Biologically Active Synthetic Peptides as Probes of Embryonic Development: A Competitive Peptide Inhibitor of Fibronectin Function Inhibits Gastrulation in Amphibian Embryos and Neural Crest Cell Migration in Avian Embryos

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ABSTRACT   We describe a new method for analyzing embryonic events dependent on a specific peptide recognition signal. A short, specific amino acid sequence in fibronectin has been implicated as a recognition site in fibronectin-mediated interactions. Fibroblast adhesion to fibronectin is competitively inhibited by certain synthetic peptides, including the decapeptide Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro, which appears to contain the cell recognition sequence. We found that this peptide inhibited both amphibian gastrulation and avian neural crest cell migration in vivo, as well as the attachment and migration of neural crest cells in vitro. These processes are major cell migratory events previously suggested to involve fibronectin. Negative controls included another conserved fibronectin peptide from the collagen-binding region containing the sequence Cys-Gln-Asp-Ser-Glu-Thr-Arg-Thr-Phe-Tyr and another peptide. Our results demonstrate the feasibility of using synthetic peptides directed at recognition sites in extracellular proteins as probes of morphogenetic processes, and they provide further support for the hypothesis that fibronectin is involved in gastrulation and neural crest cell migration.

Morphogenetic events in embryonic development appear to require the precise, sequential functioning of specific macromolecules. The developmentally regulated appearance and disappearance of a molecule has often been correlated with a possible developmental role for the molecule (9, 10, 17, 37). However, the direct demonstration of causal roles for specific molecules in vertebrates has been hampered by difficulties in specifically ablating individual molecules to be able to prove causation.

Treatment with antibodies is the most obvious approach to obtaining specific inhibition of the function of a molecule. For example, neurite fasciculation can be prevented in vitro by the presence of monovalent antibodies to the neural cell-adhesion molecule (N-CAM)[1] (4, 33). Migration of external

granule cells is inhibited in organotypic cultures of cerebellum by the addition of antibodies to the cell surface L1 glycoprotein (24). Injection of anti-N-CAM antibodies into the eye cup of avian embryos causes misrouting of optic nerve axons (36). In *Xenopus* tadpoles, local application of anti-N-CAM antibodies to the tectum disrupts retinotectal projections (14). Microinjection of antibodies to fibronectin has suggested a role for fibronectin in amphibian gastrulation (2).

All of these types of antibody inhibition experiment can strongly suggest a specific function for a molecule, but they suffer from the theoretical limitations of an immunological approach. That is, it may never be possible to prove definitively that the effect of an antibody is due to direct inhibition or ablation of a molecule's function, rather than being due to

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[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; N-CAM, neural cell-adhesion molecule; P1, Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro; P2, Gly-Arg-Gly-Asp-Ser-Pro-Cys; P3, Cys-Gln-Asp-Ser-Glu-Thr-Arg-Thr-Phe-Tyr.

deleterious effects of the antigen-antibody complex itself, or inhibition of another process because of antibody cross-reaction with another regulatory or structural molecule present at low concentrations, or other indirect effects.

We suggest a complementary approach to this problem: the use of a synthetic peptide that competitively inhibits the recognition system of a molecule involved in embryonic development. Such peptides would be expected to diffuse more readily through embryonic tissues than antibodies, and to function by a different type of inhibitory mechanism than immunological inhibitors. Such peptides should provide an independent approach to evaluating the role of a specific molecule in development.

We have used two very different experimental systems, amphibian gastrulation and avian neural crest cell migration, to test the feasibility of this peptide inhibitor approach. Although these two systems provide differing views of the mechanism and sequelae of embryonic cell movements, previous studies have suggested that these and certain other morphogenetic events require the multifunctional glycoprotein fibronectin for cell migration (1, 5, 7, 8, 18–20, 23, 32, 38, 43). Certain synthetic peptides of fibronectin appear to inhibit the mechanism by which cells recognize fibronectin, and they block its in vitro functions in assays for fibroblast adhesive activity using NRK (normal rat kidney) cells (30), as well as BHK (baby hamster kidney) or CHO (Chinese hamster ovary) cells (44). In the latter study, a conserved decapeptide sequence from the cell-binding region of fibronectin was found to be a noncytotoxic, competitive inhibitor of fibronectin function on artificial plastic or collagenous substrates, whereas another completely conserved decapeptide sequence had no inhibitory activity (44). We now report that these peptides can be used to probe the possible involvement of this fibronectin recognition sequence in gastrulation and in the migration of neural crest cells in vivo and in vitro.

MATERIALS AND METHODS

Peptides

The fibronectin synthetic peptides Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro (P1), Gly-Arg-Gly-Asp-Ser-Pro-Cys (P2), and Cys-Gln-Asp-Ser-Glu-Thr-Arg-Thr-Phe-Tyr (P3) were purchased from Peninsula Laboratories, Inc. (Belmont, CA) and were purified and characterized as described (44). Purified ACTH peptide (4–11) was also from Peninsula Laboratories. P1 corresponds to a highly conserved, hydrophilic sequence from the cell-binding site of fibronectin and the P2 sequence is closely related to P1; in contrast, P3 is a hydrophilic sequence from the collagen-binding domain that is identical in chicken and bovine fibronectin sequences.

Monoclonal Antibody NC-1

An IgM monoclonal antibody that identifies migrating avian neural cells was obtained after immunization with quail ciliary ganglia from 8-d-old embryos (41, 42). This antibody recognizes a carbohydrate epitope at the surfaces of all migrating neural crest cells, as well as on the neurons and glia of the central and peripheral nervous system and on a subpopulation of leukocytes (40). NC-1 was coupled directly to fluorescein isothiocyanate as previously described (40).

Amphibian Embryos

Pleurodeles waltlii eggs were collected from natural matings during the breeding season. Different batches of eggs were pooled, and the eggs were manually dejellied and allowed to develop at 18°C in sterile Steinberg's solution. Staging was performed according to Gallien and Durocher (15). Micropipets were produced from 100-μl disposable pipets (Drummond Scientific Co., Broomall, PA) pulled in a Leitz (Weitzlar, Federal Republic of Germany [FRG]) horizontal pipet puller. They were broken to a diameter of ~20 μm with watchmaker's forceps under high magnification using a stereo microscope.

Microinjections were performed at blastula and early gastrula stages. Each embryo was injected with 200 nl of 10 or 20 mg/ml solutions of P1, P2, or P3 peptides, which were the lowest initial concentrations at which complete, synchronous blockade of gastrulation was observed; the ACTH fragment and the Steinberg's solution alone were used as controls. Injected embryos were fixed 6 or 24 h later. Blockage of gastrulation was characterized by the failure of embryos to internalize the endodermal mass corresponding to the vegetal hemisphere. The vegetal region is readily identified because it is nonpigmented and contains large cells, whereas the animal hemisphere is pigmented. Embryos were scored by visual inspection under a dissecting microscope, supplemented by examinations of transverse sections.

