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Evidence for the role of fibronectin in amphibian gastrulation

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SUMMARY

In amphibian embryos, fibronectin (FN) assembles as a fibrillar network on the roof of the blastocoel cavity, preceding mesodermal cell migration. Local inversion of the ectoderm to produce a site where no FN is available prevents mesodermal cell migration. Microinjection of monovalent antibodies to FN arrests gastrulation. A complete inhibition of mesodermal cell migration is obtained after microinjection of a synthetic peptide containing the cell binding site sequence of FN. Prevention of interactions between receptors and FN appears to be the primary cause for blockage of gastrulation.

INTRODUCTION

Gastrulation represents a fundamental event in the course of development. During this phase, integrated movements of cells permit new interactions which in turn promote the formation of three germ layers: ectoderm, mesoderm, and endoderm. In amphibians the first sign of gastrulation is the formation of the blastoporal groove in the future dorsal region of the embryo. It involves endodermal cells which are beginning to invaginate (Holtfreter, 1943; Baker, 1965; Perry & Waddington, 1966). Subsequently, invagination proceeds inwards with active migration of mesodermal cells (Nakatsuji 1974, 1975*a,b*). However, so far, the cellular and molecular basis of gastrulation movements remain largely unknown.

In amphibians the initiation of morphogenetic movements correlates with changes in the synthesis of extracellular components (Tarin, 1973). Extracellular

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materials containing galactose and glucosamine residues are synthesized and secreted by ectodermal cells at the onset of gastrulation (Johnson, 1977*c*). Furthermore, when coupled to Sepharose beads this extracellular material enhances the adhesion of isolated mesodermal cells (Johnson, 1981). These results suggest that the locomotion of mesodermal cells is in some way triggered by an extracellular substrate. At the blastula stage, extracellular fibrils cover the entire surface of the blastocoelic roof (Nakatsuji, Gould & Johnson, 1982).

Recently, the high molecular weight glycoprotein fibronectin (FN), has been revealed as one of major components of extracellular matrices (Yamada, 1983). Its ability to bind both to cell surfaces and to the other extracellular components argues for a key role in cell migration in various embryonic systems. FN has been localized in embryos of a number of species (Zetter & Martin, 1978; Critchley, England, Wakely & Hynes, 1979; Spiegel, Burger & Spiegel, 1980; Newgreen & Thiery, 1980; Duband & Thiery, 1982). *In vitro* studies have indicated that FN is required for attachment and migration of neural crest cells and primordial germ cells (Wylie & Heasman, 1982; Newgreen *et al.* 1982; Rovasio *et al.* 1983). In early amphibian embryos FN has now been detected prior to gastrulation (Boucaut & Darribère, 1983*a*; Lee, Hynes & Kirschner, 1984). This paper reviews these data as well as a series of perturbation experiments strongly suggesting that FN promotes mesodermal cell migration during amphibian gastrulation.

MATERIALS AND METHODS

Egg collection

Pleurodeles waltl and *Ambystoma mexicanum* eggs were collected from natural matings. They were manually dejellied and maintained at 18 °C in sterile Steinberg's medium. Embryonic stages of embryos were determined by reference to Gallien & Durocher (1957), and Schreckenberg & Jacobson (1975).

Fluorescence microscopy

For cryostat sections (10 μm), embryos were fixed for 2 h in 4 % formaldehyde and then washed in Steinberg buffer overnight. They were impregnated with increasing sucrose solutions and embedded in Cryo M Bed compound (Bright). For whole-mount observations, pieces of selected areas were dissected from living embryos and fixed in 4 % formaldehyde (4°C) for 10 min. Sections or pieces of embryos were incubated with 1/100 dilution of anti-FN in 0·5 % BSA for 1 h. After washing with Steinberg buffer, fluorescein-labelled sheep anti-rabbit IgG 1/100 (Institut Pasteur) was incubated with the sections or specimens for 1 h. They were mounted after rinsing in 90 % glycerol, 10 % Steinberg buffer, pH 7·4. Immunofluorescence photomicroscopy was performed on a Leitz Dialux 20. Preparation of rabbit antibodies against *Ambystoma mexicanum* plasma FN have been previously described (Boucaut & Darribère, 1983*b*).

Colloidal gold preparation and labelling for FN

Gold colloids were prepared as described by Horisberger (1979) for 12 nm diameter particles and Frens (1973) for 40 nm diameter particles. Protein A (*Staphyloccocus aureus*, Sigma)-labelled colloids were obtained according to Horisberger & Rosset (1977). 200 nl anti-FN antibody (1/20 diluted IgG) was introduced in the blastocoelic cavity by microinjection and the embryos incubated for 1 h at 18 °C. Then the blastocoel was rinsed with sterile Steinberg's solution and injected with 12 or 40 nm gold-labelled protein A ($10\,\mu g\,ml^{-1}$, 200 nl). After 1 h, embryos were bisected, washed, and areas were selected for electron microscopy.

Electron microscopic techniques

Embryos or specimens were fixed in 2·5 % glutaraldehyde (TAAB) in 0·05 M-cacodylate buffer (Serva) pH 7·4 for one day. They were rinsed and postfixed in 1 % osmium tetroxide. For scanning electron microscopy (SEM), they were dehydrated, critical-point dried with liquid CO_2, coated with gold and examined in a JEOL-JSM-35. Samples for transmission electron microscopy (TEM) were embedded in epon. Sections were stained with uranyl acetate and lead citrate. Finally they were observed with a Philips 301 electron microscope.

