Extracellular Matrix Receptors and Their Effects on Cell Behaviour During Gastrulation in the Sea Urchin

by

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We accept this thesis as conforming to the required standard

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ABSTRACT

During gastrulation in sea urchins cells at the tip of the archenteron extend filopodia that attach to the wall of the blastocoel and are thought to assist in the elongation of the archenteron. At the completion of gastrulation these cells ingress and migrate into the blastocoel. Time-lapse video records were made of preparations in which ectodermal cells were removed, leaving the archenteron, mesenchyme cells and blastocoelar extracellular matrix (BECM). In preparations of late gastrulae, cells at the tip of the archenteron extend filopodia which 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. Increased migratory behaviour was observed 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 behaviour was not observed in preparations treated with whole laminin, fibronectin, or Arg-Gly-Asp-Ser (RGDS) peptides. Cells in BECM preparations incubated in YIGSR extend significantly more processes than those incubated in RGDS, laminin, fibronectin or BSA. This effect is titratable between 8 μM and 1 mM. Laminin has a significant inhibitory effect on the number of cell processes observed. Double labelling experiments with biotinylated laminin or biotinylated Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg (CDPGYIGSR) and a monoclonal antibody against mesenchyme cells (Sp12) reveal that laminin and CDPGYIGSR label specific cells within the blastocoel,

some of which are mesenchyme. A laminin affinity column binds several ^{125}I-labelled cell surface components, one of which elutes with YIGSR and has an M_r of 70 k on SDS-PAGE. Eluent from a CDPGYIGSR affinity column contains only a 70 k protein. I propose that cells at the tip of the archenteron attach to, and exert a force on the basal lamina during archenteron elongation, and that YIGSR containing domains of laminin at the roof of the blastocoel interact with a 70 k cell surface receptor which stimulates migratory behaviour in these cells at the completion of gastrulation.

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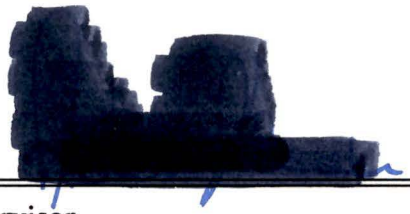

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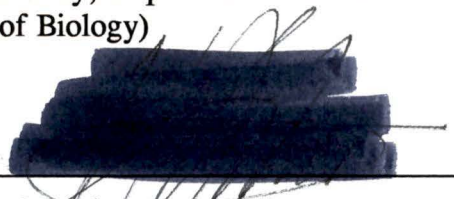

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LIST OF ABBREVIATIONS

BECM	Blastocoelar extracellular matrix
BIM	Bag isolation media
BSA	Bovine serum albumin
CMFSW	Calcium magnesium free sea water
CPM	Counts per minute
DEA	Diethylamine
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol-bis-N-N'-tetraacetic acid
FITC	Fluoresceine isothiocyanate
FN	Fibronectin
FSW	0.22 μm Filtered sea water
k	Relative molecular mass in kilodaltons
kDa	Kilodaltons
LN	Laminin
mAb	Monoclonal antibody
NHS	N-hydroxysuccinimide
PMSF	Phenylmethylsulfonylfluoride
RAM	Rabbit anti-mouse IgG
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TRITC	Tetramethylrhodamine-ß-isothyocyanate

ACKNOWLEDGEMENTS

The individuals who have played important roles in my development as a student are numerous. But of these I am most indebted to Robert Burke, who, perhaps as one of his famous long-shot experiments, accepted me as a student with little supportive data. I hope he is satisfied with the results. I would also like to thank my fellow denizens of Cunningham 066, who have been both enlightening and enjoyable comrades. My family has also been most supportive over the years, and without their encouragement I would certainly have given up long ago. However, my greatest gratitude goes to Robyn Shortt, who makes life fun.

