Development 120, 3227-3234 (1994)
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YIGSR domain of laminin binds surface receptors of mesenchyme and stimulates migration during gastrulation in sea urchins

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SUMMARY

During gastrulation in sea urchins, cells at the tip of the archenteron extend filopodia that attach to the blastocoel wall and are thought to assist in the elongation of the archenteron. Upon completion of gastrulation, these cells migrate into the blastocoel. Time-lapse video records were made of preparations from which ectodermal cells have been removed, leaving the archenteron, mesenchyme cells and blastocoelar extracellular matrix (ECM) bounded by the basal lamina. In preparations of late gastrulae, cells at the tip of the archenteron extend filopodia that attach to the basal lamina and pull it inward, collapsing the preparation. This collapse does not occur in preparations made prior to the elongation phase and can be inhibited with cytochalasin B and azide, but not with colchicine. Migratory behavior increased in preparations treated with the laminin-derived peptide Tyr-Ile-Gly-Ser-Arg (YIGSR). Cells extend and retract filopodia, collapse the ECM and migrate out of the preparation. This behavior was not observed in preparations treated with whole laminin, fibronectin or Arg-Gly-Asp-Ser (RGDS) peptides. Cells in preparations treated with YIGSR extend significantly more processes than those incubated in RGDS, laminin, fibronectin or BSA. This effect is titratable between 10^{-3} and 10^{-6} M. Whole laminin has a significant inhibitory effect on the number of cell processes observed. Double labelling experiments with biotinylated laminin or biotinylated CDPGYIGSR and a mesenchyme-specific monoclonal antibody (Sp12) reveal that laminin and CDPGYIGSR label mesenchymal and non-mesenchymal cells. A CDPGYIGSR affinity column binds a ^{125}I-labelled cell surface component, which elutes with YIGSR and has an M_r of about 80×10^3 on SDS-PAGE. We propose that cells at the tip of the archenteron attach to the basal lamina during archenteron elongation, and that domains of laminin containing YIGSR in the basal lamina of the target region stimulate migratory behavior in these cells.

Key words: gastrulation, sea urchin, secondary mesenchyme, laminin receptors, extracellular matrix, morphogenesis, YIGSR

INTRODUCTION

Studies of the mesenchyme cells of sea urchin embryos have revealed that these migratory cells play several fundamental roles in morphogenesis (Harkey, 1983; Wilt, 1987; Ettensohn, 1991; McClay et al., 1992). Most of these studies have dealt with primary mesenchyme cells, which form the larval skeleton; however, a second group of mesenchyme, which ingresses after the skeleton-forming cells, are also proving instructive (Ettensohn and Ingersol, 1992). Secondary mesenchyme includes the chromogenic mesenchyme, the myogenic mesenchyme and a population of cells that ingress upon completion of archenteron extension to form a complex network of fibroblast-like cells within the blastocoel (Gibson and Burke, 1985; Ishimoda-Takagi et al., 1984; Tamboline and Burke, 1992).

Secondary mesenchyme cells are thought to play important roles in morphogenesis. One of the original proposals was that secondary mesenchyme at the tip of the archenteron assist with the elongation of the archenteron by pulling on the blastocoel roof (Gustafson and Kinnander, 1956; Dan and Okazaki, 1956). These cells extend long filopodia when the archenteron is only partly extended which attach to the blastodermal wall and maintain this attachment as the archenteron continues its elongation. The presence of microfilaments in the filopodia (Tilney and Gibbins, 1969) suggests that the cells at the tip of the archenteron are pulling or guiding the archenteron to its target. Laser ablation of these cells indicates that they are essential to completion of archenteron elongation (Hardin, 1988). During elongation the filopodia undergo cycles of extension, attachment to the blastocoelar wall and retraction (Hardin and McClay, 1990). When the tip of the archenteron nears the animal pole, filopodia make contact with a region of the blastocoel wall to which they make long-lived attachments. Eventually the cells that contact this region release from the tip of the archenteron and migrate into the blastocoel. Experimental manipulations of embryos have led to the hypothesis that the target region is qualitatively different from other regions of the inner blastoderm (Hardin and McClay, 1990).

The nature of the target substance that alters the behavior of secondary mesenchyme cells is not known, nor is there direct evidence for the presence of specific receptors on the surface of the mesenchyme.

Here we describe preparations of embryos from which the ectodermal cells have been removed. In these preparations, specific aspects of the behavior of secondary mesenchyme can be monitored and altered by treatment with peptides from the cell-binding domains of laminin. We provide evidence that the cells at the tip of the archenteron attach to the basal lamina, and that interactions between a domain of laminin and a cell surface receptor appear to initiate migratory behavior.