Embryonic grafts

Ectodermal grafting experiments were performed in sterile Steinberg's solution. Donor ectodermal fragments were isolated at early gastrula stage with tungsten needles. They were inverted and grafted to a recipient embryo (early gastrula) at a site close to the dorsal lip of the blastopore. The cicatrization occurred within 30 min at 18 °C. The blastocoel roof of grafted embryos was bisected 3 to 6 h later and fixed for SEM.

Microinjections of Fab′ or synthetic peptides

Fab′ monovalent fragments were prepared from purified rabbit IgG directed against *Ambystoma mexicanum* plasma FN. They were obtained according to Brackenbury, Thiery, Rutishauser & Edelman, (1977). Their purity was tested on 10 % polyacrylamide gel electrophoresis before use.

The synthetic peptides: Arg - Gly - Asp - Ser - Pro - Ala - Ser - Ser - Lys - Pro (P_1), Gly - Arg - Gly - Asp - Ser - Pro - Cys (P_2) and Cys - Gln - Asp - Ser - Glu - Thr - Arg - Thr - Phe - Tyr (P_3) were purchased from Peninsula Laboratories, Inc., Belmont, CA (Yamada & Kennedy 1984). P_1 corresponds to a conserved and hydrophilic sequence from the cell-binding site of FN. P_2 sequence is closely related to P_1. P_3 is a hydrophilic sequence from the collagen-binding domain that is identical in chicken and bovine FN sequences (see Yamada & Kennedy (1984) and Pierschbacher & Ruoslahti (1984) for further characterization of the peptides). Purified ACTH (2–11) peptides were obtained from Peninsula Laboratories.

Microinjections were carried out with micropipettes (10 μm diameter) produced

with a Leitz (Weitzlar, FRG) horizontal pipette puller. They were performed into the blastocoel of late blastula, early or middle gastrula. Each embryo was injected with 200 nl of 10 or 20 mg ml^{-1} solution of Fab′, P_1, P_2, or P_3. Controls were injected with ACTH peptides or BSA under the same conditions. Embryos were kept at 18 °C in sterile Steinberg's solution for 6 h; the appearance of ectodermal furrows and of a circular blastoporal slit were taken as evidence of blockage of gastrulation. Embryos were freed from their vitelline membrane and fixed for SEM either intact or after sagittal sectioning.

RESULTS

Distribution of fibronectin (FN)

As a prerequisite to understanding the possible role of FN in morphogenetic movements, its spatial distribution was determined before and during gastrulation. Specific antibodies directed against amphibian FN were applied either to sections or whole-mount specimens.

Light microscopy

In early stages of development, amphibian cells may synthesize FN but do not deposit it on their surfaces (Darribère, Boucher, Lacroix & Boucaut, 1984; Lee *et al.* 1984). Indirect immunofluorescence staining for FN is first noticed at the early blastula stage. Significantly, FN is exclusively confined to the roof of the blastocoel. This particular pattern is clearly apparent with frozen sections prepared from late blastulae (stage 7). As gastrulation proceeds, FN remains restricted to the inner surface of the blastocoel roof over which mesodermal cells are migrating (Fig. 1). Mesodermal cells themselves are not stained for FN.

The question arose as to how FN is organized on the surface of the roof of the blastocoel. The answer was found by studying the occurrence of FN in whole-mount specimens of the roof of the blastocoel that were explanted from early blastula to late gastrula stages. As shown in Fig. 2, fluorescent strands of extracellular FN cover the entire surface of the roof of the blastocoel. First, at early blastula stage, some extracellular FN fibrils begin to appear. These FN fibrils are mostly radially ordered and diverge toward the cell periphery (Fig. 2A). Later, this initial pattern gives rise to a dense FN-rich extracellular matrix. At midgastrula stage FN persists between migrating mesodermal cells and ectodermal cells forming the roof of the blastocoel (Fig. 2B). It is clear that at the level of the leading edge of migration, mesodermal cells which do not stain for FN, nevertheless connect with some extracellular fluorescent FN fibrils (Fig. 2C).

The above immunofluorescence data are in good agreement with the idea that the FN matrix may provide a substrate for migrating mesodermal cells.