INTRODUCTION

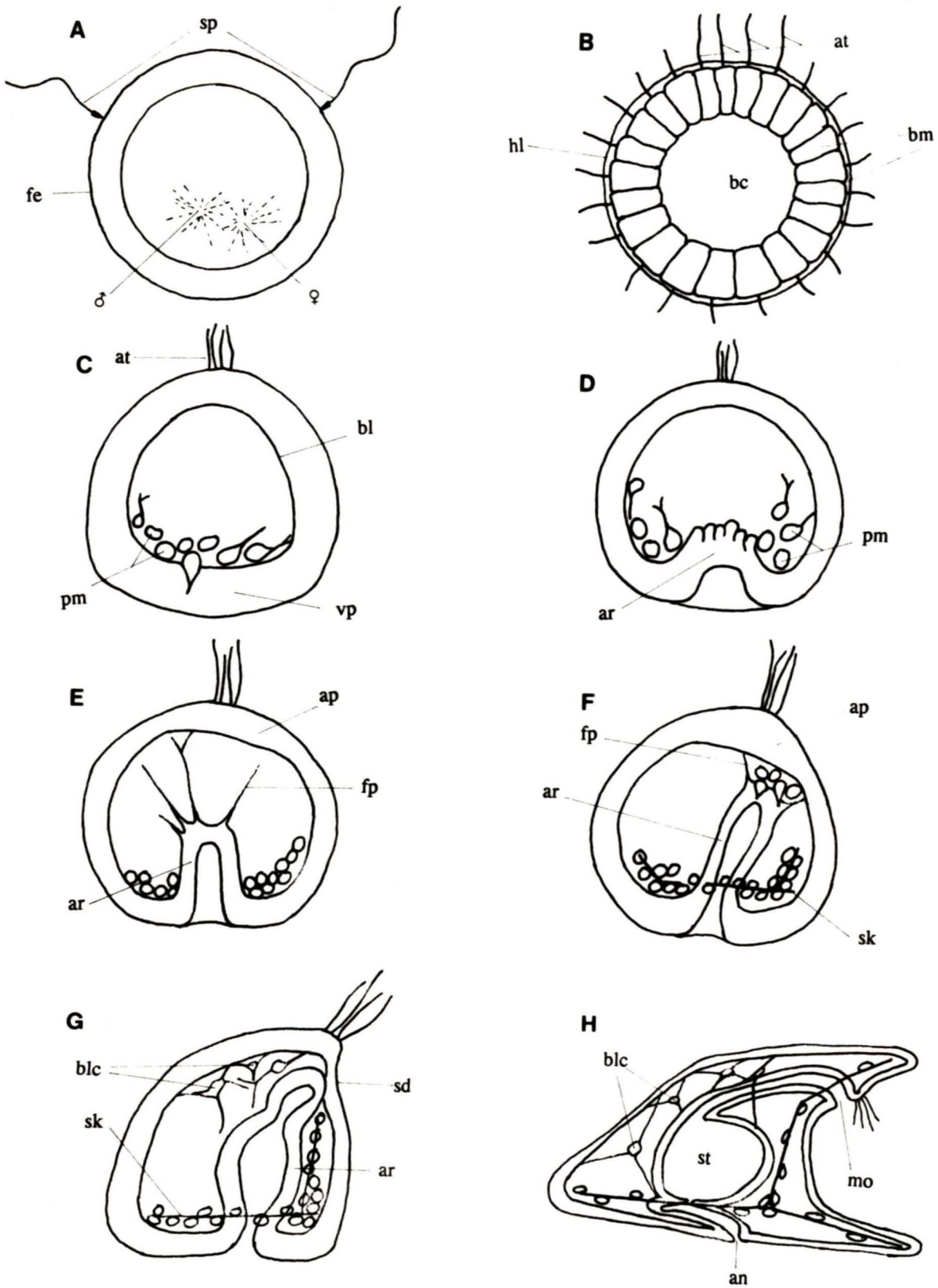
The sea urchin embryo has many attractive features which have made it an enduring model for the study of morphogenesis. For almost a century scientists have been observing sea urchin gastrulation in order to better understand the mechanisms which underlie embryogenesis. These investigations have provided the foundations upon which, with modern molecular techniques, contemporary developmental biologists are building a comprhensive understanding of one of the most fundamental processes in biology - morphogenesis.