MATERIALS AND METHODS

Embryonic bag preparations

Bag preparations were based on Harkey and Whitely (1980). Gametes were collected from adult *S. purpuratus* by intracoelomic injection of 0.55 M KCl. Eggs were rinsed with filtered sea water (FSW) and fertilized with a dilute suspension of sperm. Embryos were incubated in FSW at 12˚C, collected by gentle centrifugation and resuspended in calcium-/magnesium-free sea water (CMFSW) containing 1 mM EDTA (pH 8.0) at 4°C. Embryos were pelleted at 250 ***g*** and resuspended in CMFSW+1 mM EDTA twice more, before pelleting and resuspending in bag isolation media (BIM) (40% CMFSW+1 mM EDTA, 40% 1 M dextrose, 20% distilled H_2O, pH 8.0).

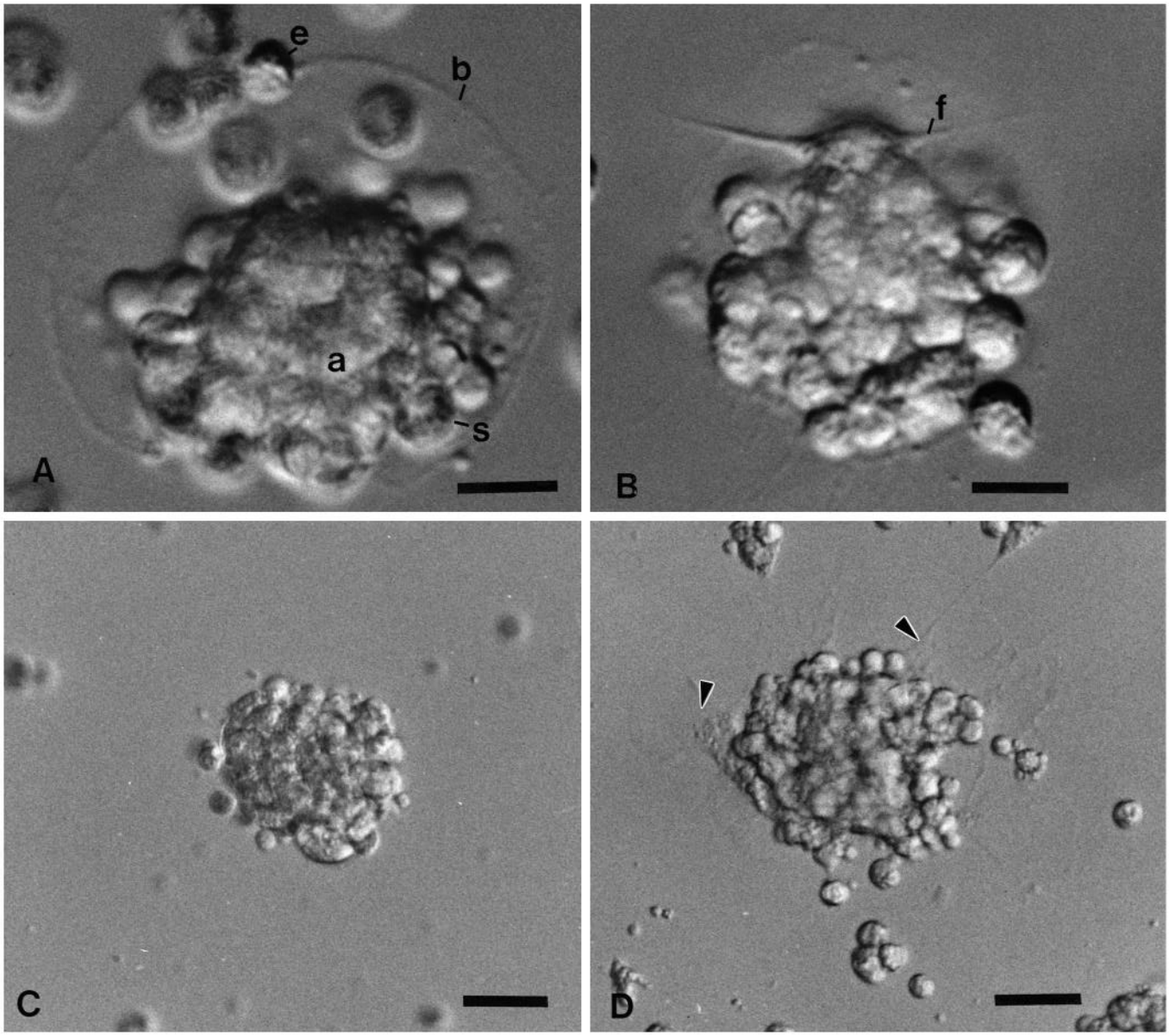
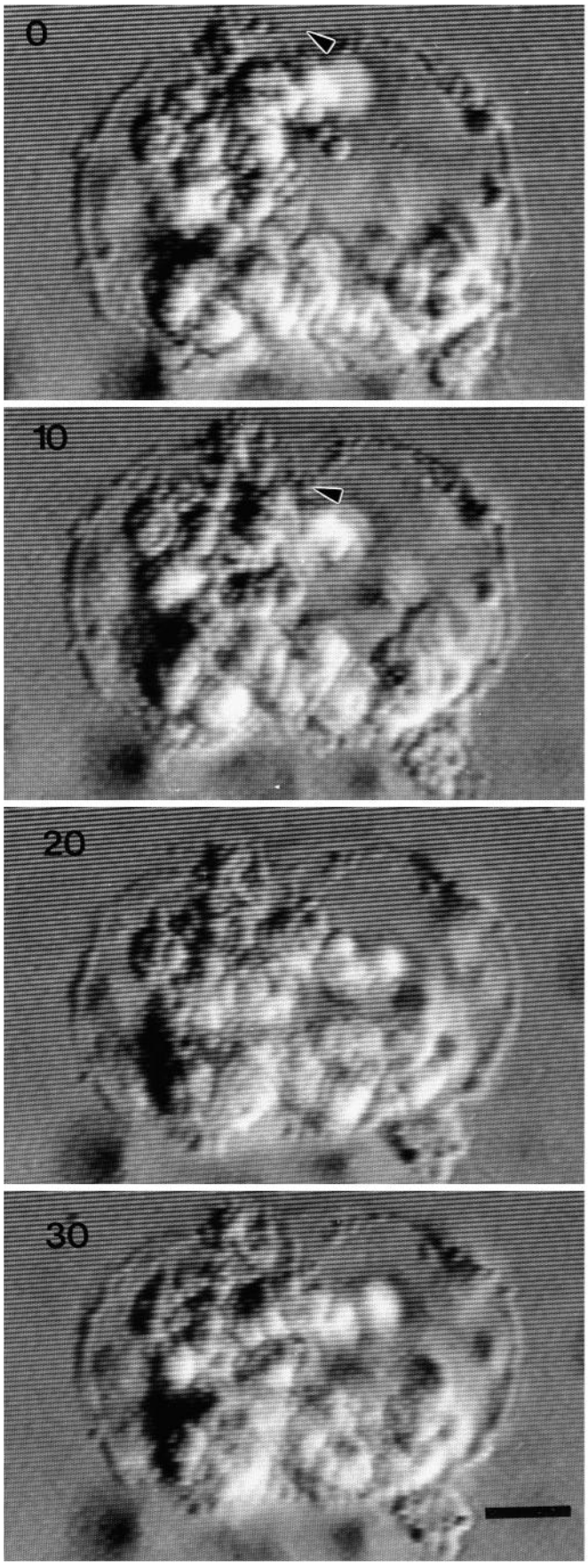