Electron microscopy

To gain more insight into the mechanisms by which FN controls the locomotion

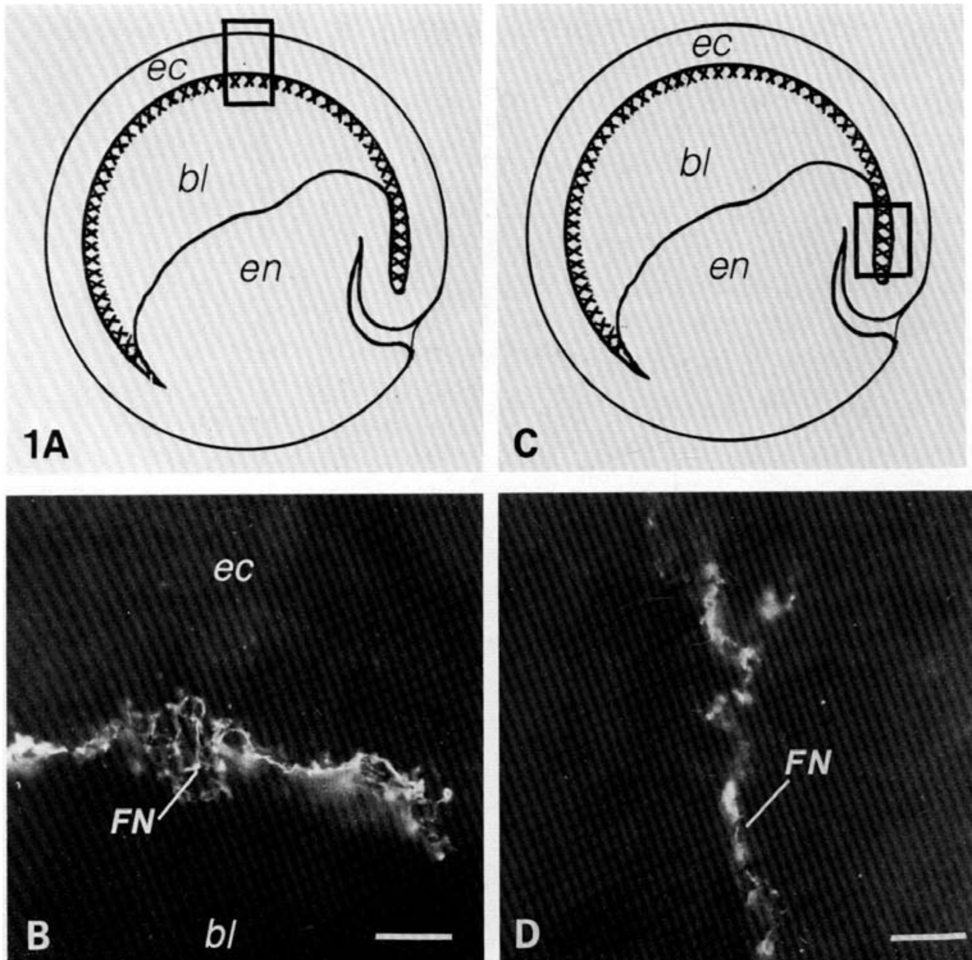

Fig. 1. Indirect immunofluorescent staining for FN at early gastrula stage (stage 10) in *Ambystoma mexicanum*. (A) Schematic representation of the area presented in (B). (B) Sagittal frozen section. A FN-rich extracellular matrix lines the inner surface of the roof of the blastocoel (C) Schematic representation of the area presented in (D). (D) Sagittal frozen section. The FN-rich extracellular matrix is present between migrating mesodermal cells and the overlying cells facing the blastocoel roof. *bl*, blastocoel; *ec*, presumptive ectoderm; *en*, presumptive endoderm; *FN*, fibronectin; crosses show immunofluorescent staining for FN. Scale bar: 10 μm.

of mesodermal cells, we investigated the distribution of FN using indirect immunogold staining.

At the late blastula stage, scanning electron microscope examination of the blastocoel roof showed a regulat arrangement of ectodermal cells from which extended numerous filopodia. The flattened surface of ectodermal cells is coated with a meshwork of extracellular fibrils of 50 nm to 100 nm thickness. From the middle to late blastula stage, extracellular fibrils are branched and progressively associate in strands with no defined orientation. As shown in Fig. 3A, during

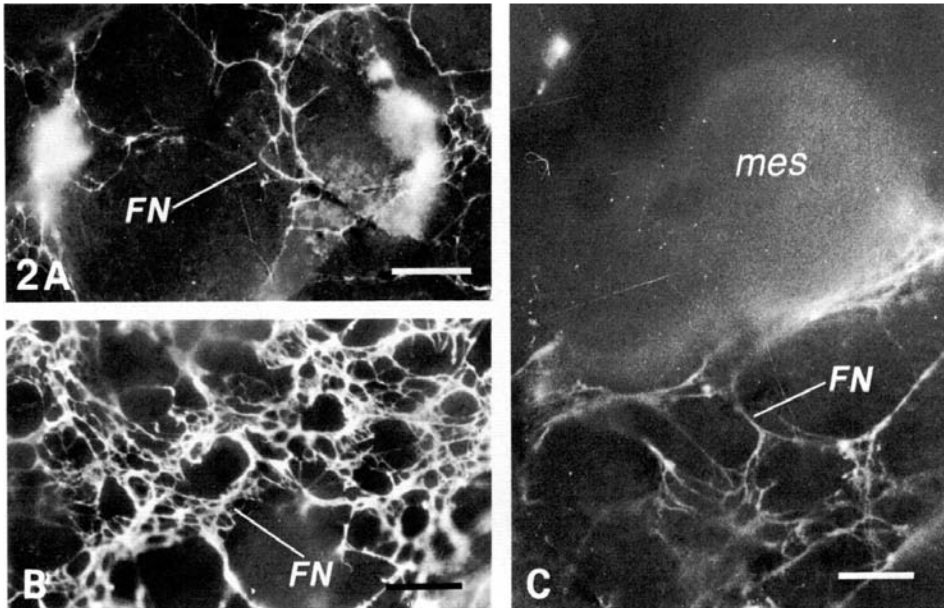

Fig. 2. Indirect immunofluorescent staining for FN in whole mounts from blastula and early gastrula stages in *Ambystoma mexicanum* (A) Blastula (stage 8) – FN fibrils are found in the regions of cell-to-cell contacts. They are confluent and cover the inner surface of the ectoderm cells. (B) Early gastrula (stage 9). FN fibrils form a network that extends ahead of migrating mesodermal cells. (C) Midgastrula (stage 11). Front zone of migration. Elongated FN fibrils connect the edge of migrating mesodermal cells to the fibrillar matrix. *FN*: fibronectin; *mes*: mesoderm. Scale bars: A,B, 20 μm; C, 10 μm.