For scanning electron microscopy, embryos were fixed for 1.5 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and postfixed for 1 h in 1% OsO₄ in the same buffer. Embryos were dehydrated in a graded ethanol series, then substituted with amyl acetate. Some embryos were sectioned into halves at the mid-sagittal plane with tungsten needles. Specimens were critical-point dried, coated with gold, and examined with a JEOL JSM-35 scanning electron microscope at 25 kV.

Quail Embryos—In Vitro Culture

ISOLATION OF NEURAL PRIMORDIA: Neural primordia (neural tube and neural crest) were isolated from 2.5-d-old quail embryos (25- to 30-somite stage) as described by Rovasio et al. (32). The caudal 6-somite region of embryos was excised by a scalpel. The trunk fragments were incubated for 30–60 min at room temperature with 500 U/ml Dispase (Godo Shusei, Tokyo, Japan) in Dulbecco's modified Eagle medium (DME, Gibco Ltd., Paisley, Scotland). The enzyme was inactivated by dilution with excess DME according to the manufacturer's instructions. The neural tube segments that contained neural crest cells were freed of ectoderm, somites, endoderm, and notochord with a pair of sharpened tungsten needles under a stereo microscope. The isolated neural tube fragments were maintained in DME supplemented with 1% fetal calf serum (Gibco, Ltd.) to prevent attachment to the bottom of dishes.

NEURAL CREST CELL CULTURES: 20 μl of human plasma fibronectin (12 μg/ml in DME) purified from outdated plasma as previously described (32) was incubated on a Falcon petri dish (1008; Falcon Labware, Oxnard, CA) in an air-CO₂ incubator at 37°C overnight. The outline of the area covered with the fibronectin solution was marked with indelible ink, and the dishes were then rinsed twice with PBS. A 4-mm-diameter polyethylene tube was mounted with silicon grease on the fibronectin-coated surface, and the neural primordia were introduced into the polyethylene well filled with 50 μl of DME supplemented with 10% fetal calf serum from which fibronectin had been removed by a gelatin-Sepharose affinity column (11). The dorsal side of the neural tube was placed in contact with the substratum to facilitate crest cell emigration.

Neural crest cells were able to emigrate as a monolayered cell sheet on substrates coated with fibronectin. The minimal concentration of fibronectin solution for coating dishes that still permitted maximal migration was determined to be 12 μg/ml; i.e., higher concentrations of fibronectin did not elicit any further increase in the area covered by the halo of emigrating cells. No migration was observed in the absence of fibronectin.

Cultures were incubated for 5 h in an air-CO₂ incubator at 37°C to permit crest cells to migrate out of the neural tube onto the fibronectin substrate. The following aliquots of peptide stock solutions were then added to the standard 50-μl culture solutions to yield the indicated final concentrations of peptide: 10 μl of 18 mg/ml peptide (final concentration of 3 mg/ml), 5 μl of 18 mg/ml (1.6 mg/ml), 5 μl of 9 mg/ml (0.8 mg/ml), 5 μl of 4.5 mg/ml (0.4 mg/ml), 5 μl of 2.25 mg/ml (0.2 mg/ml), or 5 μl of DME without peptide (control). For reference, 1.6 mg/ml of peptide P1 is 1.6 mM, compared to 1.3 mM for peptide P3. After a further 12 or 16 h of incubation, cultures were fixed with 2.5% glutaraldehyde solution in 10 mM HEPES-buffered saline, pH 7.4. The petri dishes were then completely filled with PBS in order to produce a planar surface above the wells suitable for phase-contrast microscopy. To obtain quantitative dose-response data, we photographed cultures with phase-contrast optics at 12 h after adding the peptides. After 100-fold enlargement of the image onto uniform-thickness paper, the total area of cell migration was determined for each culture by cutting out and weighing the area covered by the halo of migratory cells (minus the area covered by the neural tube). Results were expressed as area (mm²) per length (mm) of neural tube; in these cultures, 1 mm corresponded to approximately six somites.

Chick Embryos—In Vivo Microinjection

Chicken eggs containing embryos of 5 to 10 somites were windowed over the air space. Embryos were visualized by subblastodermal injection of drawing ink (Pelican waterproof, Hanover, FRG). Several drops of Tyrode's solution were added and a small hole was torn in the vitelline membrane with finely sharpened watchmaker's forceps. Embryos were injected with 2–5 nl of a 10

mg/ml peptide solution containing phenol red using a 20-μm diameter micropipet. This quantity of P1 peptide was the lowest that produced highly reproducible inhibition of crest cell migration; lower concentrations yielded variable results. In 5-somite stage embryos, the peptides were injected into the cell-free space into which crest cells would shortly thereafter migrate. The windows were covered with cellophane tape and the eggs were allowed to develop for an additional 8–9 h in a humidified forced-draft egg incubator (37°C). The embryos were cut from the yolk and fixed in Bouin's before embedding in paraffin. Sections cut at 6.5 μm were deparaffinized by brief exposure to toluene, passed through a graded ethanol series to PBS, and stained for 1 h with a 1:400 dilution of a 5 mg/ml solution of NC-1 directly coupled to fluorescein isothiocyanate. After a 15-min PBS wash, sections were mounted in 90% glycerol in PBS supplemented with p-phenylenediamine (1 mg/ml) and examined as previously described (40).

RESULTS

Inhibition of Gastrulation in Urodeles

In amphibian embryos, previous studies have documented the formation of an extracellular matrix containing fibronectin on the inner surface of the blastocoel before gastrulation, followed by highly active cell migration on this substrate during gastrulation (Fig. 1) (1, 23). Microinjection of antifibronectin antibodies at blastula or early gastrula stages produces a complete arrest of invagination (2). We performed similar experiments with synthetic peptides from two functional domains of fibronectin that are implicated in its binding to cells or to collagen; the latter protein is another possible component of the extracellular matrix of early amphibian embryos.

Fig. 2A is a scanning electron micrograph of a control $P.$ $waltlii$ embryo injected 6 h previously with Steinberg's solution, then fixed and sectioned sagittally. In such control embryos, as in untreated embryos, presumptive mesodermal cells migrated dorsad on the inner surface of the blastocoel roof. Endodermal and mesodermal cells contributed to the formation of the archenteron. When gastrulation occurred normally (Fig. 2B), the embyro remained spherical with a smooth, pigmented ectodermal surface; the last endodermal cells to invaginate formed a yolk plug.

Embryos injected at the blastula stage with the P1 peptide displayed a strikingly abnormal morphology. 24 h after the injection (Fig. 2C), the animal half was severely distorted by a series of deep ectodermal furrows. In the vegetal region, a blastopore-like structure remained around the egg circumference, and presumptive mesodermal and endodermal cells developed an everted, oversized, nonpigmented yolk plug. All of the embryos injected at this stage with P1 at an initial concentration of 10 mg/ml displayed similar defects (Table I).

