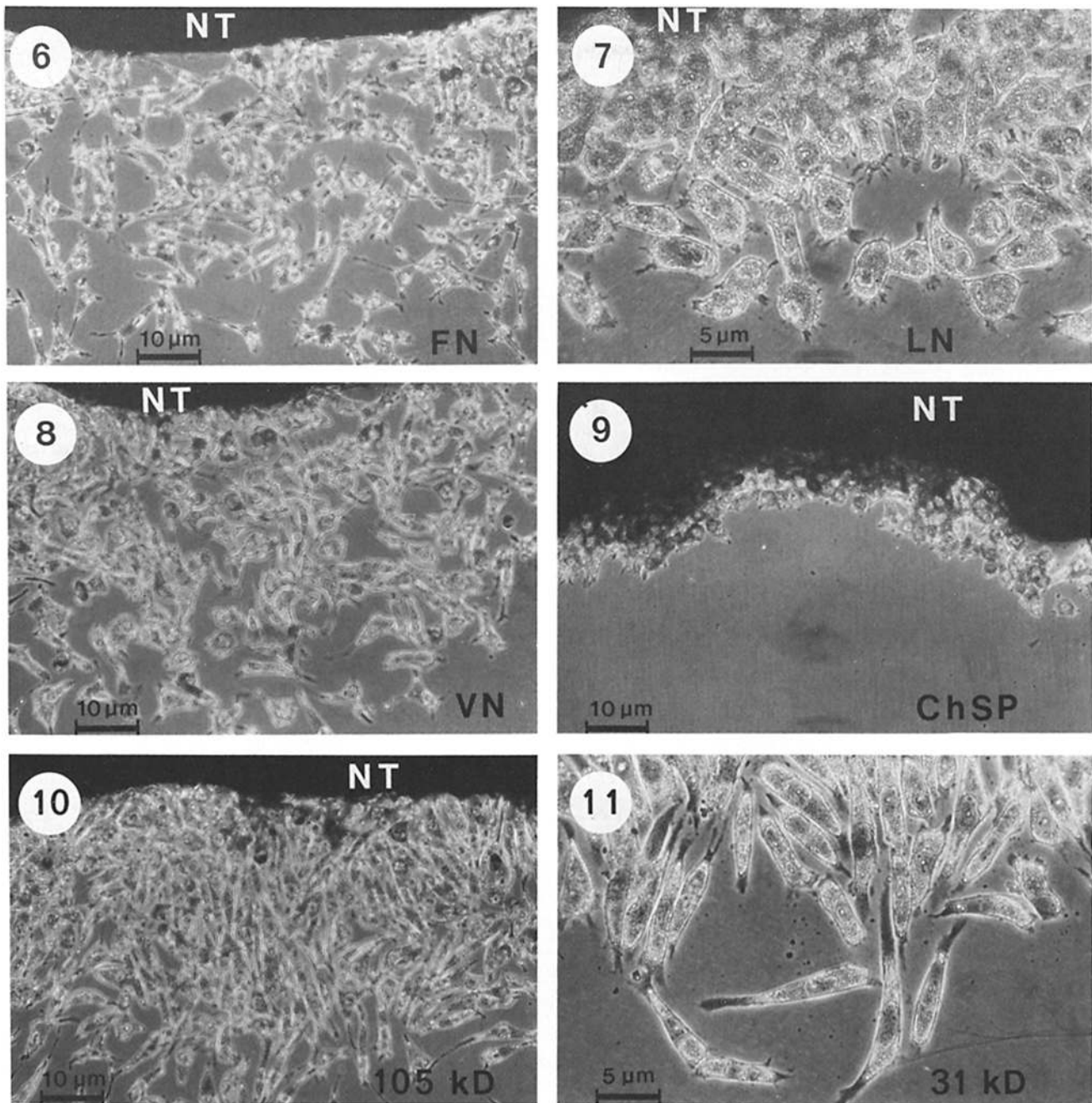
### Neural Crest Cell Migration on Cell Adhesion Proteins