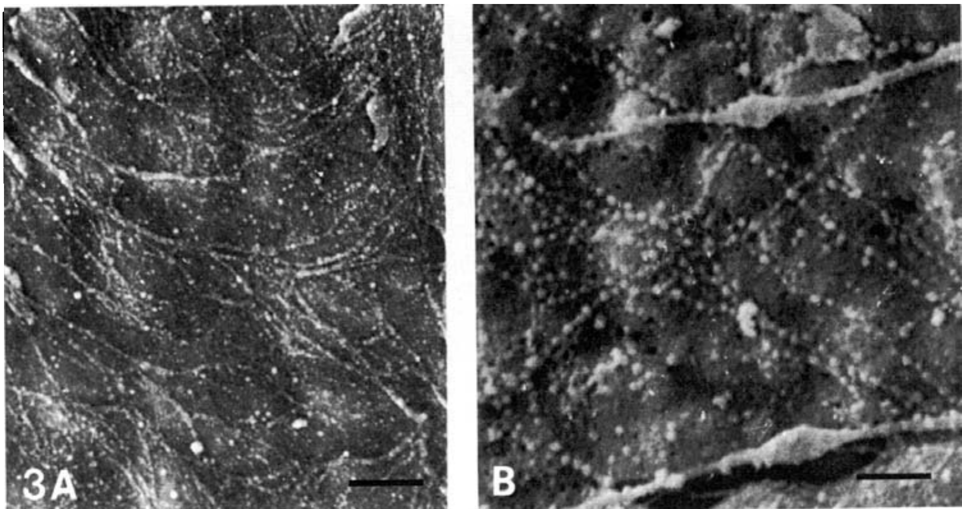

Fig. 3. Scanning electron micrographs of the inner surface of the blastocoelic roof in *Ambystoma mexicanum* embryos. At late blastula stage (stage 8^{+}) (A) Fibrils facing an extracellular matrix cover the entire surface of ectodermal cells. Scale bar: 0·2 μm. (B) Immunogold staining for FN, extracellular fibrils contain FN, because they are decorated with 40 nm gold particles linked to protein A when treated with anti-FN antibodies. Gold particles are aligned; they are distributed regularly along every extracellular fibril. Scale bar: 0·1 μm.

gastrulation the fibrillar matrix is fully developed. Fibrils observed at higher magnification display a fine granular structure. These fibrils are selectively labelled after applying anti-FN antibodies and gold-coupled protein A. As shown in Fig. 3B gold particles are bound at regular intervals along FN-containing fibrils. By contrast, only very few gold particles are found on ectodermal cell surfaces. This pattern is consistent with that previously observed with immunofluorescent staining for FN.