A sagittal section through an embryo injected with P1 shown in Fig. 2D displays early signs of gastrulation, since a blastopore slit had formed; however, only one cavity corresponding to the blastocoel was visible in all of the experimental embryos examined (Fig. 2D). No migrating cells were found on the blastocoel roof along the basal surface of the ectoderm; however, rounded cells were dispersed along the blastocoel floor in contact with endodermal cells. The morphology of the ectoderm differed strikingly from that of control embryos; its external surface area was considerably increased through multiple foldings of the simple cell sheet (Fig. 2D). The inner surface facing the blastocoel still appeared relatively smooth, but many of the ectodermal cells became included into the highly convoluted ectodermal cell layer. In contrast, at the vegetal pole, the external surface of the endoderm remained smooth (Fig. 2D).
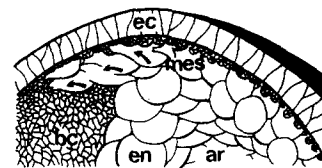

FIGURE 1   Fibronectin and cell migration in amphibian gastrulation. A current model of amphibian gastrulation based on recent immunofluorescence and scanning electron microscopic studies. Schematic drawing of part of the blastocoelic roof at the level of the migration front. Mesodermal cells are located along the basal surface of the ectoderm and above endodermal cells. The roof of the blastocoel (the only cavity shown in the drawing) is covered with a dense meshwork of fibronectin-containing fibrils (#). Adherence and active migration of mesodermal cells is thought to occur through cell surface receptors of filopodia binding to fibronectin present in the three-dimensional, non-oriented fibrillar matrix (arrows indicate the direction of active cell migration).

Table I summarizes the results obtained at two different embryonic stages. At an initial concentration of 10 mg/ml, P1 produced a complete, synchronous arrest of gastrulation within 6 h after injection. This blockage of gastrulation was still obvious at 24 and 48 h; after 48 h, the ectoderm had differentiated into typical ciliated epidermis, but the embryos lacked a neural plate. The embryos degenerated without any evidence of gastrulation after 3 d. Microinjection of a twofold higher concentration of P1 (20 mg/ml) did not result in significant differences. However, microinjection of 5 mg/ml P1 produced results varying from egg to egg: a majority (about three quarters) were able to gastrulate on time, whereas a quarter displayed a delay in development of several hours. Nevertheless, after 24 h, all of the embryos were similar in appearance, and all displayed a normal neural plate.

The P2 peptide produced full inhibition at a concentration of 20 mg/ml; at 10 mg/ml, only one third of the embryos were blocked (Table I). The P3 peptide from the collagen-binding domain, chosen like P1 for its highly conserved sequence in vertebrates and its hydrophilicity, did not perturb the migration of mesodermal cells at any concentration tested. The same negative results were obtained with a similar-sized irrelevant peptide from ACTH (Table I). It should be noted that these concentrations of injected peptides are initial values, and that the final concentrations are much lower after the injected solutions are diluted into the varying volumes of blastocoelic fluid in each of the eggs. For reference, the molarity of a 10 mg/ml stock solution of P1 is 10.0 mM, compared to 14.5 mM for P2 and 8.0 mM for P3.

Inhibition of Avian Neural Crest Cell Migration In Vitro

Neural crest cells from avian embryos can be cultured routinely in vitro by explanting a neural tube segment from the most caudal region of the embryo. Pure fibronectin or fibronectin-containing extracellular matrices provide the best substrates for both attachment and migration of these neural crest cells (13, 27, 28, 32). In the culture system used here, neural crest cells migrated from neural tube explants in a consistent manner only when the bacteriological plastic substrate was precoated with fibronectin at a concentration of 12 μg/ml or higher.

Fig. 3, A and B, shows that the halos of migrating crest cells around the neural tubes increase dramatically between 5 and
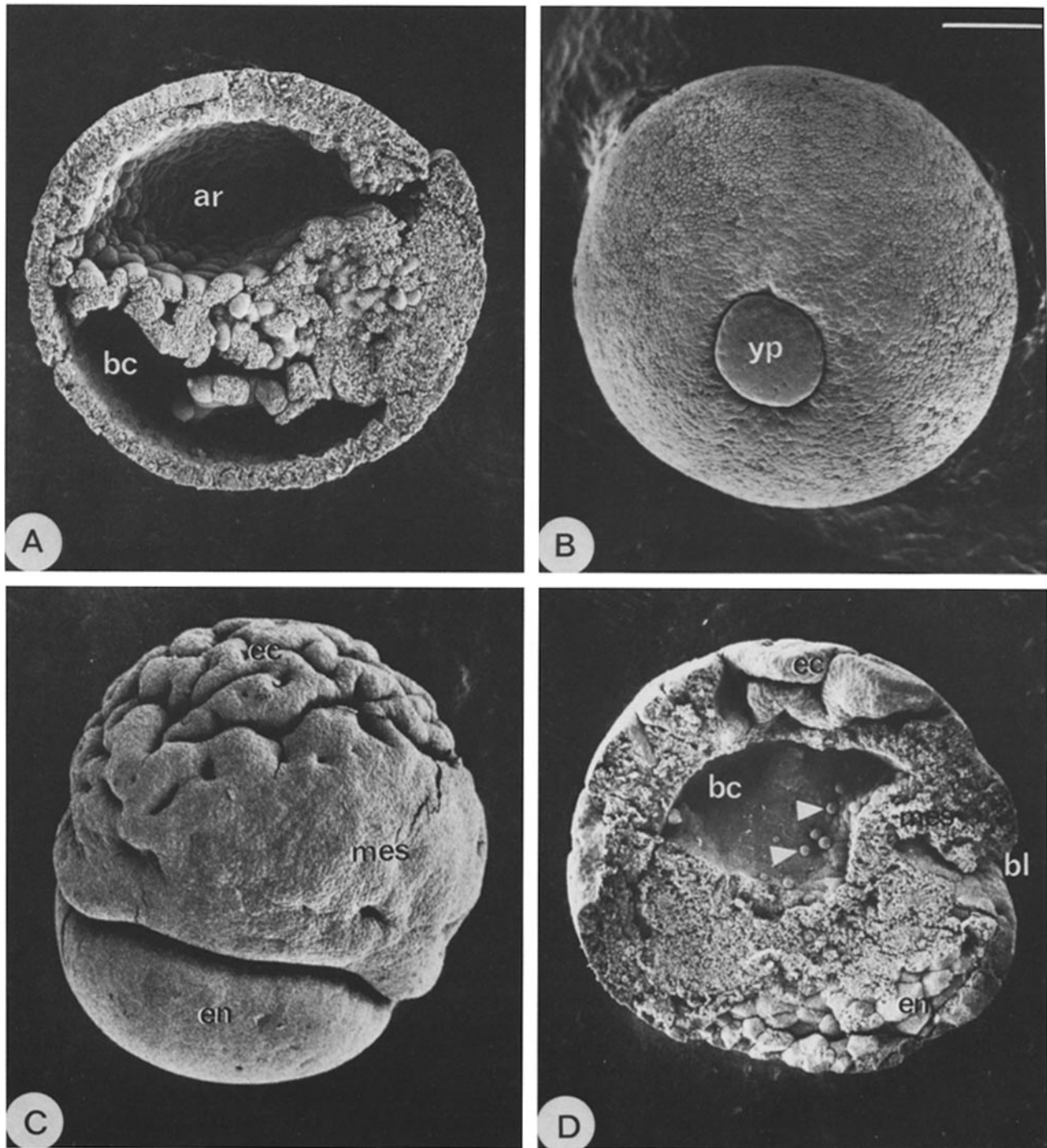
FIGURE 2   Scanning electron micrographs of normal and arrested gastrulation of *P. waltlii* embryos. (*A*) Sagittal section of a mid-gastrula embryo injected with Steinberg's solution 6 h before fixation (control). During gastrulation, mesodermal cells migrate under the ectodermal cell layer, and together with the endodermal cells obliterate the blastocoel cavity (*bc*). The latter is displaced ventrally, while the archenteron (*ar*) develops dorsally. (*B*) At the end of gastrulation, a similar embryo exhibits a yolk plug (*yp*) corresponding to the last cells to invaginate. (*C*) An embryo injected with P1 peptide (10 mg/ml) at the blastula stage and then fixed 24 h later. When compared with *B*, inhibition of gastrulation is clearly discernible. The embryo displays a highly convoluted ectodermal cap (*ec*) with deep furrows, associated with a voluminous, noninvaginated mesodermal (*mes*) cell mass. The endodermal mass (*en*) remains segregated on the other side of the slit. (*D*) Sagittal section of an embryo corresponding to *C*. Mesodermal cells (*mes*) did not migrate along the blastocoel roof; isolated cells (arrowheads) are found on the blastocoel floor, near the site of invagination. Despite the presence of a small blastopore (*bl*), the archenteron did not form; therefore only one cavity, corresponding to the blastocoel, occupies the central part of the embryo. Note the highly convoluted aspect of the ectodermal cap (*ec*); furrows are mostly restricted to the external part of the blastocoel roof, which becomes much thicker than in controls. Bar, 200 μm. × 40.