Fertilization of the sea urchin egg initiates a series of events which activates cellular metabolism and the onset of cleavage. Twenty-four hours of rapid cell division yields a hollow ball of cells, the blastula, which digests its way free of the fertilization envelope and becomes a free swimming member of the plankton. This spherical epithelium then begins a well characterized series of cell movements which results in the formation of a triploblastic larva (Fig. 1). The primary mesenchyme loose their affinity for the hyaline layer and their neighbouring cells, and ingress into the blastocoel. These cells will migrate within the blastocoel, form a syncytial ring, and begin secretion of the larval skeleton. After the ingression of the primary mesenchyme the vegetal epithelium flattens and thickens forming the vegetal plate. This region of the embryo buckles inward, forming the primary invagination of the archenteron. At this time, the chromogenic mesenchyme cells begin releasing from the archenteron, and migrating through the blastocoel, eventually to invade the ectoderm and differentiate into pigment cells (Gibson and Burke, 1985; Gibson and

Figure 1: Sea urchin embryogenesis

Schematic drawings of normal development in *Strongylocentrotus purpuratus*. **1A)** fertilized egg, 0 h of development **1B)** hatched blastula, 24 h of development **1C)** mesenchyme blastula, 32 h of development **1D)** primary archenteron invagination, 36 h of development **1E)** archenteron elongation, 40 h of development **1F)** full gastrula, 44 h of development **1G)** prism stage larva, 72 h of development **1H)** pluteus stage larva, 5 days of development.

Abbreviations: **fe** fertilization envelope, **sp** sperm, ♂ male pronucleus, ♀ female pronucleus, **at** apical tuft, **hl** hyalin layer, **bm** blastomeres, **bc** blastocoel, **bl** basal lamina, **pm** primary mesenchyme, **vp** vegetal plate, **ar** archenteron, **ap** animal plate, **fp** filopodia, **sk** skeletal rod, **blc** blastocoelar cells, **sd** stomodeum, **st** stomach, **mo** mouth, **an** anus.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Burke, 1987). After invagination, archenteron extension proceeds initially by cell rearrangement, the short squat cylinder becoming longer and narrower as it progresses across the blastocoel (for recent reviews see Burke, 1990; Hardin, 1990; McClay, *et al.*, 1991; Ettensohn and Ingersoll, 1992). Embedded within the tip of the archenteron are a number of cells which extend long filopodia across the blastocoel, making brief contact with the blastocoelar wall. When these processes make contact with the presumptive stomodeum, near the animal pole, they make more stable attachments, and appear to pull or guide the archenteron to this target region.

The nature of the interactions between the cells at the tip of the archenteron and the region of the presumptive stomodeum, and their role in this last phase of gastrulation have been the topic of a number of investigations. The notion that the contraction of filopodia attached to the roof of the blastocoel pulls the archenteron across the blastocoel was initially suggested simultaneously by two independent groups (Dan and Okazaki, 1956; Gufstafson and Kinnander, 1956). Supporting this theory is the observation that the processes extending from the tip of the archenteron are very rich in microfilaments (Tilney and Gibbins, 1969), and that the process is microtubule independent (Hardin 1987). More evidence for a mechanical role are the results of experiments conducted by Hardin and McClay (1990), in which gastrulating embryos were physically manipulated forcing the animal pole toward the extending archenteron, which causes precocious attachment. When allowed to spring back to its normal location, the blastocoel roof frequently draws a number of cells out of the tip of the archenteron with it. The authors' interpretation of this result is that the

attachment of the cells at the tip of the archenteron to the target region is so stable that it is strong enough to pull the attached cells out of the epithelium. More compelling are the results of laser ablation experiments in which the rate of archenteron elongation was seen to be proportional to the number of filopodial contacts with the animal pole left unablated (Hardin, 1988). However, none of these observations preclude the possibility that the filopodial contacts provide guidance for a force intrinsic to the archenteron. The existence of exogastrulae, in which the archenteron evaginates rather than invaginates, demonstrates that the archenteron has the intrinsic ability to extend. Furthermore, a mechanical analysis suggests that, unless the ectoderm is significantly stiffer than the archenteron, any force sufficient to pull the archenteron toward the target would cause an observable deformation in the curvature of the animal pole ectoderm (Hardin and Cheng, 1986).

Further complexity is added to the analysis of this phenomenon by our lack of information about the target region. What might the cells be attaching to? The basal lamina which lines the blastocoel consists of a complex array of extracellular matrix molecules (reviewed in Pedersen, 1991). Intriguingly, laminin has been shown to have an uneven distribution in the basal lamina of the sea urchin embryo (McCarthy and Burger, 1987), with maximal concentrations in the animal and vegetal poles. However, ultrastructural examination has demonstrated that at least some of the processes extending from cells at the tip of the archenteron pass through the basal lamina to make contact with ectodermal cells, although it is not clear that this is true of the processes which anchor the archenteron to the target region (Spegiel and

Spegiel, 1992). Thus, at the moment, it is unclear what these cell processes attach to, or what their function is once they make these attachments.