The emigrating NC cell population moved rapidly on FN substrates. Cells at the leading front of the moving cell mass migrated with relatively low persistency in directionality. The average directionality ( $D$ ) was calculated at  $0.53 \pm 0.16$  (11 cells analyzed) as revealed by video time-lapse tracings and vector analysis. The degree of cell–cell association was also low (Fig. 6) and cells at the periphery of the outgrowth migrated away from each other to become markedly dispersed. Under optimal migratory conditions, i.e., on substrates coated with 0.1–1 mg FN/ml, ~14% of the emigrated cells exhibited a bipolar elongated shape characteristic of migrating NC cells (30, 46), compared with 5% on BSA substrates. The locomotory response on FN substrates was dose dependent (Table I, Fig. 12), reaching a maximum at a coating concentration of 100  $\mu\text{g}/\text{ml}$ . Reduction of the coating concentration from 100 to 10  $\mu\text{g}/\text{ml}$  caused almost a 50% reduction of cell migration (Fig. 12), even though the amount of FN bound to the plastic was only diminished by ~10% (Fig. 1). At coating concentrations <0.1  $\mu\text{g}/\text{ml}$  the migration on FN was indistinguishable from the minimal movement on BSA. When studied at its optimal coating concentration (10  $\mu\text{g}/\text{ml}$ ; Dr. K. Gebb, Pharmacia AB, Uppsala, Sweden, personal communication), VN stimulated dispersion equally as well as FN (Table I, Fig. 8).

NC cells migrated less far on LN than on FN or VN, but LN proved to be more efficacious than FN at low concentrations. A substrate produced by coating with 0.1  $\mu\text{g}/\text{ml}$  of LN sustained a pronounced dispersion comparable to the one on dishes coated with 10  $\mu\text{g}/\text{ml}$  of FN (Fig. 7). Further augmentation of the LN concentration significantly reduced the extent of dispersion (Table I, Fig. 12). On LN substrates the randomness of movement was somewhat lower than on FN ( $D = 0.68 \pm 0.15$ , 12 cells analyzed). NC cells at the margins



**Figures 4 and 5.** (Fig. 4) Micrograph illustrating a representative optimal NC cell dispersion obtained on a substrate containing the FN cell-binding region (105 kD; coating concentration, 100  $\mu\text{g}/\text{ml}$ ). *A*, anterior; *P*, posterior; *NT*, neural tube. (Fig. 5) A selected area of the same culture as shown in Fig. 4 after vital staining with carboxylfluorescein diacetate succinimyl ester and viewed under fluorescence optics. Note the minimal nuclear overlapping exhibited by migrating NC cells.



**Figures 6–11.** NC cell migration on cell adhesion proteins and ChSP. (Fig. 6) On FN (coating concentration, 100 µg/ml), NC cells migrated extensively. The cells frequently attained an elongated shape and were loosely associated with each other. (Fig. 7) On LN (0.1 µg/ml), NC cells moved less rapidly, were preferentially rounded, and were more closely connected one to another. (Figs. 8 and 9) The dispersion after 18 h of culture on VN (10 µg/ml) and ChSP (40 µg/ml) substrates, respectively. (Fig. 10) The prominent NC cell migration observed on the 105-kD FN peptide (100 µg/ml). Note the high proportion of elongated cells and, despite the large number of cells, their scattered distribution within the outgrowth. (Fig. 11) The parallel alignment of elongated cells was predominant on substrates composed of the heparin-binding FN fragment (31 kD; 10 µg/ml).

of the outgrowth tended to flatten out on the substrate and remained connected one to another (Fig. 7). About 9% of the emigrated cells were observed in an elongated phase after 18 h of culture, probably reflecting a gradual retardation of locomotion.

#### **Neural Crest Cell Migration on Proteolytic FN Fragments**

A 105-kD peptide containing the cell-binding domain of the

FN molecule was found to promote NC cell migration even better than intact FN (Fig. 13). However, changes in directionality during single cell movement occurred more frequently than on FN substrates ( $D = 0.43 \pm 0.22$ , 12 cells analyzed). Cells were found to be more scattered and exhibited a lower overall degree of cell–cell contact (Fig. 10). The percentage of elongated cells was markedly high (17%; Fig. 10).

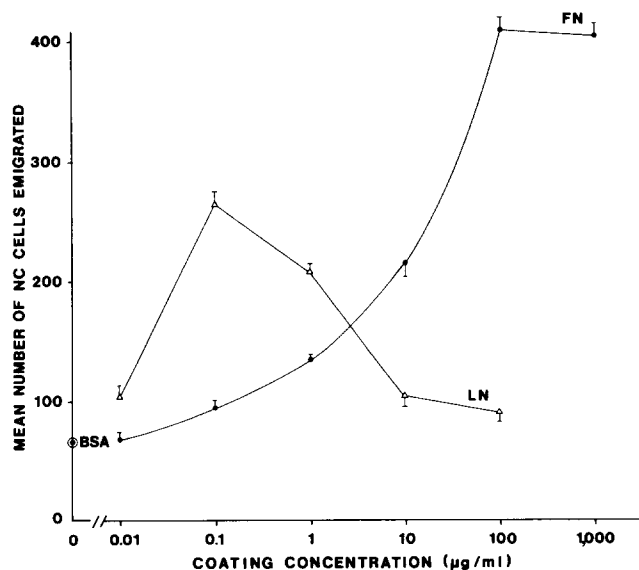
The 31-kD heparin-binding FN fragment also supported