21 h of culture. Within the halo, crest cells are closely juxta-posed to each other and often overlap, whereas at the periph-ery (Fig. 3*C*) some of the cells migrate in advance of the leading edge. Crest cells in this culture system are well spread and exhibit a stellate morphology with many thin filopodia. When P1 peptides were added to the medium after 5 h of

Microinjection of Synthetic Peptides into the Blastocoelic Cavity of P. waltlii Embryos

	Stages	6 h		24 h	
		Normal	Blocked	Normal	Blocked
		%		%	
P1 (10 mg/ml)	Blastula	—	100	—	100
	Early gastrula	—	100	—	100
P1 (20 mg/ml)	Blastula	—	100	—	100
	Early gastrula	—	100	—	100
P2 (10 mg/ml)	Blastula	68	32	68	32
	Early gastrula	70	30	70	30
P2 (20 mg/ml)	Blastula	—	100	—	100
	Early gastrula	—	100	—	100
P3 (10 mg/ml)	Blastula	100	—	96 (4)	—
	Early gastrula	100	—	96 (4)	—
P3 (20 mg/ml)	Blastula	100	—	88 (12)	—
	Early gastrula	100	—	88 (12)	—
ACTH (10 mg/ml)	Blastula	100	—	100	—
	Early gastrula	100	—	100	—
ACTH (20 mg/ml)	Blastula	100	—	100	—
	Early gastrula	100	—	100	—
Steinberg	Blastula	100	—	100	—
	Early gastrula	100	—	100	—

50 embryos were injected in all experiments. In some injected embryos, partial necrosis was observed after 24 h (percent indicated in parentheses).

culture at 0.2–3.0 mg/ml (Fig. 3D), a drastic reduction in the final size of the halo of crest cells was noted. This effect was observed consistently when the substrate was coated with the standard fibronectin concentration (12 µg/ml). Crest cells that had attached and started to migrate progressively rounded up and either detached or formed two- and three-dimensional aggregates in the neighborhood of the neural tube. This situation is reminiscent of the behavior of crest cells cultured on thermally denatured fibronectin (32). In contrast, P3 at the same concentrations lacked this inhibition of crest cell migration (Fig. 3E). The morphology of P3-treated cells differed slightly in that crest cells appeared more epithelial-like.

Fig. 4 provides a quantitative analysis of the effects of peptides on neural crest cell migration in vitro. The total area covered by migrating crest cells was determined for each culture in triplicate sets treated with increasing concentrations of P1. Inhibition became half-maximal at ~0.4 mg/ml (Fig. 4). In contrast, peptide P3 from the collagen-binding domain was without effect at even 1.6 mg/ml (Fig. 4).

Inhibition of Neural Crest Cell Migration In Vivo

Fig. 5A shows the normal distribution of neural crest cells in a histological section at the level of the mid-mesencephalon in an 11-somite chick embryo, as indicated by staining with monoclonal antibody NC-1, which identifies migrating crest cells.

The neural crest cells have migrated into a large, cell-free, hyaluronate-rich space at this level (31) into which, in this case, a PBS solution has been injected. The effect of the 2–5-nl injection is minimal or nonexistent, as this and other sections are indistinguishable from similar sections from non-

experimental embryos (Fig. 5B) (41).

Fig. 5, B–D, shows transverse sections taken from a serially sectioned 11-somite chick embryo injected within the cell-free space with the P1 peptide at the 5-somite stage. The P1 peptide retarded the forward advance of the crest cellular stream at the level of the mesencephalon bilaterally, with the most prominent effects at the site of injection (Fig. 5C). Moreover, neural crest cells appear to have been forced into the lumen of the neural tube. However, only the most ventral portion of these misplaced crest cells were stained with the NC-1 monoclonal antibody; in normal cases, the NC-1 epitope appears only after crest cells have emigrated from the neural tube (41, 42). The areas affected by the peptide included both sides of the embryo to a distance of ~200 µm on either side of the point of injection, as judged by the partial recovery of neural crest cell migration (Fig. 5, B and D). Highly consistent patterns of inhibition were obtained in a total of 20 embryos injected in this manner.

DISCUSSION

Direct proof of the role of any specific molecule in vertebrate embryonic development has been difficult to obtain. Recent approaches include obtaining fortuitous retrovirus insertion mutations (34) and microinjection of specific antibodies (2, 4, 14, 36). We suggest a complementary approach based on the microinjection of a specific molecular recognition sequence, to competitively inhibit the function of molecules that depend on this sequence.