Upon completion of gastrulation, the cells at the tip of the archenteron ingress, and begin a period of migration. They move about within the blastocoel, forming a complex network of cell processes of unknown function, which persists throughout larval life (Tamboline and Burke, 1992). These cells have not been well studied, and the mechanism which triggers their release from the tip of the archenteron is unknown. The release and migration of mesenchymal cells, followed by differentiation into specific cell types is a common theme in dueterostome development. For example, the neural crest cells of vertebrate embryos ingress from the neural epithelium, migrate extensively, and differentiate into a wide variety of cell types (reviewed in Weston, 1970; LeDouarin, 1982). Apart from being an interesting phenomenon in and of itself, understanding the mechanism which causes the cells at the tip of the archenteron to release and migrate may provide insight into similar phenomena in embryos of a number of other species. Furthermore, the behaviour of migrating mesenchyme is very similar to that of invasive tumour cells, and therefore its investigation is of relevance to our understanding of the biology of metastasis.

The objective of my research is to address two of the unresolved questions about morphogenesis in sea urchins. Firstly, what are the filopodia which extend from the tip of the archenteron during gastrulation attaching to, and do they exert a force on it? And secondly, what are the molecular mechanisms which cause the release and migration of the cells at the tip of the archenteron as they reach the target

region at the animal pole of the blastocoel?

MATERIALS AND METHODS

Blastocoelar extracellular matrix (BECM) preparations.

BECM preparation was 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 FSW and fertilized with a dilute suspension of sperm. Embryos were incubated in FSW at 12 °C, until they were collected by centrifugation at 200 x G, and resuspended in CMFSW containing 1 mM EDTA (pH 8.0) on ice. Embryos were then 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). Embryos were incubated in BIM on ice for 5 minutes, or until all remaining ectodermal cells had come away from the matrix. BECM preparations were then collected by centrifugation at 650 × G for 3 min 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 then observed in FSW containing 250 μg/ml streptomycin sulphate (pH 8.0) at 12 °C.

Drug inhibition of matrix collapse.

BECM preparations made from 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 h at 12 °C, and then 50 preparations from each treatment were scored for contraction of the blastocoelar matrix. This procedure was repeated 4 times using different batches of eggs and sea water.

Cell behaviour.

BECM 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. Individual preparations were recorded for 8 to 12 h using a Nikon inverted DIC microscope fitted with a controlled temperature stage and a time-lapse video camera. Tracings were made from the monitor at 5 minute intervals of individual cells as they migrated across the substrate.

Quantification of the effects of compounds on cell behaviour.

BECM preparations made from 2/3 gastrulae were incubated on coverslips sealed with celloseal in 50 μl FSW + 250 μg/ml streptomycin sulphate containing either 1 mM YIGSR, 1 mM RGDS, 0.085 mg/ml laminin, 0.025 mg/ml fibronectin, or 0.1 mg/ml BSA. Preparations were incubated for 4 h at 12 °C then equal numbers of collapsed preparations were scored for numbers of cell processes protruding through the basal lamina. This experiment was repeated using several different batches of embryos, until the total number of preparations scored for each treatment was 87. Significance of these data was determined using pair-wise t-tests.

Cell labelling with biotinylated ligand.

Whole mouse laminin (Boehringer Mannheim) and CDPGYIGSR-NH_2 (Sigma) were biotinylated using a sulfo-NHS-biotin kit (Pierce). BECM 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 h at 12 °C, then spun at 500 × G and resuspended in FSW three times before fixation in 4% formaldehyde (20 min at room temperature). In double labelling experiments, fixed cells were then incubated with Sp12 acities fluid (Tamboline and Burke, 1989) at 1:100 in PBS for 2 h, rinsed 3 times in PBS, incubated in rabbit anti mouse IgG-TRITC at 1:500 in PBS for 1 h at room temperature, and rinsed three times in PBS. Preparations were then incubated in avidin-FITC at 1:500 in PBS for 1 h at room temperature, rinsed thrice in PBS and viewed with a Ziess epifluorescence microscope.