**Table I. Neural Crest Cell Migration on Cell Adhesion Proteins**

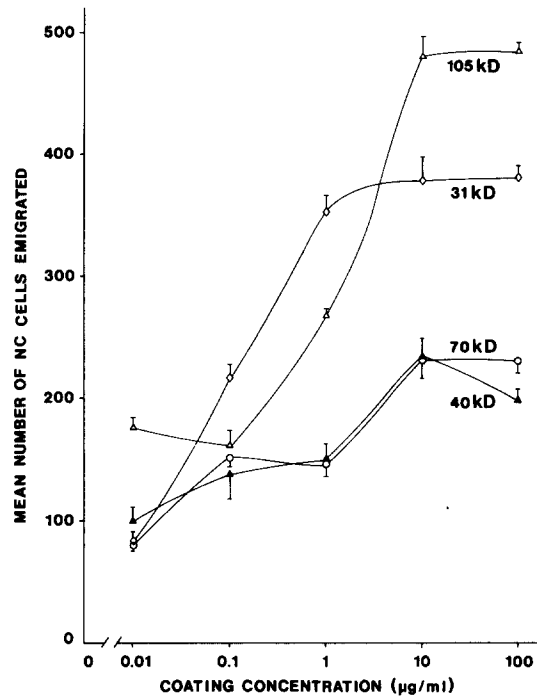
Substrate	No. of cultures	Localization zone 0-4 (mean)	No. of NC cells emigrated
BSA (control)	16	1 (1.3)	66.4 ± 17.0
FN (1,000 µg/ml)	14	4 (3.6)	406.7 ± 21.9
FN (100 µg/ml)	16	4 (3.6)	411.5 ± 20.7
FN (10 µg/ml)	16	2 (2.4)	215.3 ± 22.2
FN (1 µg/ml)	16	2 (2.0)	136.9 ± 8.2
FN (0.1 µg/ml)	16	1 (1.2)	99.4 ± 13.7
FN (0.01 µg/ml)	16	1 (1.0)	68.5 ± 11.4
VN (10 µg/ml)	12	3 (3.4)	408.6 ± 38.7
LN (100 µg/ml)	16	1 (1.3)	91.3 ± 14.5
LN (10 µg/ml)	16	2 (1.9)	104.0 ± 16.2
LN (1 µg/ml)	16	2 (2.4)	208.7 ± 15.0
LN (0.1 µg/ml)	16	3 (2.6)	204.4 ± 22.3
LN (0.01 µg/ml)	16	2 (1.8)	103.8 ± 23.0
ChSP (40-80 µg/ml)	13	1 (0.7)	17.2 ± 5.2

VN and ChSP were used at coating concentrations giving maximal adsorbance onto the plastic. The variation in migration distance (zones) is indicated by mean values in parentheses. The number of NC cells emigrated from the NT explant after 18 h of culture is indicated in the right column as mean ± SD.

prominent NC cell migration, although maximal emigration was not as extensive as that observed on the 105-kD fragment (Fig. 11). During displacement from the NT explant on the 31-kD peptide, individual NC cells showed a slightly higher degree of mean directionality than was found on FN or the 105-kD fragment ( $D = 0.59 \pm 0.17$ , 11 cells analyzed). Cell-cell associations appeared to be more numerous. Many cells stretched and aligned themselves perpendicular to the NT explant. A significantly greater portion of NC cells migrating on the 31-kD heparin-binding peptide (27%) attained an elongated shape than of cells moving on the 105-kD fragment (Fig. 11).



**Figure 12.** Dose-dependent NC cell migration on FN and LN. Mean number of NC cells emigrated from the NT explant after 18 h of culture is presented as a function of the concentration of protein used for coating of the substrates.



**Figure 13.** Dose-dependent locomotion of NC cells on substrates prepared from functionally distinct portions of the FN molecule. The cell-binding domain (105 kD), the heparin-binding domain (31 kD), the collagen-binding domain (40 kD) and the collagen-binding plus the NH<sub>2</sub>-terminal regions (70 kD) were coated onto dishes at the indicated concentrations and the numbers of NC cells emigrated were determined as described in Materials and Methods.