The adhesive function of fibronectin in fibroblasts is dependent on a cell-recognition sequence (cell-attachment determinant) that may be as small as four to five amino acids
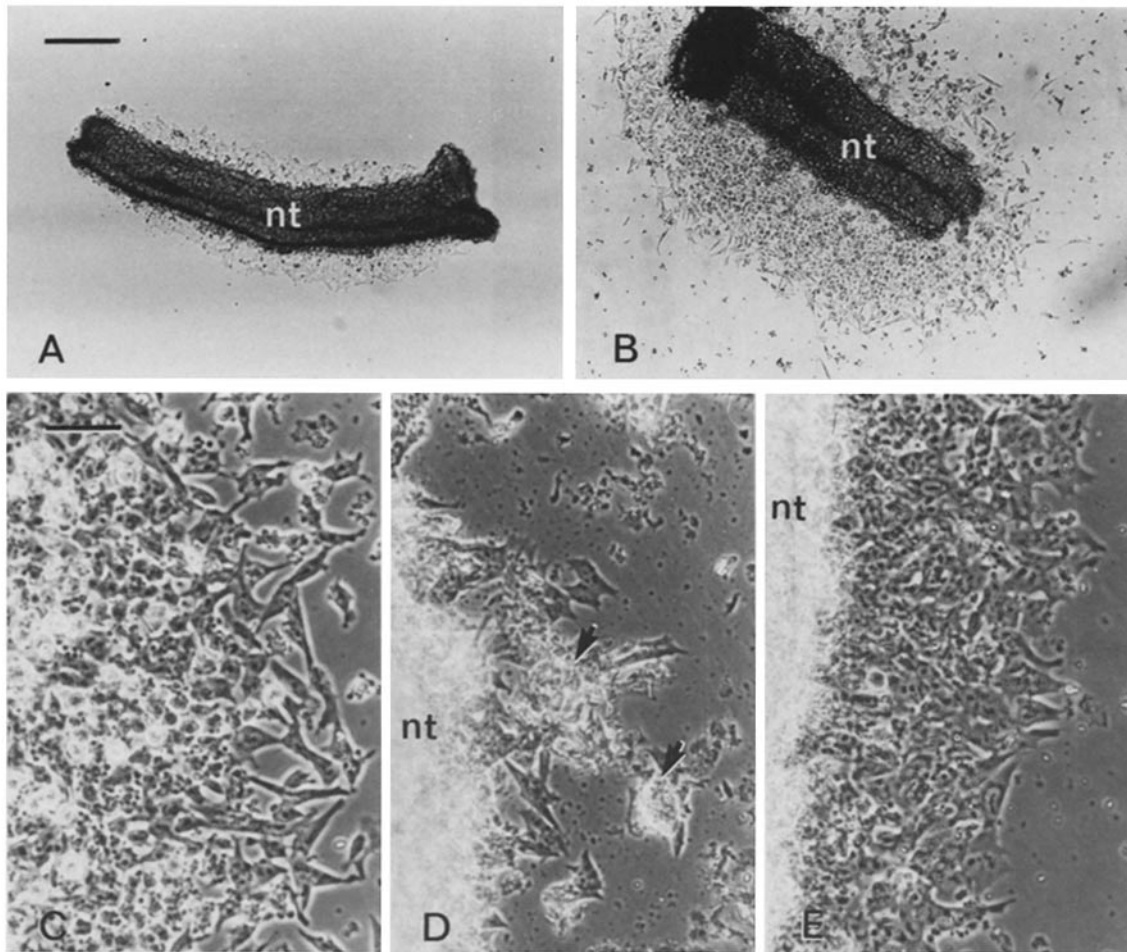
FIGURE 3 In vitro neural crest cell migration. (A) In the presence of DME supplemented with fibronectin-free fetal calf serum, neural crest cells emigrated onto the fibronectin substrate to form a monolayered cell sheet around the explant; the crest cell halo is already visible at low magnification after 5 h; nt, neural tube. (B and C) After 21 h of culture, cells at the periphery of the halo are well spread with several filopodia per cell; the morphology of cell sheet differs from that of epithelial cells or fibroblastic cells. (D) P1 at 1.6 mg/ml final concentration was added 5 h after beginning the culture (see A); 16 h later, further migration of cells was strongly inhibited; moreover, many of the cells which had been in a monolayered cell sheet before adding P1 detached from the substrate, some forming aggregates (arrows). (E) In contrast, in the presence of P3 added 5 h after initiating the culture, neural crest cells continued to migrate as in controls. (A and B) Bar, 200 μm; × 50. (C–E) Bar, 50 μm; × 200.
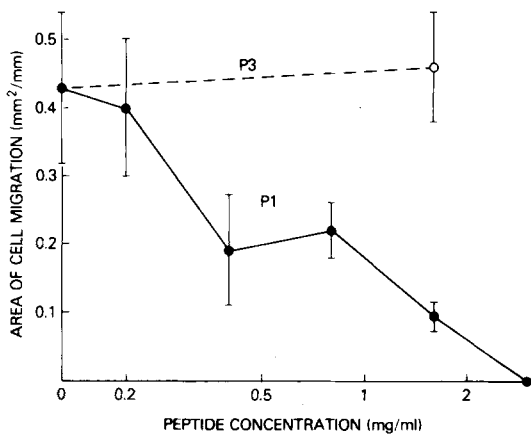

FIGURE 4 Dose-response curve for peptide inhibition of neural crest cell migration. Quail neural crest cell cultures were incubated for 5 h to permit cell attachment to the fibronectin substrate, then the indicated concentrations of peptides P1 or P3 were added for an additional 12 h, during the period of extensive cell migration. The surface area covered by the halos of crest cells migrating away from the neural tubes was quantitated from phase-contrast micro-

in length (29, 30, 44). Synthetic peptides containing this highly conserved region are competitive inhibitors of adhesion, and a series of over 15 other peptides tested as controls showed no inhibitory activity (30, 44) (Yamada, K. M., and D. W. Kennedy, unpublished observations). Even though the key amino acids Arg, Asp, and Ser are also present in a different sequence in a synthetic decapeptide from the collagen-binding region of fibronectin, it displays no inhibitory activity (44).