Laminin affinity chromatography.

2/3 gastrulae were dissociated by suspension in CMFSW + 2 mM EDTA and passage through 20 μm nitex. $1x10^7$ dissociated cells were surface labelled with 0.5 μCi ^{125}I (Amersham) using Iodobeads (Pierce), pelleted at 14000 × G, frozen, and extracted with 100 mM n-octylglucoside + PMSF in FSW. A laminin affinity column was made by linking 4 mg of laminin to 1 ml CNBr activated Sepharose 4B (Pharmacia). ^{125}I-labelled cell surface components were applied to the column and incubated 1 h at room temperature. Unbound radioactivity was rinsed off with 20

volumes of FSW. 4 volumes of 1.5 mM YIGSR in FSW was applied to the column. Eluted radioactivity was collected in 1 ml fractions. The column was then rinsed with 4 volumes of CMFSW + 2 mM EDTA, and finally with 4 volumes 50 mM diethylamine pH 11.5. Radioactive fractions were dialysed overnight against distilled H_2O, concentrated by vacuum centrifugation, and run on a 10% acrylamide gel using SDS-PAGE under reducing conditions. The gel was dried and autoradiographed for 8 days.

CDPGYIGSR affinity chromatography

An affinity column was made by linking 2 mg of CDPGYIGSR-NH_2 (Sigma), to 2 ml of SulfoLink gel (Pierce). 2/3 gastrulae were dissociated by suspension in hyaline extraction medium (Fink and McClay, 1985) (300 mM Glycine, 300 mM NaCl, 10 mM KCl, 10 mM magnesium sulphate, 10 mM Tris, 2 mM EGTA, pH 8.0), and passage through 20 μm nitex. Dissociated cells were rinsed thrice in FSW, pelleted at 14000 x G, frozen, and extracted with 100 mM n-octylglucoside + PMSF in FSW. Extract was applied to the affinity column and incubated 1 h at room temperature, before unbound material was rinsed off with 20 ml of FSW. 2 ml of 1.5 mM YIGSR in FSW was then applied to the column and incubated for 30 min at room temperature. Eluted material was forced off the column with 6 ml of FSW. Eluent was desalted and concentrated to 0.5 ml using a Centriprep-10 concentrator (Amicon). 1 mg/ml myosin was added before desalting in order to prevent loss due to non-specific binding of protein to the concentrator membrane. 500 μl of desalted

sample was further concentrated using a microcon 30 (Amicon), before being loaded onto a 10% gel and subjected to PAGE. The gel was then silver stained.

RESULTS

Normal behaviour of BECM preparations in FSW

BECM preparations consist of the matrix and the cells which were in the blastocoel at the time the embryos were collected. Thus BECM preparations made from 2/3 gastrulae contain primary mesenchyme cells, and the cells of the archenteron, surrounded by the BECM (Fig. 2). Typical embryos prepared at this stage undergo the following sequence: cells at the tip of the archenteron extend filopodia which attach to the basal lamina, the basal lamina indents at the point of attachment and as the filopodia contract the ECM collapses (Fig. 2 B, arrow). The entire sequence takes about 3 to 5 h at 12 °C. Preparations made from embryos after the primary mesenchyme have ingressed, but before the invagination of the archenteron, do not collapse (Fig. 2, E and F). Contraction of the basal lamina is blocked by incubation of the preparations in 0.05% sodium azide (11.0 $\pm$ 6.0% collapsed), or 1 mM cytochalasin B (21.0 $\pm$ 3.8% collapsed), but not by incubation in 1 mM colchicine (88.5 $\pm$ 10.3% collapsed). In untreated preparations 95.5 $\pm$ 3.0% collapsed (n = 200 for all treatments).