Fig. 1. Nomarski DIC images of bag preparations of *S. purpuratus* embryos. The ectodermal cells are removed leaving the archenteron and mesenchyme within the blastocoelar ECM. (A) Bag preparation of a 2/3 gastrula. The basal lamina (b) contains the archenteron (a) and skeletogenic mesenchyme (s). A few ectodermal cell (e) remain attached. Bar, 10 μm. (B) A similar preparation to that shown in A, the filopodial (f) processes of secondary mesenchyme cells at the tip of the archenteron are apparent. Bar, 10 μm. (C) Untreated bag preparation which has undergone contraction. Bar, 20 μm. (D) Bag preparation treated with 1 mM YIGSR. Following contraction, processes extend from the periphery and attach to the substratum. Bar, 20 μm.



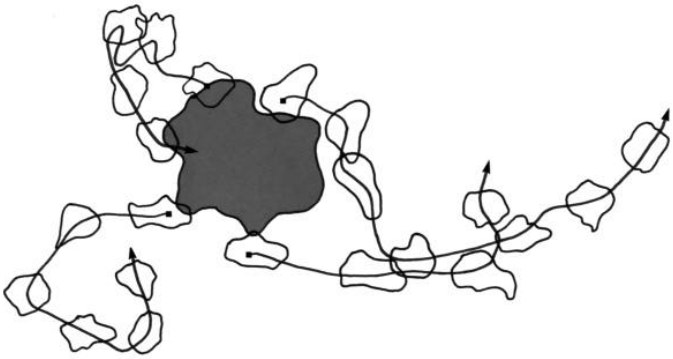


Fig. 3. Composite tracing from time-lapse video records of cell migrations from a collapsed bag preparation treated with 1 mM YIGSR. Outlines of individual cells are separated by 10 minute intervals. The migrations of the cells overlap in time, but are not synchronous. Scale bar, 20 μm.

Embryos were incubated in BIM on ice for 5 minutes or until ectodermal cells had come away from the matrix. Bag preparations were then collected by centrifugation at 650 ***g*** for 3 minutes over a sucrose step gradient. Gradients consisted of a sucrose stock pad (1 M Sucrose, 100 μM EDTA, 1 mM tris Base, pH 8.0) overlaid with 50% and 30% sucrose layers (diluted with BIM). Preparations were then rinsed in FSW and observed in FSW containing 250 μg/ml streptomycin sulphate (pH 8.0) at 12˚C.