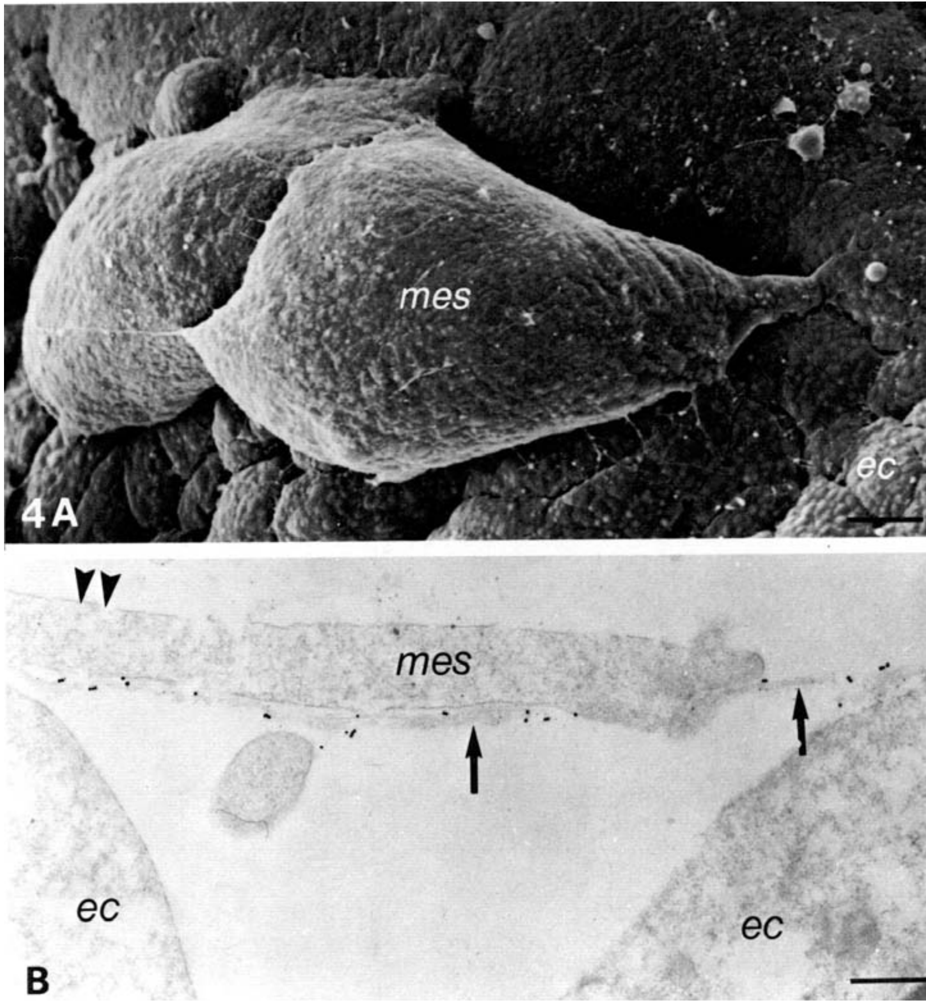

Fig. 4. Scanning (SEM) and transmission (TEM) electron micrographs of the inner surface of the blastocoelic roof in *Pleurodeles waltl* embryos at midgastrula stage (stage 10). (A) View of two pioneer migrating mesodermal cells. They extend lamellipodia and filopodia at their leading margin. Scale bar: 1·4 μm. (B) A filopodium from a migrating mesodermal cell contacts an electron dense extracellular fibril attached to the surface of two ectodermal cells. Note that the extracellular fibril is well-decorated by gold particles, whereas the mesodermal cell surface is totally free of labelling. Scale bar: 0·5 μm. Double arrow heads: mesodermal cell surface. Single arrow: extracellular fibril (FN fibril). *ec*, ectoderm; *mes*, mesoderm.

It clearly indicates that FN is a major component of extracellular fibrils.

During gastrulation, pioneer mesodermal cells move as a stream of loosely packed cells on the roof of the blastocoel. They extend lamellipodia and filopodia at their leading margin, whereas the trailing edge remains round or associates with

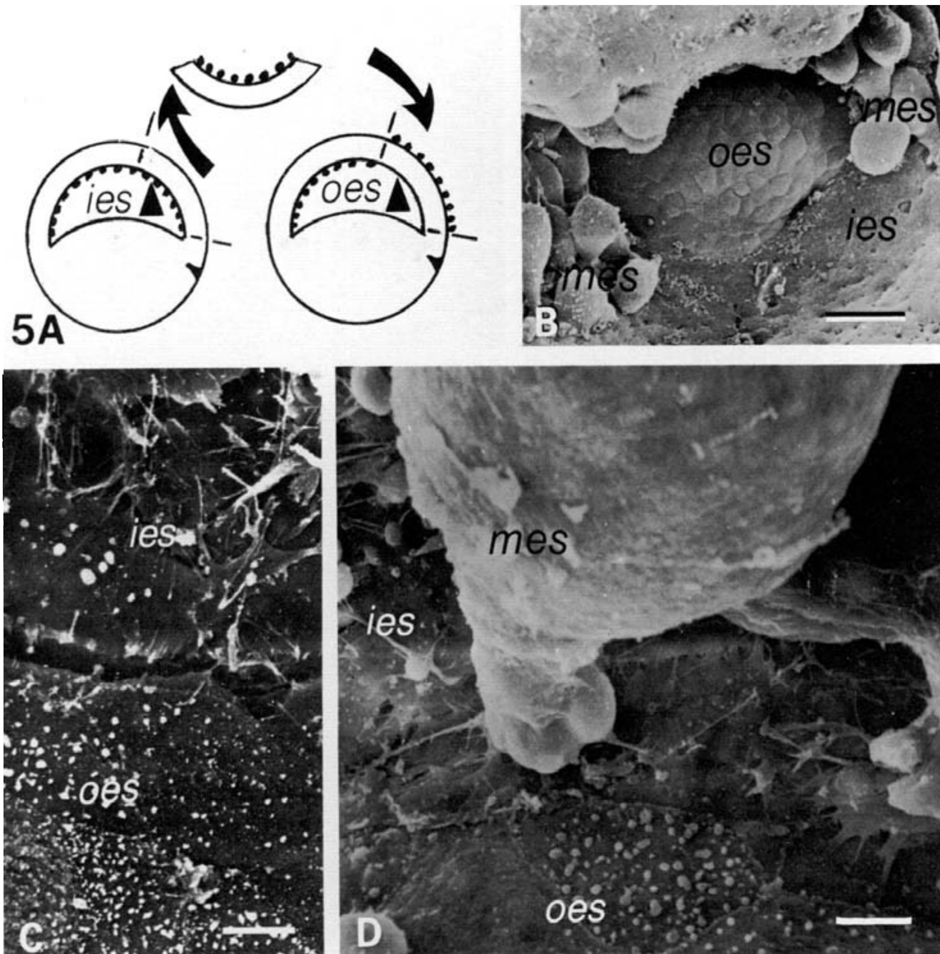

Fig. 5. Involvement of the extracellular matrix in morphogenetic movements of gastrulation. (A) Schematic representation of the grafting procedure. Inverted ectodermal explant is grafted on the future dorsal region of the embryo, above the blastoporal lip. (B–D) Scanning electron micrographs of the blastocoelic roof in gastrulae bisected 2 h after grafting. (B) Pioneer mesodermal cells are migrating laterally to the inverted ectodermal explant. Mesodermal cells adhere to the inner surface of ectodermal cells but do not contact the surface of the grafted explant. (C) Boundary between the inner ectodermal surface of the blastocoelic roof and the outer ectodermal surface of the grafted explant. Note the presence of numerous filopodia on the inner ectodermal surface, whereas the outer ectodermal surface is covered by short microvilli. (D) A pioneer mesodermal cell is migrating near the boundary of the grafted explant. Lamellipodia and filopodia adhere to the inner surface of ectodermal cells, but not to the inverted explant. Scale bars: (B) 100 μm; (C,D) 10μm. *ies*, inner ectodermal surface; *oes*, outer ectodermal surface; *mes*, mesoderm.