FN fragments composed of the NH<sub>2</sub>-terminal domain and/or the collagen-binding domain (70 and 40 kD, respectively) were weak supporters of NC cell migration. Maximal dispersion on the 70- and 40-kD peptides reached only 30% of that obtained on the 105-kD peptide. However, both molecules were able to support NC cell migration clearly distinguishable from that on BSA substrates at concentrations as low as 0.01 µg/ml. Directionality of individual cell migration was found to be higher than on LN, reaching the  $D$  values  $0.71 \pm 0.18$  (nine cells analyzed) for the 70-kD fragments and  $0.76 \pm 0.19$  (nine cells analyzed) for the 40-kD fragments, respectively. The degree of cell-cell association on these fragments was similar to that observed on the 31-kD peptide. The portion of cells exhibiting a bipolar shape was 14 and 11% for the 70- and 40-kD peptides, respectively.

Further evidence for the involvement of the cell- and heparin-binding domains in NC cell migration on FN was obtained by using antibodies against different parts of the molecule. As expected, antibodies against the NH<sub>2</sub>-terminal region of the FN molecule had no effect on NC dispersion on FN (Table II). In contrast, antibodies against the heparin-binding domain (31 kD) or the cell-binding region (105 kD) both reduced NC cell locomotion on FN. Their alteration of NC cell movement was progressively abolished at lower antibody concentrations (Table II). Used in combination on FN substrates, anti-31- and 105-kD antibodies had an additive inhibitory effect. The antibodies completely inhibited NC cell migration on the isolated 31- and 105-kD FN peptides, respectively.

**Table II. Inhibition of Neural Crest Cell Migration on FN Substrates by Antibodies against Different Domains of the Molecule**

Substrate	No. of cultures	Local-ization zone 0-4 (mean)	Emigrated
			NC cells %
FN + anti-29 kD (700 µg/ml)	16	3 (3.3)	90
FN + anti-31 kD (470 µg/ml)	16	2 (2.4)	59
FN + anti-31 kD ( 47 µg/ml)	16	3 (2.8)	51
FN + anti-31 kD (1.6 µg/ml)	16	3 (3.4)	89
31 kda + anti-31 kD (470 µg/ml)	16	1 (1.4)	19
FN + anti-105 kD (120 µg/ml)	16	2 (2.4)	58
FN + anti-105 kD ( 12 µg/ml)	16	3 (2.6)	65
FN + anti-105 kD (4.1 µg/ml)	14	3 (3.3)	84
105 kD + anti-105 kD (120 µg/ml)	10	1 (1.5)	17
FN + anti-105 kD + anti-31 kD (120 µg/ml, 47 µg/ml)	14	2 (2.0)	35

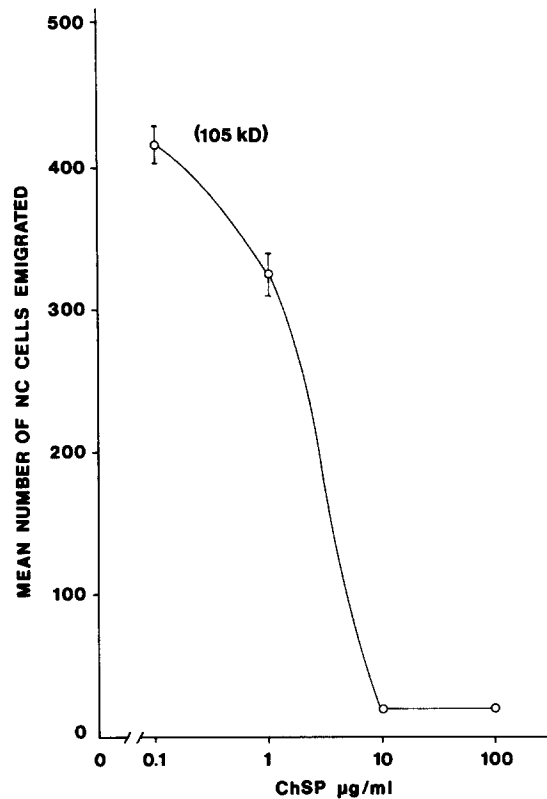
Substrates produced by coating with 100-150 µg/ml of the proteins were incubated with various concentrations of antibodies directed against specific regions of the FN molecule (see Materials and Methods). The extent of NC cell dispersion obtained after each antibody incubation is indicated as percentage of the migratory response observed in the absence of antibodies.