Cell behavior

Bag preparations made from 2/3 gastrulae were incubated in 1 mM YIGSR or 1 mM RGDS (Sigma) in FSW+250 μg/ml streptomycin sulphate at 12˚C. Preparations were recorded for 8 to 12 hours using a Nikon inverted DIC microscope fitted with a controlled temperature stage and a time-lapse videocamera.

In inhibition experiments, bag preparations of 2/3 gastrulae were incubated in FSW+250 μg/ml streptomycin sulphate containing 1 mM cytochalasin B, or 0.05% sodium azide, or 1 mM colchicine, or without additives. Preparations were incubated for 4 hours at 12˚C, and then 50 preparations from each treatment were scored for contraction.

Cell motility was quantified on the basis of numbers of cell processes observed extending from collapsed bags under various conditions. Processes occurred as either long narrow projections extending through the basal lamina and making contact with the substratum (filopodia), or as broad flattened projections through the basal lamina, spreading onto the substratum (lamellapodia). Preparations were scored blind. Comparison of the effects of different compounds on cell motility was carried out using bag preparations made from 2/3 gastrulae incubated in FSW+250 μg/ml streptomycin sulfate containing either 1 mM YIGSR, 1 mM RGDS, 0.085 mg/ml EHS laminin, 0.025 mg/ml fibronectin, or 0.1 mg/ml BSA. Preparations were incubated for 4 hours at 12˚C, after which equal numbers of preparations were scored from each treatment. Analysis of the effect of YIGSR concentration on cell motility was conducted similarly, but with each preparation incubated in FSW+250 mg/ml streptomycin sulfate plus varying concentrations of YIGSR.

Fig. 2. Time-lapse video preparations of a bag preparation of *S. purpuratus* embryo (2/3 gastrula). The initial stages of contraction and collapse of the preparation are shown in this sequence. The basal lamina associated with the secondary mesenchyme cells at the tip of the archenteron deforms suggesting processes of the secondary mesenchyme cells attach to it and contract during collapse Scale bar, 10 μm.

Cell labelling

EHS laminin (Boehringer Mannheim) and CDPGYIGSR-NH_2 (Sigma) were biotinylated using a sulfo-NHS-biotin kit (Pierce). Bag preparations were made from 2/3 gastrulae, and dissociated by passing through 20 μm nitex. Dissociated cells were incubated in the presence of one or the other biotinylated ligand in FSW for 1 hour at 12˚C, then spun at 500 ***g*** and resuspended in FSW three times before fixation in 4% formaldehyde (20 minutes at room temperature). In double labelling experiments, fixed cells were then incubated with Sp12 ascites fluid (Tamboline and Burke, 1989) diluted at 1:100 in PBS for 2 hours, rinsed 3 times in PBS, incubated in rabbit anti-mouse IgG-TRITC diluted at 1:500 as well as avidin-FITC diluted at 1:1000 in PBS for 1 hour at room temperature, and rinsed three times in PBS. Preparations were then viewed using a Zeiss epifluorescence microscope.

Affinity chromatography

A laminin affinity column was made by linking 4 mg mouse EHS laminin (Boehringer) to 1 ml CNBr-activated sepharose 4B (Pharmacia). ^{125}I-labelled membrane extract was then prepared as follows. Mid-gastrulae were dissociated using hyaline extraction medium (Fink and McClay, 1985) (300 mM glycine, 300 mM NaCl, 10 mM KCl, 10 mM $MgSO_4$, 10 mM Tris-base, 2 mM EGTA, pH 8.0) and passage through 20 μm Nitex. 10^7 cells were collected, rinsed thrice in FSW, and surface iodinated (0.5 mCi Amersham) using Iodobeads (Pierce). Labelled cells were checked for viability using trypan blue exclusion, pelleted at 14000 ***g***, lysed by freezing, rinsed thrice in FSW, and finally extracted with 2 ml of 100 mM n-octyl-glucoside. Aqueous rinse and detergent fractions were counted in a gamma-counter to determine efficiency of labelling, and surface specificity. Labelled membrane extract was applied to the equilibrated column and incubated for 1 hour at room temperature before eluting unbound material with 20 volumes of Tris-buffered FSW. Four volumes of 1.5 mM YIGSR in buffered FSW was then applied, followed by four volumes of buffered CMFSW+2 mM EDTA and finally four volumes of 50 mM diethylamine, pH 11.5. Radioactive fractions were dialyzed overnight against distilled water and concentrated by vacuum centrifugation.

A second affinity column was made by linking 2 mg of CDPGYIGSR-NH_2 to 2 ml of SulfoLink gel (Pierce). An ^{125}I-labelled membrane extract was prepared from 2.1×10^8 2/3 gastrulae cells as described above, using 1 mCi of ^{125}I. Extract was applied to the affinity column and allowed to bind for 1 hour at room temperature, before unbound material was rinsed off with 10 volumes of FSW. After all unbound material was removed, YIGSR-binding material was eluted using 4 ml of 1.5 mM YIGSR in FSW into tubes containing horse heart myoglobin (1 mg/ml) as a carrier. This material was concentrated and desalted using a centriprep-10 ultrafiltration apparatus (Amicon), and further concentrated using vacuum centrifugation. Concentrated sample was then run on a 10% polyacrylamide gel under reducing conditions. The gel was dried and autoradiographed.