Effects of YIGSR on BECM preparations

BECM preparations incubated in 1 mM YIGSR undergo contraction normally, however cellular motility is enhanced (Fig. 3). In time-lapse video records individual mesenchyme cells migrate out of the BECM preparations. The cells extend numerous

Figure 2: Blastocoelar ECM collapse

Blastocoelar ECM preparations were made from 2/3 gastrulae and mesenchyme blastulae. Time-lapse video recordings of these preparations show that in preparations made from 2/3 gastrulae, cells at the tip of the archenteron extend filopodia, attach to the basal lamina, then contract, pulling the lamina inward. This was not observed in preparations made from mesenchyme blastulae. **2A)** blastocoelar ECM preparation of a 2/3 gastrula **2B)** same preparation 20 minutes later, note deformation of the basal lamina (arrow) **2C)** same preparation 35 minutes later **2D)** same preparation at 50 minutes **2E)** blastocoelar ECM preparation of a mesenchyme blastula **2F)** same preparation 50 minutes later.

Abbreviations: **bl** basal lamina **sm** secondary mesenchyme **ar** archenteron **pm** primary mesenchyme. Scale bars = 10 μm.

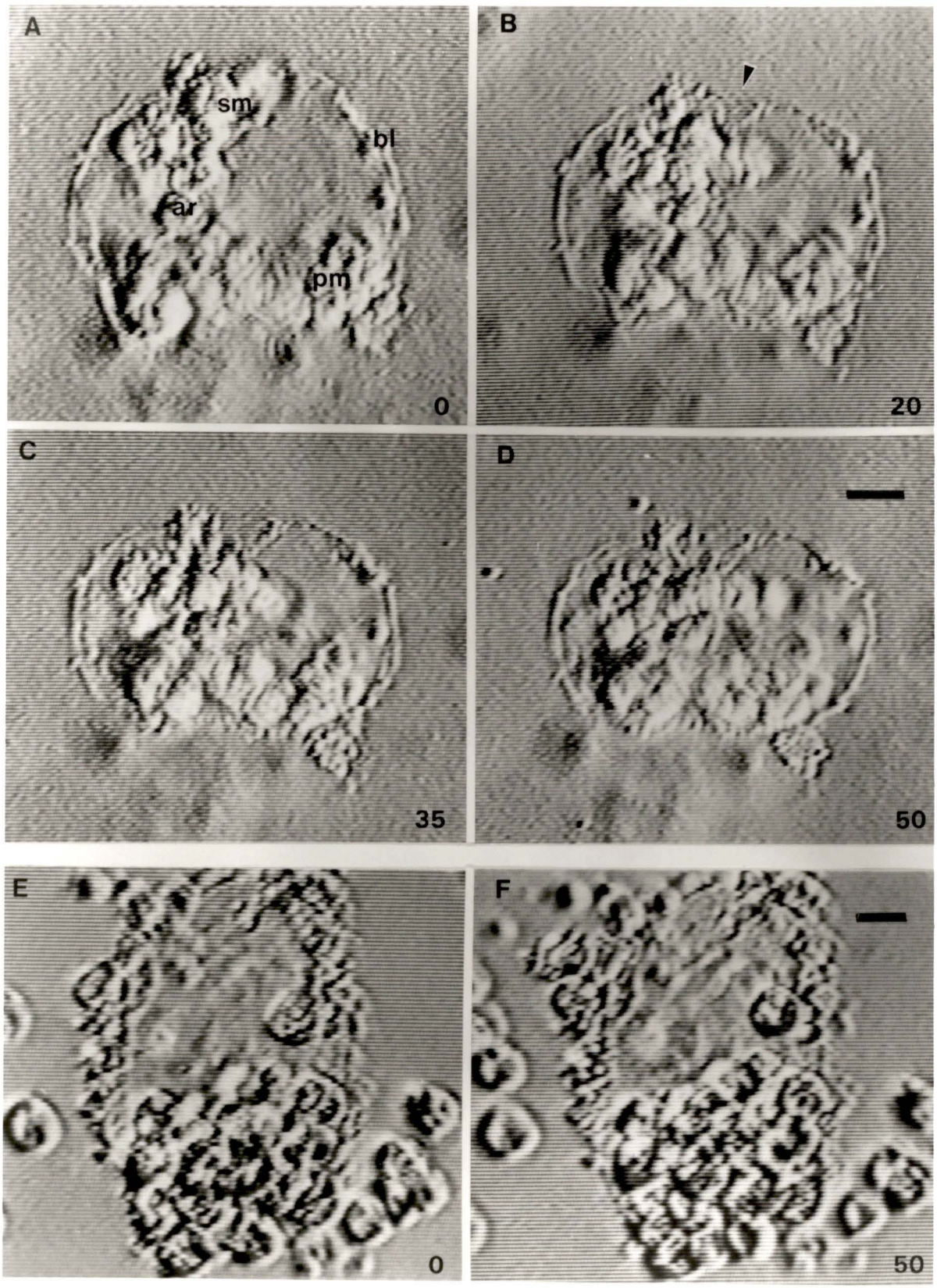
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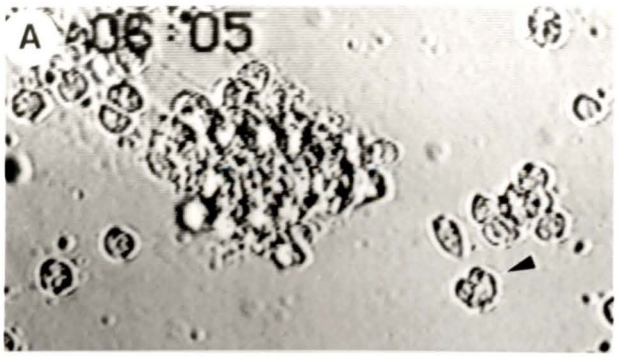
Figure 3: Cell migration in 1 mM YIGSR