### Effects of ChSP and Heparin on NC Cell Migration

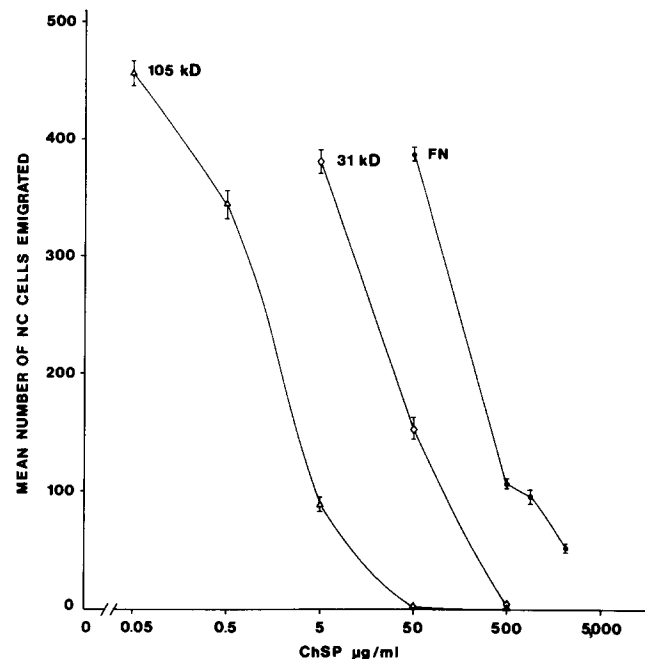
NC cells were entirely unable to migrate on substrates of ChSP (Table I, Fig. 9). On mixed substrates containing different relative amounts of the ChSP and the 105-kD peptide, the ChSP impeded emigration of NC cells from the NT explant in a dose-dependent manner (Fig. 14). When half of the culture dish was coated with the 105-kD peptide and the adjacent half with ChSP, NC cells migrated rapidly on the 105-kD fragment but stopped abruptly as they reached the ChSP-coated surface (Fig. 18). Similarly, NC cells were constrained and moved solely along the tracks of the 105-kD fragment when such were produced on ChSP substrates (Fig. 19).

ChSP was also found to interfere with NC cell migration on FN substrates when added to the culture medium. Efficient inhibition of cell dispersion on intact FN occurred at concentrations of >0.5 mg ChSP/ml (Fig. 15) while 0.05 mg/ml of ChSP was sufficient to totally block NC cell migration on the 105-kD peptide (Figs. 15-17). A 10-fold lower concentration of ChSP still prevented dramatic emigration from the NT explants, whereas further diminutions of the amount of ChSP in the medium progressively abrogated the inhibitory effect. Soluble ChSP also obstructed NC cell locomotion on substrates of the 31-kD peptide as well as of the 70- and 40-kD peptides.

To determine whether ChSP affected NC cell motility by binding to the cell surface and thereby modulating attachment of the cells to the substrate, premigratory cells were incubated in a solution of ChSP, rinsed, and then deposited on a substrate of the 105-kD peptide. Such preincubation with ChSP, however, did not alter NC cell migration (Table III) and neither did a similar preincubation of the 105-kD peptide substrates. Furthermore, addition of optimal inhibitory amounts of ChSP to NC cells that had been migrating on the