RESULTS

Bag preparations

Bag preparations consist of the blastocoelar extracellular matrix and the cells which were in the blastocoel at the time the embryos were collected. Thus, bag preparations made from 2/3 gastrulae contain primary mesenchyme cells and the cells of the archenteron, surrounded by the blastocoelar extracellular matrix including its basal lamina (Fig. 1). Typically, embryos prepared at this stage undergo this sequence: cells at the tip of the archenteron extend filopodia that attach to the

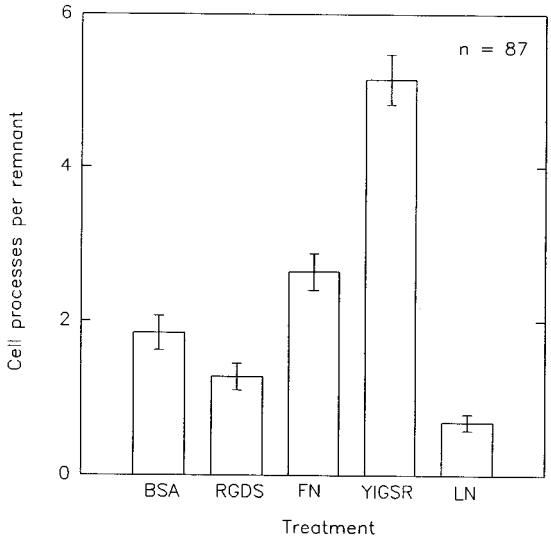


Fig. 4. Quantification of the response of cells to YIGSR. Blastocoelar ECM preparations of 2/3 gastrulae were incubated on sealed coverslips in FSW containing 0.1 mg/ml BSA, 1 mM RGDS, 25 μg/ml fibronectin (FN), 1 mM YIGSR, or 85 μg/ml laminin (LN). Equal numbers from each treatment were then scored for cell processes extending from the collapsed preparations. YIGSR-treated preparations extended significantly (P<<0.01) more processes than preparations in any other treatment. Laminin-treated preparations extended significantly (P<<0.01) fewer processes. Error bars represent standard error.

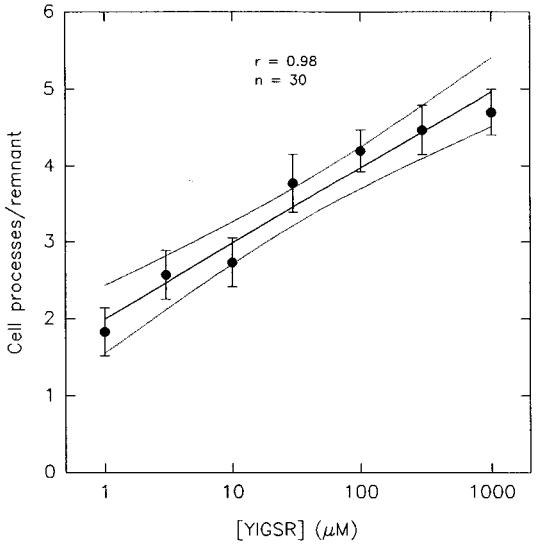


Fig. 5. Correlation of cell process counts to the concentration of YIGSR. Blastocoelar ECM preparations of 2/3 gastrulae were incubated in FSW containing YIGSR and 30 preparations from each treatment were scored for cell processes (see Fig. 1D). The solid line represents a linear regression of the data, with the dotted lines showing 95% confidence intervals. Error bars represent standard error. Preparations treated with control compounds had the following numbers of cell process (mean±s.d.): BSA 0.91±0.34, laminin 0.24±0.15,RGDS 1.33±0.41, and fibronectin 0.65±0.31.