Blastocoelar ECM preparations of 2/3 gastrulae were videotaped in FSW containing 1 mM YIGSR, 1 mM RGDS, 25 μg/ml fibronectin, 85 μg/ml laminin, or 0.1 mg/ml BSA. In the presence of YIGSR cells migrate out of the preparations at 3.59 ± 0.95 μm/min. No migratory activity was observed in other treatments. **3A)** still from video recording of cell migration (arrow indicates migrating cell) **3B)** tracing of migration at 5 minute intervals (arrows indicate the same cell at the same time as indicated in 3A). **3C - F)** tracings of other cells migrating from the same preparation (small numbers inside cells indicate time since cell first left preparation. Note that cells reverse direction (3D)). Scale bar = 20 μm.



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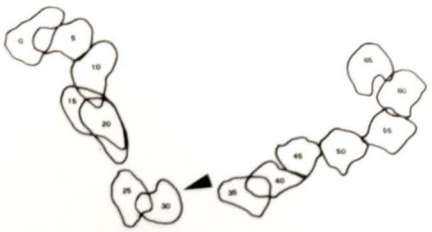

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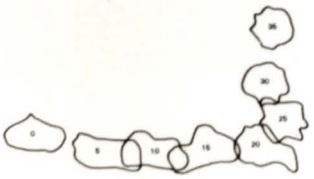

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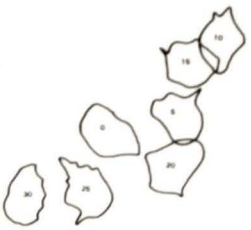

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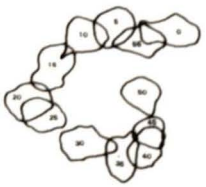

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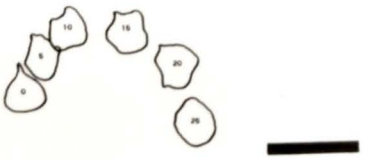


processes through the basal lamina which attach to the substrate and draw the cells out of the preparation. The mean rate of movement is 3.59 $\pm$ 0.95 μm/min (n = 5). In similar records of preparations not treated with YIGSR or treated with other peptides, the basal lamina collapses, but cells do not migrate.

After 4 hours in 1 mM YIGSR, BECM preparations have significantly more ($p < 0.0001$, $n = 87$) cell processes extending from them than preparations incubated in RGDS, BSA, fibronectin, or laminin. The preparations incubated in laminin have significantly fewer ($p < 0.0001$, $n = 87$) cell processes than any of the other treatments (Fig. 4). The number of cell processes extending out of the preparations correlates with the concentration of YIGSR over a range of 8 μM to 1 mM ($r = 0.85$) (Fig. 5).

Cell labelling with biotinylated laminin and CDPGYIGSR

Biotinylated laminin binds to a subset of dissociated cells (Fig. 6). 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, which frequently cap the ligand. Intensity of labelling varied considerably from cell to cell, with some cells being so strongly positive that fluorescence was easily detectable even under bright field illumination, whereas labelling on other cells could only be observed under darkfield illumination.