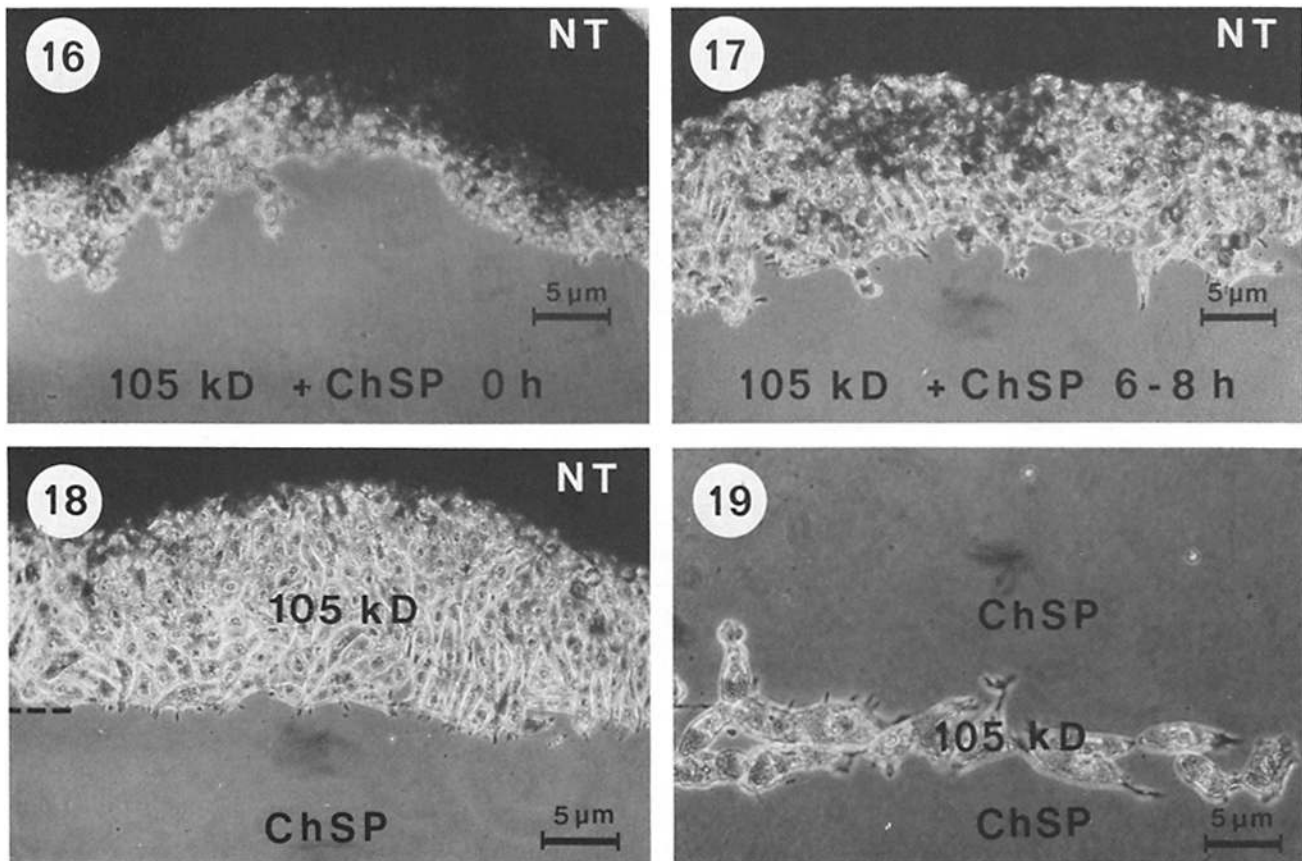


**Figure 14.** Migratory response of NC cells plated on mixed substrates containing the ChSP and the 105-kD peptides in different relative proportions. The mean number of NC cells emigrated is shown as a function of the concentration of ChSP employed in the first coating step (see Materials and Methods). SD are not indicated unless they extended beyond the width of the symbols.



**Figure 15.** Inhibited NC cell migration on substrates of FN, 105-, and 31-kD proteolytic fragments by addition of ChSP to the culture medium. Mean number of NC cells emigrated is indicated as a function of the concentration of ChSP administered. FN and proteolytic fragments were used at their optimal coating concentrations. In cases where the SD did not exceed the width of the symbols they are not indicated.





**Figures 16-19.** (Fig. 16) Inhibited NC cell migration on the 105-kD FN fragment (coating concentration, 100  $\mu\text{g}/\text{ml}$ ) by inclusion of soluble ChSP (50  $\mu\text{g}/\text{ml}$ ) from the start of the culture (0 h). (Fig. 17) Arrest of NC cell dispersion by addition of soluble ChSP (50  $\mu\text{g}/\text{ml}$ ) to NC cells that had been displacing for 8 h on a substrate identical to that in Fig. 16. (Fig. 18) NC cells moving on the 105-kD FN fragment interrupt their migration at the border of a surface coated with ChSP (80  $\mu\text{g}/\text{ml}$ ). The boundary between the two types of substrate is indicated at the middle-left side of the micrograph. (Fig. 19) NC cells proceeding along a track of the 105-kD FN fragment generated on a ChSP substrate (see Materials and Methods). The cells are restrained to the 105-kD peptide route during their emigration from the NT.

105-kD fragment for 6-8 h arrested movement (Table III, Fig. 17), without causing detachment of cells from the substrate.

Addition of heparin to the culture medium affected NC cell migration differently than did administration of ChSP. Heparin strongly inhibited the movement of NC cells on the 31-kD heparin-binding peptide, but had no effect on migration on the 105-kD fragment (Table III).