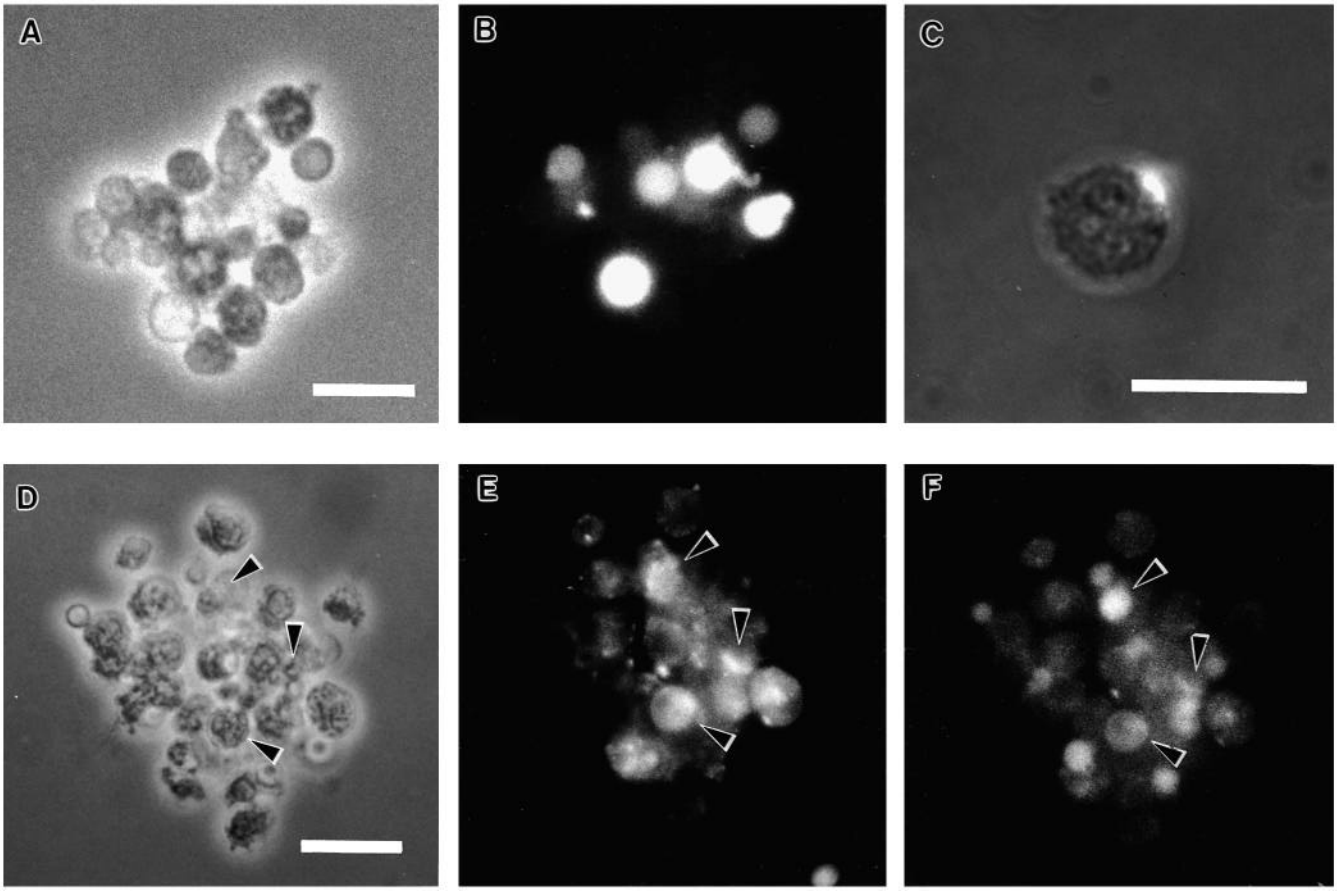


Fig. 6. Cell labelling with biotinylated ligands. Dissociated cells from BECM preparations were incubated in FSW containing either biotinylated laminin or biotinylated CDPGYIGSR for 1 hour. Cells were then rinsed and fixed. Biotinylated label was visualized using avidin-FITC and epifluorescence microscopy. In double labelling experiments, cells were probed with Sp12, a mesenchyme-specific MAb, after fixation, and RAM-TRITC was uses as well as avidin-FITC. (A) Phase contrast image of cell aggregate; (B) laminin labelling of same aggregate; (C) capping of laminin labelling on dissociated cell; (D) phase contrast image of cell aggregate; (E) Sp12 labelling of same aggregate; (F) CDPGYIGSR labelling of same aggregate (arrows indicate cells that are labelled by both Sp12 and CDPGYIGSR). Scale bars, 20 μm.

basal lamina, the basal lamina indents at the point of attachment and, as the filopodia contract, the ECM collapses (Fig. 2). The entire sequence takes about 3 to 5 hours at 12˚C. Preparations made from embryos after the primary mesenchyme have ingressed, but before the invagination of the archenteron, do not collapse. Contraction of the basal lamina is blocked by incubation of the preparations in 0.05% sodium azide (11.0±6.0% collapsed, s.d. n=50), or 1 mM cytochalasin B (21.0±3.8% collapsed, s.d. n=50), but not by incubation in 1 mM colchicine (88.5±10.3% collapsed, s.d. n=50). In untreated preparations 95.5±3.0% collapsed (s.d. n=50).

Effects of YIGSR

YIGSR does not interfere with the normal collapse of bag preparations, however cellular motility is enhanced (Fig. 1D). In time-lapse video records, individual mesenchyme cells migrate out of the preparations (Fig. 3). The cells extend numerous processes through the basal lamina, which attach to the substratum and draw the cells out of the preparation. The mean rate of movement is 3.59±0.96 μm/minute (s.d., n=5). In similar records of preparations not treated with YIGSR or treated with other peptides, the basal lamina collapses, but cells do not leave the preparation.