an elongated process (Fig. 4A). Transmission electron microscopy show that filopodia are aligned with gold-labelled extracellular fibrils (Fig. 4B). FN is not detected on mesodermal cell surface by immunogold staining (Fig. 4B).

These results clearly show the presence of a FN fibrillar network prior to gastrulation: the FN fibrils cover the entire surface of the roof of the blastocoel on which mesodermal cells will migrate. These results also indicate a possible direct interaction between mesodermal cells processes and FN during active migration.

Grafting experiments

Preliminary evidence for the involvement of the extracellular matrix in mesodermal cell migration has been obtained by grafting an inverted part of the roof of the blastocoel. In grafted gastrulae, migrating mesodermal cells avoid the inverted explant which is denuded of FN fibrils (Fig. 5). Scanning electron microscopic observations showed that neither filopodia nor lamellipodia which extended from mesodermal cells are able to adhere to the outer surface of ectodermal cells (Fig. 5). These findings are consistent with a direct role for the FN-rich extracellular matrix in mesodermal cell adhesion and spreading.

Injection of monovalent anti-FN antibodies or synthetic peptides

A much more direct demonstration of the involvement of FN in cell migration comes from the inhibition of the function of this molecule (Fig. 6). One possibility is to inhibit cell interactions with the cell-binding domain, using monovalent antibodies to FN (Fig. 7A,B). A more sophisticated procedure uses a specific peptide taken from the cell-binding site of FN to prevent cell surface receptor

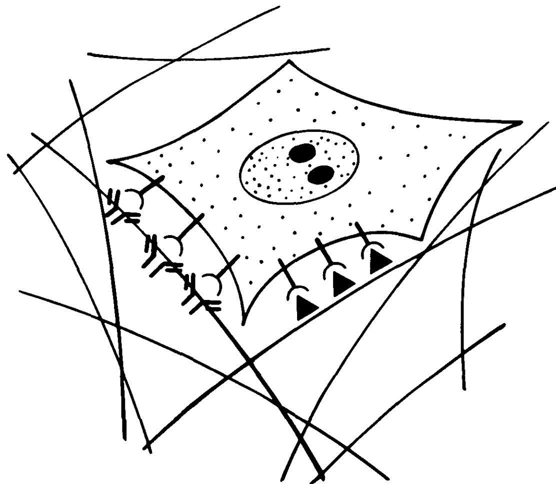

Fig. 6. Perturbation of cell migration in a FN substrate. Antibodies to FN, or specific peptides (dark triangles) taken from the cell-binding site, prevent cell surface receptors from interacting with the cell-binding domain of FN. Both reagents provide the same results through steric hindrance or competitive inhibition.