We examined whether this putative recognition signal is used by migratory nonfibroblastic cells. A synthetic peptide containing the recognition signal was found to inhibit the interaction of quail neural crest cells with fibronectin. These cells attached poorly and did not migrate in vitro on fibronectin substrates, which were previously shown to be required for neural crest cell migration in vitro (32). This result indicates that the putative recognition mechanism is also present in a nonmesodermally derived cell type, and in birds as well as mammals.

graphs of each culture as described in Materials and Methods. Points indicate mean of triplicate samples ± standard deviation.
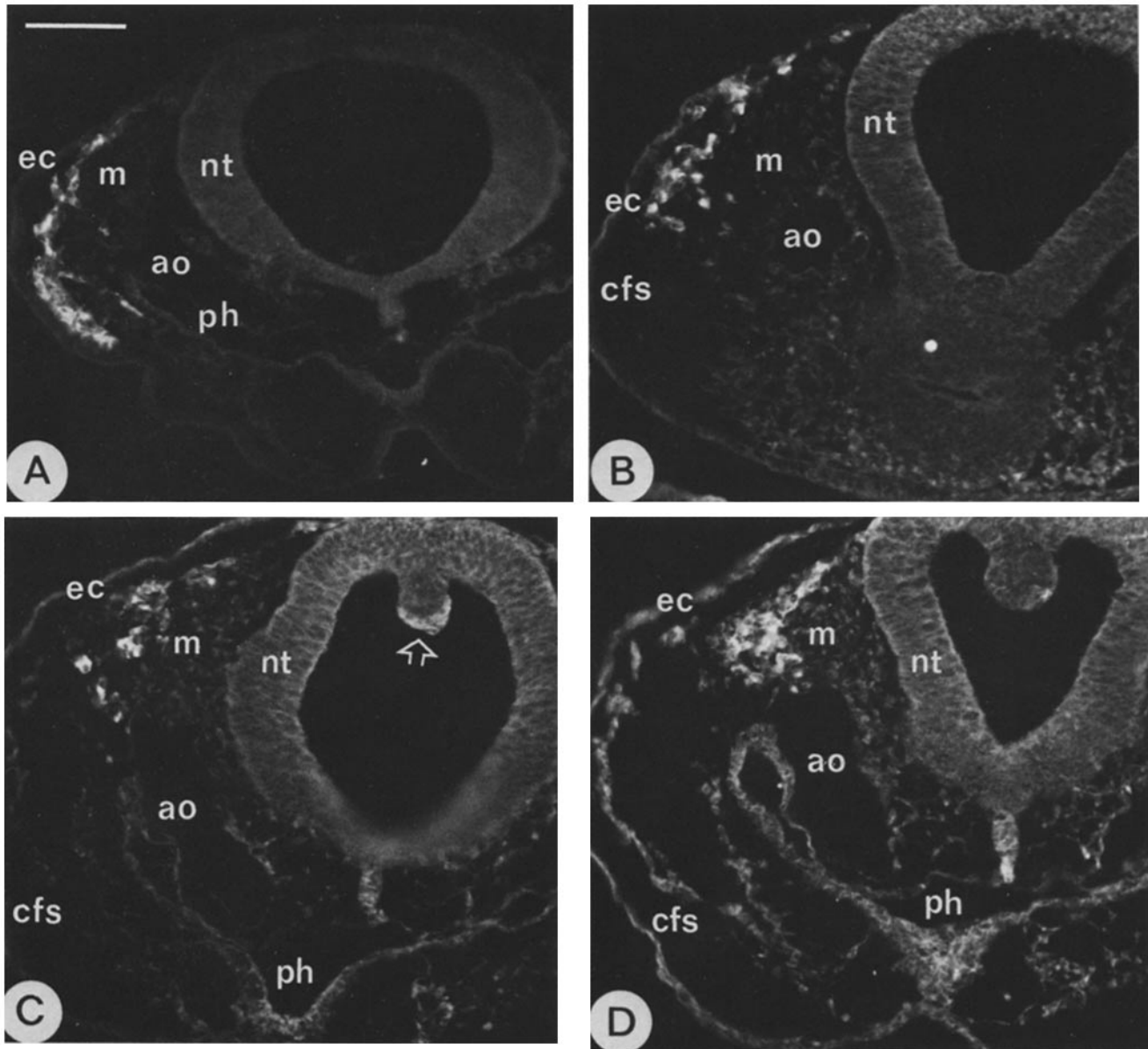
FIGURE 5 Sections of embryos fixed in ovo after microinjection. (A) NC-1 immunofluorescence of a section of an 11-somite chick embryo cut through the level of mid-mesencephalon. The large, lateral cell-free space is filled with stained neural crest cells. This embryo was injected 8 h earlier with a PBS–phenol red solution. ao, aorta; nt, neural tube; ec, ectoderm; m, mesenchyme; ph, pharynx. (B–D) Sections of an embryo injected with the P1 peptide into the cell-free space at the 5-somite stage, fixed, serially sectioned, and stained with NC-1 at the 11-somite stage. (B) This section, at the level of the anterior mesencephalon ~150 μm cranial to the site of injection, displays a fairly normal pattern of crest cell emigration into the cell-free space. The dorsal portion of the neural tube appears normal, but in control embryos, the cell-free space should be almost completely filled at this level. cfs, cell-free space. (C) Section of the same embryo as in B at the level of the mid-mesencephalon. This was the site of injection, and it displays the most dramatic inhibition of neural crest cell migration and proliferation. Neural crest cells have been forced into the lumen of the neural tube (arrow). (D) Section of the same embryo as in B at the level of the posterior mesencephalon. This section, 100 μm caudal to the site of injection, shows some recovery, but crest cells are still displaced into the neural tube lumen, and there is a large, empty cell-free space. Bar, 100 μm. × 150.

The synthetic peptide was microinjected into amphibian and avian embryos to evaluate its effects on cell migration and morphogenesis in vivo. Gastrulation in *P. waltlii* embryos was inhibited by the same synthetic decapeptide shown to be active in vitro, but not by control peptides including a conserved decapeptide from the collagen-binding region. Embryos appeared to undergo abortive cell migration, with a dramatic piling up in the superficial layer, which normally undergoes invagination into the blastocoel cavity.

The convoluted ectodermal layer probably results from a blockade of invagination rather than a direct modification of the extracellular matrix at the basal surface, although more work will be necessary to define the precise state of the matrix in peptide-injected embryos. Interestingly, P3 had no effect on gastrulation; this peptide, so far, has not been shown to inhibit cell adhesion or to modify the structure of the extracellular matrix in vitro or in vivo. In particular, it does not disrupt the interaction between collagen and fibronectin. Par-
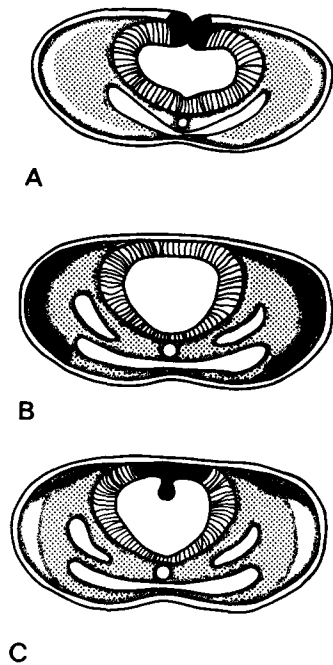
FIGURE 6 Normal and perturbed pattern of mesencephalic crest cell migration in avian embryos. (A) Diagram of a 5-somite chick embryo, cross-sectioned at the level of the mid-mesencephalon. Neural crest cells (solid black areas) originate at the junction of the fusing neural folds. A cell-free space has already formed laterally under the ectoderm. A fibronectin-rich basement membrane (finely stippled areas) lies under the ectoderm adjacent to the cell-free space, which is the presumptive crest cell pathway. (B) 11-somite chick embryo, cross-sectioned at the level of the mid-mesencephalon. Neural crest cells have reached their furthest lateral extent occupying the cell-free space, but will subsequently continue to migrate ventrally. (C) 11-somite chick embryo injected at the 5-somite stage with the peptide P1. Drawing summarizes the results of experiments as shown in Fig. 5C. The majority of the migrating neural crest cells have been forced into the neural tube lumen. However, some cells are also found within the mesenchymal mesoderm.

enthetically, collagen(s) have not been identified definitively in early amphibian embryos by either immunological or biochemical criteria.