### Discussion

A large number of cell types, including NC cells, attach to and migrate extensively on FN substrates (12, 18, 42, 58). The attachment of cells to FN is confined to a limited portion of the molecule known as the cell-binding region, in which the tetrapeptide RGDS has a key role (52). Cell surface receptors mediating the attachment to FN have recently been isolated and characterized in different cell types (1, 8, 10, 20, 28, 33, 54). Antibodies against the fibroblastic FN receptor, as well as high concentrations of soluble synthetic RGDS peptides, have been reported to perturb NC cell migration in chick embryos (3, 5, 6). These observations underline the importance of FN for NC cell migration *in vivo*. Furthermore, there is evidence that migratory embryonal cells dis-

play a diffuse distribution of the FN receptor on the cell surface in apparently labile adhesions, whereas in stationary cells the FN receptor accumulates at specific cell substrate attachment sites linked to the cytoskeleton (14). A similar diffuse distribution of FN receptors has been demonstrated in virally transformed cells (9). In such cells it has also been reported that the FN receptor is phosphorylated on a tyrosine residue (23), which indicates that dynamic interactions of the receptor with extracellular FN or the cytoskeleton may be regulated by phosphorylation of the FN receptor.

In the present investigation we attempted (a) to localize the domains of the FN molecule responsible for the motility-promoting activity exerted by the intact protein and (b) to define the effects of a high molecular mass ChSP on NC cell migration on FN. These objectives were approached by using an *in vitro* migratory assay. The extent to which NC cells were capable of migrating on ECM-related substrates was established by quantifying the degree of dispersion of cells from the NT explant.

In agreement with previous findings in other systems, our results indicate that axolotl NC cells migrate extensively and equally well on substrates of intact FN or the isolated cell-binding domain of the molecule. In both cases the extent of NC cell dispersion was directly correlated to the amount of



**Table III. Inhibition of NC Cell Migration by ChSP and Heparin**

Substrate	No. of cultures	Local-ization zone 0-4 (mean)	No. of NC cells emigrated
31 kD + Heparin (105 µg/ml)*	12	1 (1.3)	51.5 ± 10.9
31 kD + Heparin (15 µg/ml)	12	2 (2.2)	254.4 ± 23.1
31 kD + Heparin (1.5 mg/ml)	12	4 (3.8)	382.3 ± 13.0
105 kD + Heparin (150 µg/ml)	10	4 (3.8)	476.9 ± 12.5
105 kD + ChSP (0.3 mg/ml) after 6-8 h of culture‡	10	2 (2.0)	152.8 ± 28.6
Preincubation of NC cells in ChSP (1.5 mg/ml) before plating on 105-kD substrate§	10	4 (3.9)	484.3 ± 13.3
Preincubation of 105-kD substrates with ChSP (1-3 mg/ml) before plating of the cells	10	4 (3.9)	482.7 ± 17.2

\* Various concentrations of heparin were added at time of cell plating.

‡ ChSP was added to NC cell cultures after 6-8 h, at time when displacement of some NC cells from the NT explant had started. The number of cells emigrated here should be compared with the emigration observed when identical amounts of ChSP were added at the start of the culture (Fig. 15).

§ Preincubation of freshly isolated neural primordia in ChSP, washing, and subsequent plating on 105-kD peptide substrates.

|| Preincubation of substrates composed of the 105-kD FN fragment followed by washing and plating of the neural primordia.

protein incorporated into the substrate. In contrast, cell migration on LN was optimal at low protein concentrations and markedly reduced on substrates with high quantities of LN. Contradictory results have previously been reported concerning the preference of NC cells for FN or LN as migratory substrates (18, 45, 58). Our results emphasize the importance of considering several quantitative parameters in these types of discriminative examinations. The significance of the differential migratory response observed at increasing substrate-bound concentrations of FN and LN is unclear, but the results may support the idea that NC cell migration occurs in the embryo preferentially along FN-rich paths, while LN-abundant areas (i.e., well-developed basement membranes) correspond to sites of arrest for the moving cells.

VN supported NC cell migration to an extent comparable to that of FN. This result establishes yet another common property of these two proteins, in addition to similarities in their domain structure (63), tissue distribution (21), and cell attachment-promoting properties (59). Two related but distinct cell surface receptors have been described for FN and VN, respectively, and have been shown to exhibit mutually exclusive reactivities for the two molecules (55). Thus it seems conceivable that both types of receptors may be present on early migratory NC cells and that on these cells they are functionally analogous.