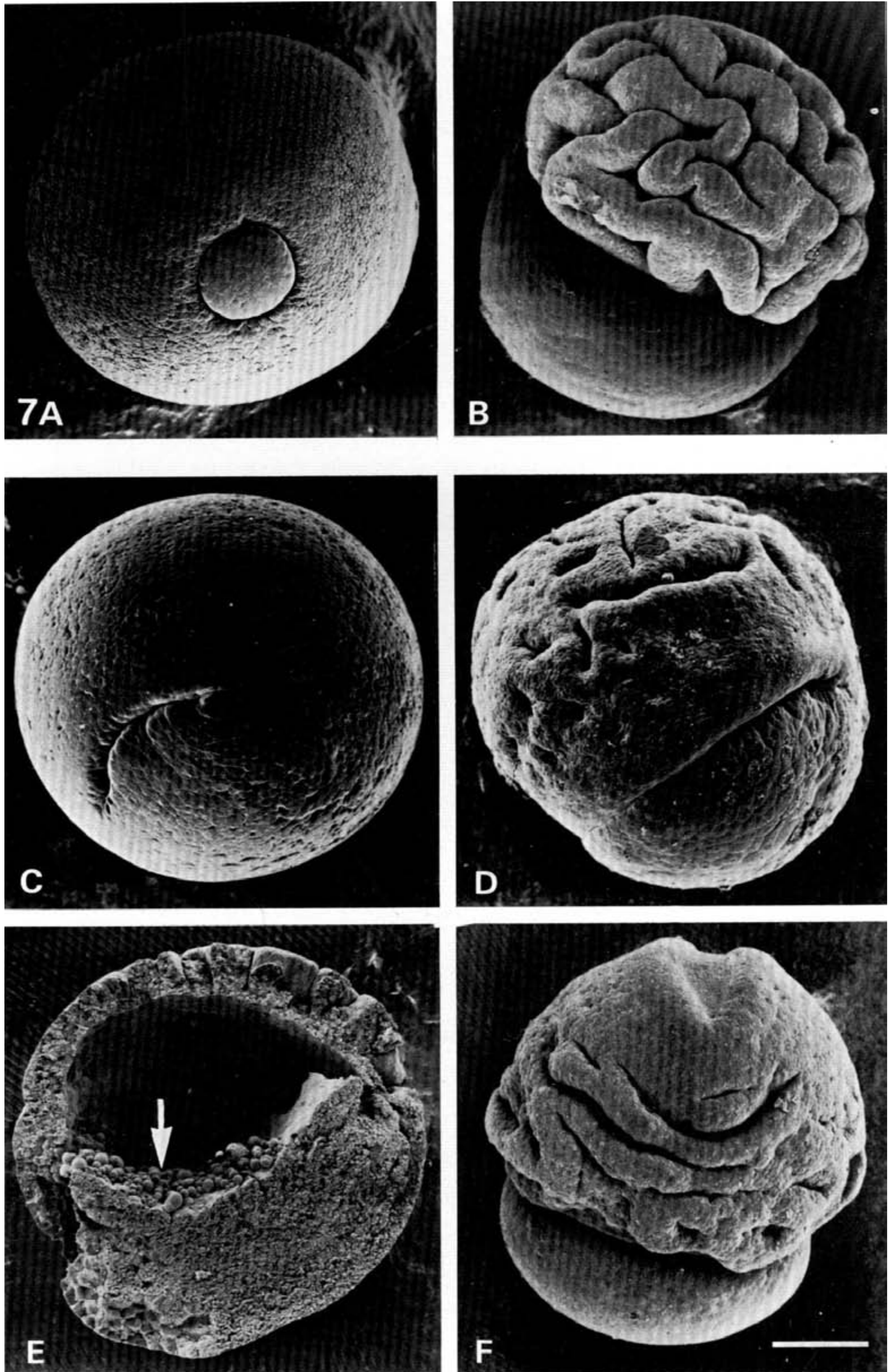

Fig. 7

interaction with FN (Yamada *et al.* 1984) (Fig. 7C,F). These two complementary approaches have been examined (Fig. 7).

When performed at the blastula or early gastrula stage, microinjections into the blastocoelic cavity of either monovalent antibodies to FN or synthetic peptides (P_1,P_2) completely inhibited the invagination of mesodermal cells. Fig. 7D shows a scanning electron micrograph of an embryo injected with P_1 peptide at the blastula stage. The blastocoel roof is highly convoluted with deep ectodermal furrows. The non-invaginated endodermal mass is segregated in the vegetal region, beneath a blastopore-like structure found around the egg circumference. Interestingly a sagittal section through such an arrested gastrula injected with P_1 reveals that, despite the presence of a small blastopore, mesodermal cells did not migrate along the blastocoel roof (Fig. 7E). Furthermore, presumed mesodermal cells are dispersed on the blastocoel floor, near the site of invagination.

Gastrulation is inhibited when embryos are injected with monovalent antibodies to FN or synthetic peptides (P_1, P_2) at midgastrula stage. Later, a partial neural plate developed in the area of contact between already invaginated mesoderm and the overlaying ectoderm: such an injected embryo observed at the corresponding neural stage is shown in Fig. 7F.

Control experiments performed with appropriate controls including preimmune monovalent antibodies, antibodies preincubated with FN, synthetic peptide P_3, or a similar size peptide from ACTH, produced normal gastrulae.

DISCUSSION

It is a remarkable fact that until very recently, there was no awareness that the amphibian gastrula contains an elaborate FN-rich extracellular matrix which permits the migration of mesodermal cells. Studies on the effect of monovalent antibodies to FN and synthetic peptides provide the first clear evidence for such a

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Fig. 7. Effects of monovalent antibodies to FN or of specific peptides on the gastrulation of *Pleurodeles waltl* embryos. (A) Injection of monovalent preimmune antibodies at the late blastula stage; the fixation procedure is performed 24h later. The embryo develops normally. Note the small yolk plug at the site of invagination. (B) Injection of monovalent antibodies to FN at the late blastula stage; the fixation procedure is performed 24h later. A complete inhibition of gastrulation is visible. Note that the blastocoel roof becomes folded whereas the non-invaginated endodermal hemisphere remains smooth. (C) Control embryo injected with P_3 ($10\,mg\,ml^{-1}$) at late blastula stage. 6 h after the injection the gastrulation occurs normally. (D) Embryo injected with P_1 ($10\,mg\,ml^{-1}$) at late blastula stage, then fixed 6 h later. A clear-cut inhibition of the gastrulation is apparent. (E) Sagittal section of an embryo corresponding to (D): the migration of cells is inhibited. Presumed mesodermal cells are found on the blastocoel floor (arrow). Note the highly convoluted aspect of the ectodermal cap, whereas the inner ectodermal surface facing the cavity of the blastocoel remains smooth. (F) Injection of P_1 ($10\,mg\,ml^{-1}$) at midgastrula stage; the fixation procedure is performed 24 h later. The gastrulation is blocked. A partial neural plate develops dorsally. Note that deep ectodermal furrows appear ventrally whereas the non-invaginated yolk plug is prominent. Scale bar: $0{\cdot}3\,\mu m$.

role. In the presence of anti-FN antibodies, mesodermal cells cannot migrate actively along the blastoporal roof. A very similar phenomenon is observed in the presence of synthetic peptides containing the cell-binding sequence of FN. Antibodies to FN and peptides are thought to prevent proper interaction between FN and specific surface receptors.