In contrast to the results obtained with mammalian cell lines (44), P2 was less effective as an inhibitor in amphibian embryos, possibly indicating that in addition to the absolutely required Arg-Gly-Asp-Ser sequence (30), additional sequences may enhance fibronectin interactions with the cell surface depending on the species. Combined with previous experiments demonstrating an inhibition of mesodermal migration by antifibronectin antibodies, these results support the role of fibronectin in gastrulation (Fig. 1).

A second, quite different morphogenetic process in vivo involves the migration of neural crest cells to a variety of separate sites in the embryo. Microinjection of the synthetic decapeptide inhibitor into chick embryos immediately before the onset of neural crest cell migration caused a dramatic inhibition of this major morphogenetic event. Most neural crest cells identified by a neural crest cell marker failed to migrate away from the dorsal border of the closing neural tube, and they instead accumulated as a compact structure at an abnormal site within the dorsal portion of the neural tube (summarizd schematically in Fig. 6). Even though the peptide was injected into only one side of the embryo, inhibition

appeared to be complete on both sides. In contrast, microinjection of antifibronectin antibodies caused effects only on the side at which the injection was performed (Poole, T. J., K. M. Yamada, and J. P. Thiery, unpublished observations). These results suggest that the peptide diffuses readily in the embryo, as would be predicted for a probe over a 100-fold smaller than an antibody molecule. These findings also implicate the fibronectin recognition sequence in a second morphogenetic event besides gastrulation.

The results now obtained both in vitro and in vivo in amphibian and in avian embryos strongly suggest a direct role for fibronectin in the mechanism of attachment and migration of cells. We have previously suggested that to migrate, these cells require exogenous fibronectin; yet they do not produce fibronectin, or at least do not efficiently retain fibronectin on the cell surface (13, 26–28, 32, 35). Indeed, cells producing and binding large amounts of fibronectin (12) or latex beads coated with fibronectin (3) injected into the crest cell migration pathway remain at the site of injection. The low affinity constant of fibronectin to fibronectin receptors (Akiyama, S. K., and K. M. Yamada, manuscript submitted for publication) also favors a transient attachment of migrating cells to the extracellular matrix.

An additional argument in favor of a key role for fibronectin rather than collagen in early vertebrate development derives from the behavior of transgenic mice (34) homozygous for a defective collagen type I gene. Such mice die at 12–13 d of gestation, long after successful completion of gastrulation and many other morphogenetic movements, including crest cell migration. In fact, these mice lacking type I collagen die of hemorrhage, perhaps due to inadequate vascular strength (25).

As exemplified by our analysis of the possible role of the fibronectin recognition sequence in certain morphogenetic events, peptide probes can provide a new approach to analyzing the role of a particular recognition signal. One reservation, which also exists when using antibodies directed against small peptide determinants, is the possibility of cross-reaction between this molecular recognition site and similar sites that might possibly be employed by other recognition systems. More generally, such probes share the problems of all inhibitors (including antibodies): uncertainty about absolute specificity. Although part of the sequence we used is present in a few other proteins that could conceivably use the same recognition signal (30), the full decapeptide sequence is thus far unique. As for all inhibitors, only extensive testing in many biological systems will indicate whether the probe is an absolute indicator of fibronectin-mediated function in vertebrate cells. In studies to date, its activity is not mimicked by other peptides, and there has been no evidence for nonspecific cytotoxicity in terms of inhibition of viability or amino acid incorporation (44).

The use of synthetic peptide inhibitors against recognition signals on other extracellular glycoproteins could provide further insights into the roles of these proteins in morphogenesis. For example, certain peptides of collagen are competitive inhibitors of the attachment of fibronectin to collagen (6, 21) and of native collagen to fibroblasts (16); in addition, a synthetic tetrapeptide inhibits the polymerization of fibrin (22). These and other site-specific peptide inhibitors may also prove to be useful as probes of the role of extracellular proteins in embryonic development.