After 4 hours in 1 mM YIGSR, preparations have significantly more (P<<0.01) cell processes extending from them than preparations incubated in RGDS, BSA, fibronectin, or laminin (Fig. 4). The preparations incubated in laminin have significantly fewer (P<0.0001) cell processes than any of the other treatments. The number of cell processes extending out of the preparations correlates with the concentration of YIGSR over a range of 10^{-3} and 10^{-6} M (r=0.98) (Fig. 5).

Cell labelling

Biotinylated laminin binds to a subset of dissociated cells. The most common type are small round cells which, in double labelling experiments, are Sp12 negative. However, many larger Sp12-positive cells are also labelled and they frequently cap the ligand (Fig. 6). Labelling with biotinylated CDPGYIGSR is weaker than with laminin, and is less frequently observed on the small Sp12-negative cells (Fig. 6). Neither label is bound by only Sp12-positive cells, nor only Sp12-negative cells.

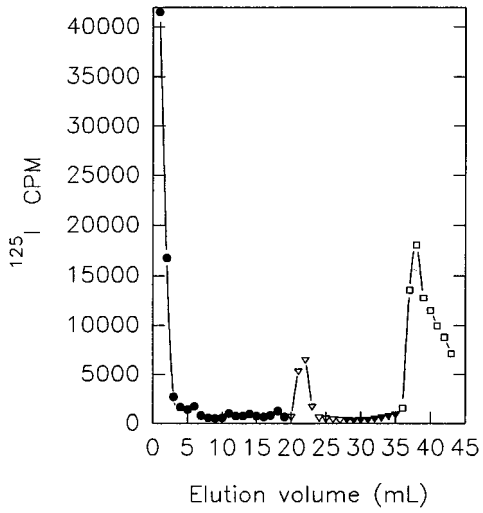


Fig. 7. Elution profile from a laminin affinity column to which ^{125}I-labelled cell surface extracts were applied. ●, flow through; ▽, 1.5 mM YIGSR; ▼, CMFSW + 2 mM EDTA; □, diethylamine ph 11.5.

Affinity chromatography

In the laminin affinity experiments, 50% of the ^{125}I was incorporated by the cells. Only 10% of incorporated radioactivity was found in the water soluble rinses of lysed cells, leaving 90% in the octylglucoside soluble membrane extract. Of the components in this extract that bind laminin, a single peak of labelled material was eluted with 1.5 mM YIGSR (Fig. 7). Subsequent rinses with CMFSW+2 mM EDTA failed to elute any radiolabelled material, but the high pH rinse eluted a large broad peak. SDS-PAGE of YIGSR eluted material failed to produce a convincing band on autoradiographs. The material eluted by high pH runs at 150, 130, 105 and 80 K (data not shown).

In experiments run on the peptide affinity column, 73% of incorporated label was found in the detergent extract. After several column volumes of FSW, all unbound radioactivity had been eluted. A single peak of labelled material was released with 1.5 mM YIGSR. Autoradiographs reveal some labelled material at the interface between the stacking and the resolving portions of the gel and a single broad band with a sharper band in it. The relative mobility of the sharp band is 80 K (Fig. 8). This material makes up 1.5×10^{-3}% of the labelled membrane extract.

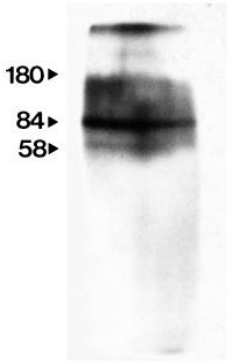


Fig. 8. CDPGYIGSR affinity chromatography. ^{125}I-labelled cell surface components were run over a CDPGYIGSR affinity column. Unbound radioactivity was eluted with FSW. A single peak of radioactivity was eluted with 1.5 mM YIGSR, run on PAGE and autoradiographed. Arrows indicate mobility of molecular weight standards.

DISCUSSION

Archenteron elongation is thought to be the result of two cooperative processes; cell rearrangement and contraction of filopodia attached to the blastocoel roof. In bag preparations, the deformation and collapse of the ECM appears to result from contraction of filopodia attached to the basal lamina. The collapse of the ECM only occurs in preparations of late stages of gastrulae, in which there are filopodia extending from the cells at the tip of the archenteron. This process is inhibited by sodium azide, indicating that metabolically active cells are necessary. As well, cytochalasin B but not colchicine interferes with collapse of the ECM, demonstrating that ECM collapse is mediated by mechanisms similar to those proposed to mediated filopodial contraction (Tilney and Gibbins, 1969, Hardin, 1988). The work of a number of authors has provided convincing evidence that the filopodia of the cells at the tip of the archenteron are necessary for the completion of archenteron extension (reviewed in McClay et al., 1991). Thus, it is reasonable to conclude that the filopodial attachment and contraction which causes the collapse of bag preparations is the same force that causes the final phase of archenteron extension in intact embryos.