In *Pleurodeles waltl* or *Ambystoma mexicanum* embryos, FN is first detected on cell surfaces at the blastula stage. FN is associated with the surface of ectodermal cells facing the blastocoel. Finally, at the end of gastrulation, staining for FN shows a fluorescent line between the basal surface of ectodermal cells and migrating mesodermal cells. Such a distribution of FN on the roof of the blastocoel has also been recently described in *Xenopus laevis* (Lee *et al.* 1984).

Both indirect immunofluorescence and immunogold staining for FN reveal that FN is organized into a three-dimensional fibrillar matrix. The FN-rich matrix exclusively coats the inner surface of the blastocoel roof from early blastula to late gastrula stages. During gastrulation it increases in complexity, and thus a dense fibrillar network is already present between migrating mesodermal cells and the roof of the blastocoel. Migrating mesodermal cells interact directly with the matrix through their filopodia that contact and follow FN fibrils. On the basis of observations on living embryos or experimentation performed *in vitro*, there is some evidence for an orientation of mesodermal cell locomotion by these fibrils (Nakatsuji *et al.* 1982; Nakatsuji & Johnson, 1984). So far we have not been able to detect any particular orientation of the fibrils: therefore more work is necessary to evaluate the possible role of the extracellular fibrils in the guidance of mesodermal cells.

Whatever the mechanism of control of the orientation of mesodermal cell locomotion, it is of importance to note that FN matrix is already deposited prior to gastrulation. We and Lee *et al.* (1984), have indeed found indications that FN is not associated with the surface of mesodermal cells, whereas these cells bind to FN fibrils. In fact it has been reported that FN is not produced and (or) retained by migrating cells. Such is the case for neural crest cells (Newgreen & Thiery, 1980) and primordial germ cells (Wylie & Heasman, 1982). Interestingly *in vitro* heart fibroblasts acquire the capacity to retain FN at their surface after a period of migration and concomitantly become stationary (Couchman, Rees, Green & Smith, 1982). To migrate efficiently, transient interactions must occur between cells and the three-dimensional matrix. This property may reflect the presence of FN receptors which could differ either in number, distribution and (or) affinity from those found on stationary cells.

It has been our working hypothesis that the FN matrix provides a substrate for mesodermal cells to migrate. Consequently, studies have been performed by disrupting the matrix and inhibiting FN-cell interactions with specific reagents (Boucaut *et al.* 1984*a,b*). First, microsurgical inversion of part of the blastocoel roof is followed by an inhibition of mesodermal cell adhesion at the site of inversion, where no FN is available. This result is consistent with the postulate that FN fibrils

are involved in the migration of mesodermal cells. Secondly, the effect of monovalent antibodies to FN indicate that the binding of mesodermal cells to FN is required for gastrulation. Further evidence for this concept comes from experimentation with synthetic peptides taken from the cell-binging domain of FN. These peptides are convenient competitive inhibitors of FN function. With these synthetic peptides as probes, it is possible to show parallel loss of binding of mesodermal cells to FN as well as inhibition of gastrulation. It is important that all these effects can be demonstrated *in vivo*. They argue very strongly for our prediction that by mediating mesodermal cells migration, FN plays a major role in amphibian gastrulation. These studies also point out that the control mechanism is probably not exerted at the level of FN synthesis and assembly, but may be linked to the behaviour of FN receptors (Darribère *et al.* 1984; Lee *et al.* 1984).

In conclusion, the results presented here show that amphibian gastrulation is of great interest to elucidate the mechanism by which FN may control embryonic cell migration *in vivo*. The amphibian gastrula provides an attractive model in which locomotion of cells can be related to spatial and temporal changes in cell surface molecules.

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DISCUSSION

Speaker: T. Darribère (Paris)

Question from D. Smith (Purdue):
You said that fibronectin is synthesized continuously through oogenesis and early cleavage. Is it secreted into the medium and have you done any fluorescent studies to see where it is localized?

Answer:
We cannot find fibronectin by fluorescence during oogenesis probably because there is too little accumulated. During cleavage, I think it is secreted into the blastocoel.

Question from R. Laskey (Cambridge):
You showed an increase in fibronectin synthesis which appeared to precede mid-blastula. Does it actually occur before the mid-blastula transition or after?

Answer:
It follows the mid-blastula transition, and I think in *Xenopus* it is the same. But fibronectin synthesis is independent of transcription and involves activation of maternal mRNA.

Question from I. Dawid (NIH, Bethesda):
Is anything known in detail about the cellular receptors for fibronectin?

Answer:
No, not in amphibian embryos.

Question from H. Woodland (Warwick):
Is it known which cells of the blastula make fibronectin?

Answer:
We think that all cells synthesize fibronectin but only ectodermal cells have the receptors which can bind it.

Question from J. Slack (ICRF, London):
When I stained some *Xenopus* gastrulae with an anti-fibronectin antibody from Chris Wylie I did see the deposit on the inside of the blastocoel as you described, but it wasn't nearly as intense as in your pictures. I also saw it around all of the other cells in the embryo. Which species were your experiments done with?

Answer:
Axolotls and *Pleurodeles*. In both we detected fibronectin containing matrix at the early blastula stage. Neither immunofluorescence nor the immunogold technique revealed any fibronectin on endodermal and mesodermal cell surfaces.