Surprisingly, the 31-kD heparin-binding FN fragment stimulated NC cell migration almost as prominently as the 105-kD peptide. It has previously been found that the isolated heparin-binding FN domain is not capable of promoting the spread of melanoma and fibroblastic cells (36, 42, 74) but induces focal contact formation and the subsequent reassembly of cytoskeletal proteins manifested by bundling of actin

filaments (74). Indirect evidence suggests that cell surface heparan sulfate proteoglycans may mediate the interaction of fibroblasts with the 31-kD fragment (36, 37, 62, 73). Since heparin efficiently inhibited locomotion of NC cells on the 31-kD fragment (but had no effect on migration on the 105-kD fragment), it is possible that membrane-bound heparan sulfate proteoglycans could also exert receptor-like activities in early migratory NC cells. Apparently, the response of the two cell types to the 31 kD is different, but the motility apparatus seems to be affected in both cases.

Further support for a model where both the heparin-binding domain and the RGDS-containing domain contribute to the stimulatory effect of FN on NC cell migration was obtained by the use of antibodies against different parts of the FN molecule. Anti-31-kD and anti-105-kD antibodies totally blocked migration on substrates of the 31- and 105-kD fragments, respectively, but were only partially inhibitory on intact FN. Used in combination, the anti-31-kD and anti-105-kD antibodies, however, markedly reduced NC cell migration on intact FN molecule.

It has been reported that soluble high molecular mass ChSP may accelerate the speed of movement of individual avian NC cells translocating within collagen gels (67). More recently it has been shown that addition of isolated chondroitin sulfate chains perturbs amphibian pigment cell migration within collagen gels (68). In spite of these observations, it was suggested in these and other *in vivo* and *in vitro* investigations (45, 48, 50, 66, 68) that enrichment of ChSP could have inhibitory effects during NC cell migration. In this study we show that NC cells are entirely unable to migrate on substrates containing the large cartilage ChSP. By examining locomotion on substrates composed of both the ChSP and the 105-kD FN peptide in various relative proportions we found that the nonpermissive effect of ChSP on motility was correlated to the amount of ChSP incorporated into the substrate. Closely related hyaluronate-binding ChSP is synthesized by a variety of embryonic and adult cell types (22, 27, 31). It is likely that this type of proteoglycan is also present along the migratory pathways of the NC in early embryos. Exogenous administration of soluble ChSP to NC cells migrating on FN substrates caused a dramatic dose-dependent reduction of locomotion. Soluble ChSP seemed to impede further progression of NC cells on FN-related substrates even when added to already moving NC cells (Figs. 16-19). Preincubation of NC cells or FN substrates with ChSP, followed by washing, did not alter the subsequent cell dispersion on the FN cell-binding domain. Hence, the inhibitory effect of ChSP could not be due to a stable binding of the molecule to the substrate or the cell surface. A low affinity binding of the ChSP to the 105-kD fragment cannot be excluded from these results but seems unlikely since: (a) interactions of glycosaminoglycans with this region of the FN molecule under physiological conditions have not been previously described and (b) heparin had no effect on NC cell migration on the 105-kD peptide. Thus, it seems plausible that a transient, rather than a stable, interaction of the ChSP with the cell surface may be the cause for inhibited migration. Exogenously administered ChSP from cartilage has been reported to inhibit adhesion of fibroblasts to FN (56) and to collagen (32), and of NC cells to FN in the presence of collagen (44). In our assay, cells arrested by the addition of ChSP never detached from the substrate, but some of them

attained a more rounded shape (Figs. 16 and 17) suggesting a partial decrease in cell substrate adhesion (67). It remains unclear whether the inhibitory effect of ChSP on NC cell motility is solely due to local alterations of cell substrate contacts, presumably involving perturbation of FN receptor functions, or whether ChSP transduces specific intracellular signals directly affecting the motility apparatus.

To conclude, our observations indicate that the inhibitory effect of high molecular mass ChSP on NC cell migration *in vivo* may counteract the motility-promoting activity of FN and may thereby influence directionality and final localization of NC cells. Thus it is important that further study be devoted to the detailed mechanisms for the inhibitory effect on cell migration as well as for the regulation of the extracellular local accumulation of ChSP along NC migratory pathways.

We are indebted to Drs. Bruce Caterson, Erkki Ruoslahti, Ulf Lindahl, and Rupert Timpl for providing the various ECM components. We would also like to thank Stine Söderström, Lars-Erik Jönsson, Vibeke Nilsson, and Henrik Olsson for technical assistance; Lena Lärkfors for advice on the procedure for the protein-binding assay; and Drs. Jan Löfberg, Marianne Bronner-Fraser, and Lena Kjellen for critical readings of the manuscript.

This study was supported by grants from the Swedish Natural Science Research Council (B-BU 3810-100), Medical Research Council (07147), Gustave V:s 80-årsfond, and Torsten & Ragnar Söderberg Foundation.

Received for publication 20 October 1986, and in revised form 29 May 1987.

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