The observation that the filopodia attach to the basal lamina and pull it inward suggests that there are components in the basal lamina that anchor the filopodia. In whole embryos, mesenchymal filopodia explore the basal lamina, occasionally penetrating it and making contact with the overlying ectoderm (Spiegel and Spiegel, 1992). This observation supports the suggestion that the attachment of filopodia from the tip of the archenteron to the roof of the blastocoel may be the result of interactions between the filopodia and the ectoderm. However, although the ectoderm may play a role in aspects of this process such as target region specification, our preparations show that, in the absence of ectoderm, filopodia still attach to, and exert a force on, the basal lamina.

When the archenteron reaches the target region near the animal pole, cells from the tip of the archenteron release and migrate within the blastocoel. In bag preparations incubated in YIGSR, the normal sequence of events leading to collapse of the ECM takes place; however, there is also a increase in migratory activity in these preparations. This migratory behavior, as quantified by the presence of cell processes protruding from the preparations, varies in a dosage-dependent way with the concentration of YIGSR. Curiously, incubation of bag preparations with laminin, which contains a YIGSR sequence in domain III of its B1 chain (Graf et al., 1987), significantly decreases observed cell processes. It may be that a domain of laminin other than YIGSR is also important in regulating cell migration in sea urchins. Alternatively, the apparent opposite effects of vertebrate laminin and YIGSR on

our preparations may reflect a non-linear dose-response curve, or a consequence of presentation context. It should be noted that due to its high molecular weight and limited solubility, the concentrations of laminin used were orders of magnitude lower than the concentrations of YIGSR which have an observable stimulating effect.

Populations of cells within the blastocoel of 2/3 gastrulae bind biotinylated laminin and CDPGYIGSR. These populations appear to include mesenchyme cells, as identified in double labelling experiments with the mesenchyme-specific mAb, Sp12. Laminin labelling is strong and labels both small round Sp12-negative cells, and fewer large irregular Sp12-positive cells. Capping, which is characteristic of multivalent ligands crosslinking surface receptors on cells capable of locomotion, was frequently observed in laminin-labelling experiments. As well, there are both Sp12-positive cells and Sp12-negative cells which are not labelled by laminin. Biotinylated CDPGYIGSR binds to fewer small round Sp12-negative cells than laminin, and similar numbers of large irregular Sp12-positive cells. There are also both Sp12-positive and Sp12-negative cells which do not bind CDPGYIGSR. These observations suggest that the population of cells which binds CDPGYIGSR is a subset of the population which binds whole laminin, and that this population includes mesenchyme cells. This suggests that there may be more than one domain of laminin bound by cell surface receptors in sea urchins.

We have observed that a single cell surface component binds to a CDPGYIGSR affinity column. This material can be eluted with 1.5 mM YIGSR and resolves as a band at 80 K on SDS-PAGE. Laminin affinity chromatography has been used to purify a 67 K laminin-binding protein from cell surface extracts of vertebrate cells (Malinoff and Wicha, 1983; Rao et al., 1983; Wewer et al., 1986; Sugrue, 1988; reviewed in Mecham, 1991). This protein appears to bind laminin with a high affinity via the YIGSR domain (Graf et al., 1987) and may be involved in tumor metastasis (Terranova et al., 1983; Wewer et al., 1986; Rao et al., 1989), establishment of membrane polarity (Salas et al., 1992), specification of positional domains (Rabacchi et al., 1990) and cell differentiation (Grant et al., 1989; Vukicevic et al., 1990). Several attempts at cloning the sequence that encodes this protein have yielded a multi-copy gene which codes a 33 kDa YIGSR-binding polypeptide (Yow et al., 1988; Rao et al., 1989; Fernández et al., 1991; Grosso et al., 1991). Further complicating the issue are the observations that the sequence of the 67 kDa laminin-binding protein is very similar, if not identical to, the sequence of a 67 kDa elastin-binding protein (Grosso et al., 1991), and that the affinity of this receptor appears to be modulated by the presence of lactose (Mecham et al., 1991). Several lines of evidence suggest that the 33 kDa laminin-binding protein is a precursor to the mature 67 kDa cell surface receptor, which may exist as a chimeric protein consisting of a YIGSR-binding domain covalently bonded to a lectin domain (Castronovo et al., 1991a,b).

Laminin in sea urchin embryos is concentrated in the basal lamina of the blastocoel, with maximal concentrations in the animal and vegetal poles (McCarthy and Burger, 1987). Thus, during gastrulation, receptors on the surface of cells at the tip of the archenteron may not be exposed to YIGSR until the archenteron reached the basal lamina at the animal pole of the blastocoel. It is conceivable that once these cells are brought into close contact with the basal lamina the YIGSR receptor is activated stimulating their release from the tip of the archenteron and subsequent migratory behavior. If this hypothesis is correct, the 80 K laminin-binding protein may provide a valuable invertebrate model for investigations of the evolution and behavior of this idiosyncratic and potentially important extracellular matrix receptor.

This research was supported by grants to R. D. B. from the Medical Research Council of Canada and the BC Health Research Foundation. We would like to thank Mike Swallow for valuable suggestions on the manuscript.

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(Accepted 8 August 1994)