REFERENCES

1. Boucaut, J. C., and T. Darribere. 1983. Fibronectin in early amphibian embryos. *Cell Tissue Res.* 234:135–145.
2. Boucaut, J. C., T. Darribere, H. Boulekbache, and J. P. Thiery. 1984. Prevention of gastrulation but not neurulation by antibodies to fibronectin in amphibian embryos. *Nature (Lond.).* 307:364–367.
3. Bronner-Fraser, M. 1982. Distribution of latex beads and retinal pigment epithelial cells along the ventral neural crest cell pathway. *Dev. Biol.* 91:50–63.
4. Buskirk, D. R., J. P. Thiery, U. Rutishauser, and G. Edelman. 1980. Antibodies to a neural cell adhesion molecule disrupt histogenesis in cultured chick neural retinae. *Nature (Lond.).* 85:488–489.
5. Critchley, D. R., M. A. England, J. Wakely, and R. O. Hynes. 1979. Distribution of fibronectin in the ectoderm of gastrulating chick embryos. *Nature (Lond.).* 280:498–500.
6. Dessau, W., B. C. Adelmann, R. Timpl, and G. R. Martin. 1978. Identification of the sites in collagen alpha-chains that bind serum antigelatin factor (cold-insoluble globulin). *Biochem. J.* 169:55–59.
7. Duband, J. L., and J. P. Thiery. 1982. Appearance and distribution of fibronectin during chick embryo gastrulation and neurulation. *Dev. Biol.* 94:337–350.
8. Duband, J. L., and J. P. Thiery. 1982. Distribution of fibronectin in the early phase of avian cephalic neural crest cell migration. *Dev. Biol.* 93:308–323.
9. Edelman, G. M., W. J. Gallin, A. Delouvee, B. A. Cunningham, and J. P. Thiery. 1983. Early epochal maps of two different cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 80:4384–4388.
10. Ekbolm, P., L. Saxen, and R. Timpl. 1982. The extracellular matrix and kidney differentiation. *In* Membranes in Growth and Development. Alan. R. Liss, New York. 429–422.
11. Engvall, E., and E. Ruoslahti. 1977. Binding of the soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer.* 20:1–5.
12. Erickson, C. A., K. W. Tosney, and J. A. Weston. 1980. Analysis of migratory behavior of neural crest cells and fibroblastic cells in embryonic tissues. *Dev. Biol.* 77:142–156.
13. Erickson, C. A., and E. A. Turley. 1983. Substrata formed by combination of extracellular matrix components alter neural crest cell motility in vitro. *J. Cell Sci.* 61:299–323.
14. Fraser, S. E., B. A. Murray, C.-M. Chuong, and G. M. Edelman. 1984. Alteration of the retinotectal map in *Xenopus* by antibodies to neural cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 81:4222–4221.
15. Gallien, L., and M. Durocher. 1957. Table chronologique du development chez *Pleurodeles waltii. Michah. Bull. Biol. France et Belgique.* 91:97–104.
16. Goldberg, B., and R. E. Burgeson. 1982. Binding of soluble type I collagen to fibroblasts: specificities for native collagen types, triple helical structure, telopeptides, propeptides, and cyanogen bromide-derived peptides. *J. Cell Biol.* 95:752–756.
17. Hay, E. D. 1981. Cell Biology of Extracellular Matrix. Plenum Press, New York. 417 pp.
18. Heasman, J., R. O. Hynes, A. P. Swan, V. Thomas, and C. C. Wylie. 1981. Primordial germ cells of *Xenopus* embryos: the role of fibronectin in their adhesion during migration. *Cell.* 27:437–447.
19. Hynes, R. O., and K. M. Yamada. 1982. Fibronectins: multifunctional modular glycoproteins. *J. Cell Biol.* 95:369–377.
20. Icardo, J. M., and F. J. Manasek. 1984. An indirect immunofluorescence study of the distribution of fibronectin during the formation of the tissue mesenchyme in the embryonic heart. *Dev. Biol.* 101:336–345.
21. Kleinman, H. K., E. B. McGoodwin, G. R. Martin, R. J. Klebe, P. P. Fietzek, and D. E. Woodley. 1978. Localization of the binding site for cell attachment in the alpha 1(I) chain of collagen. *J. Biol. Chem.* 253:5642–5666.
22. Laudano, A. P., and R. F. Doolittle. 1980. Studies on synthetic peptides that bind to fibrinogen and prevent fibrin polymerization. Structural requirements, number of binding sites, and species differences. *Biochemistry.* 19:1013–1019.
23. Lee, G., R. Hynes, and M. Kirschner. 1984. Temporal and spatial regulation of fibronectin in early *Xenopus* development. *Cell.* 36:729–740.
24. Lindner, J., F. G. Rathjen, and M. Schachner. 1983. L1 mono- and polyclonal antibodies modify cell migration in early postnatal mouse cerebellum. *Nature (Lond.).* 305:427–430.
25. Lohler, J., R. Timpl, and R. Jaenish. 1984. Lethal mutation of mouse collagen 1 gene causes rupture of blood vessels and is associated with erythropoetic and mesenchymal cell death at day 12 of gestation. *Cell.* 38:597–607.
26. Loring J., B. Glimelius, and J. A. Weston. 1982. Extracellular matrix materials influence quail neural crest cell differentiation in vitro. *Dev. Biol.* 90:165–174.
27. Newgreen, D. F. 1984. Spreading of explants of embryonic chick mesenchyme and epithelia on fibronectin and laminin. *Cell Tissue Res.* 236:265–277.
28. Newgreen, D. F., I. L. Gibbins, J. Sauter, B. Wallensels, and R. Wütz. 1982. Ultrastructural and tissue culture studies on the role of fibronectin, collagen, and glycosaminoglycans in the migration of neural crest cells in the whole embryo. *Cell Tissue Res.* 221:521–549.
29. Pierschbacher, M. D., E. G. Hayman, and E. Ruoslahti. 1983. Synthetic peptide with cell attachment activity of fibronectin. *Proc. Natl. Acad. Sci. USA.* 80:1224–1227.
30. Pierschbacher, M. D., and E. Ruoslahti. 1984. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature (Lond.).* 309:30–33.
31. Pratt, R. M., M. A. Larsen, and M. C. Johnston. 1975. Migration of cranial neural crest cells in a cell free hyaluronate-rich matrix. *Dev. Biol.* 44:298–305.
32. Rovasio, R. A., A. Delouvee, K. M. Yamada, R. Timpl, and J. P. Thiery. 1983. Neural crest cell migration: requirements for exogenous fibronectin and high cell density. *J. Cell Biol.* 96:462–473.
33. Rutishauser, U., W. E. Gall, and G. M. Edelman. 1978. Adhesion among neural cells of the chick embryo. IV. Role of the cell surface molecule CAM in the formation of neurite bundles in cultures of spinal ganglia. *J. Cell Biol.* 79:382–393.
34. Schnieke, A., K. Harvers, and R. Jaenisch. 1983. Embryonic lethal mutation in mice induced by retrovirus insertion into the α1(I) collagen gene. *Nature (Lond.).* 304:315–320.
35. Sieber-Blum, M., F. Sieber, and K. M. Yamada. 1981. Cellular fibronectin promotes adrenergic differentiation of quail neural crest cells in vitro. *Exp. Cell Res.* 133:285–292.
36. Thanos, S., F. Bonhoeffer, and U. Rutishauser. 1984. Fiber-fiber interaction and tectal cues influence the development of the chicken retinotectal projection. *Proc. Natl. Acad. Sci. USA.* 81:1906–1910.
37. Thiery, J. P., J. L. Duband, and A. Delouvee. 1982. Pathways and mechanism of avian trunk neural crest cell migration and localization. *Dev. Biol.* 93:324–343.
38. Thiery, J. P., J. L. Duband, U. Rutishauser, and G. M. Edelman. 1984. Cell adhesion molecules in early chicken embryogenesis. *Proc. Natl. Acad. Sci. USA.* 79:6737–6741.
39. Thiery, J. P., A. Delouvee, W. Gallin, B. A. Cunningham, and G. M. Edelman. 1984. Ontogenetic expression of cell adhesion molecules: L-CAM is found in epithelia derived from the three primary germ layers. *Dev. Biol.* 102:61–78.
40. Tucker, G. C., H. Aoyama, M. Lipinski, T. Tursz, and J. P. Thiery. 1984. Identical reactivity of monoclonal antibodies HNK-1 and NC-1: conservations in vertebrates on cells derived from the neural primordium and on some leukocytes. *Cell Differ.* In press.
41. Vincent, M., J. L. Duband, and J. P. Thiery. 1983. A cell surface determinant expressed early in migrating avian neural crest cells. *Dev. Brain Res.* 9:235–238.
42. Vincent, M., and J. P. Thiery. 1984. A cell surface marker for neural crest and placodal cells: further evolution on peripheral and central nervous system. *Dev. Biol.* 103:468–481.
43. Wylie, C. C., and J. Heasman. 1982. Effects of the substratum on the migration of primordial germ cells. *Phil. Trans. R. Soc. Lond. B. Biol. Sci.* 299:177–183.
44. Yamada, K. M., and D. W. Kennedy. 1984. Dualistic nature of adhesive protein function: fibronectin and its biologically active peptide fragments can auto-inhibit fibronectin function. *J. Cell Biol.* 99:29–36.