Labelling with biotinylated CDPGYIGSR is weaker than with laminin and of more homogeneous intensity from cell to cell. It is less frequently observed on the

Figure 4: Quantification of response 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. YIGSR-treated preparations extended significantly ($p < 0.001$) more processes than preparations in any other treatment. Laminin-treated preparations extended significantly ($p < 0.001$) fewer processes. Error bars represent standard error.

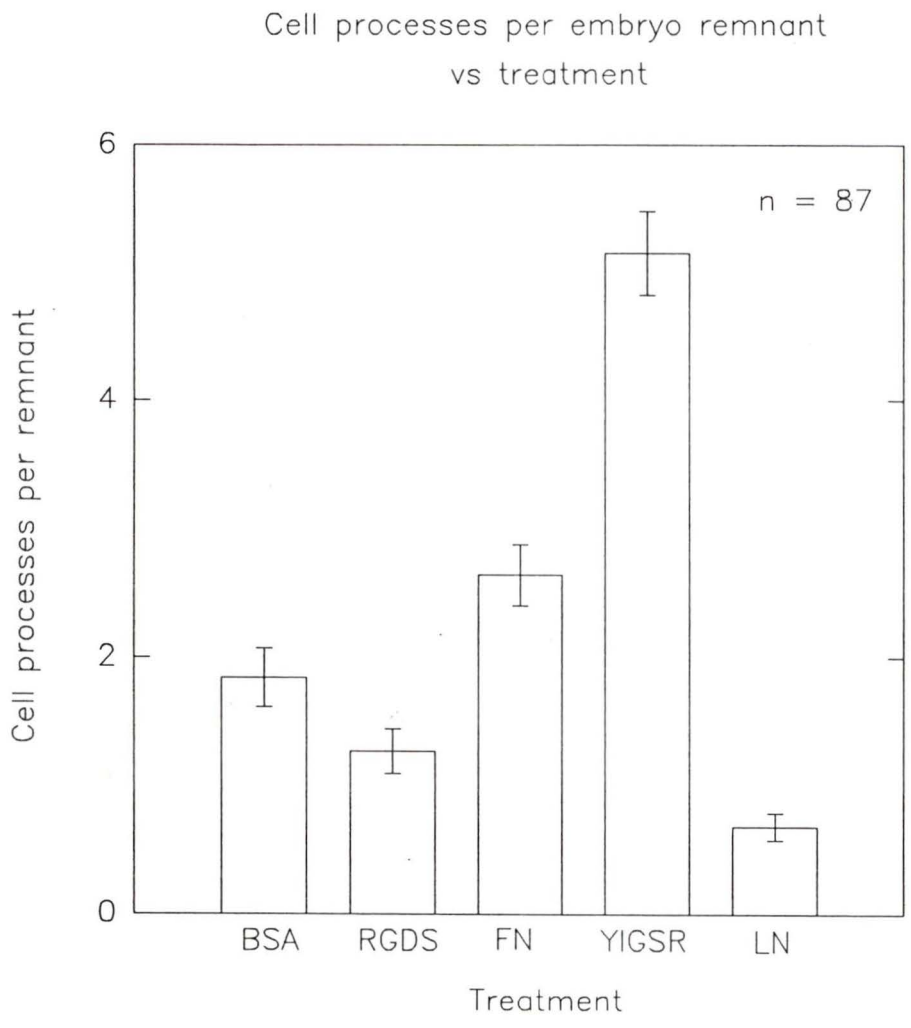

Cell processes per embryo remnant
vs treatment
n = 87
Cell processes per remnant
6
4
2
0
BSA
RGDS
FN
YIGSR
LN
Treatment

Figure 5: Correlation of cell process counts to [YIGSR]

Blastocoelar ECM preparations of 2/3 gastrulae were incubated in FSW containing 8 μM, 16 μM, 31 μM, 63 μM, 125 μM, 250 μM, 500 μM and 1 mM YIGSR, as well as the same controls as used in figure 4. 10 preparations from each treatment were scored for cell processes. Numbers of cell processes observed correlates well with the concentration of YIGSR. 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 processes (mean$\pm$S.D.): BSA 0.91$\pm$.034, laminin 0.24$\pm$0.15, RGDS 1.33$\pm$0.41, and fibronectin 0.65$\pm$0.31.

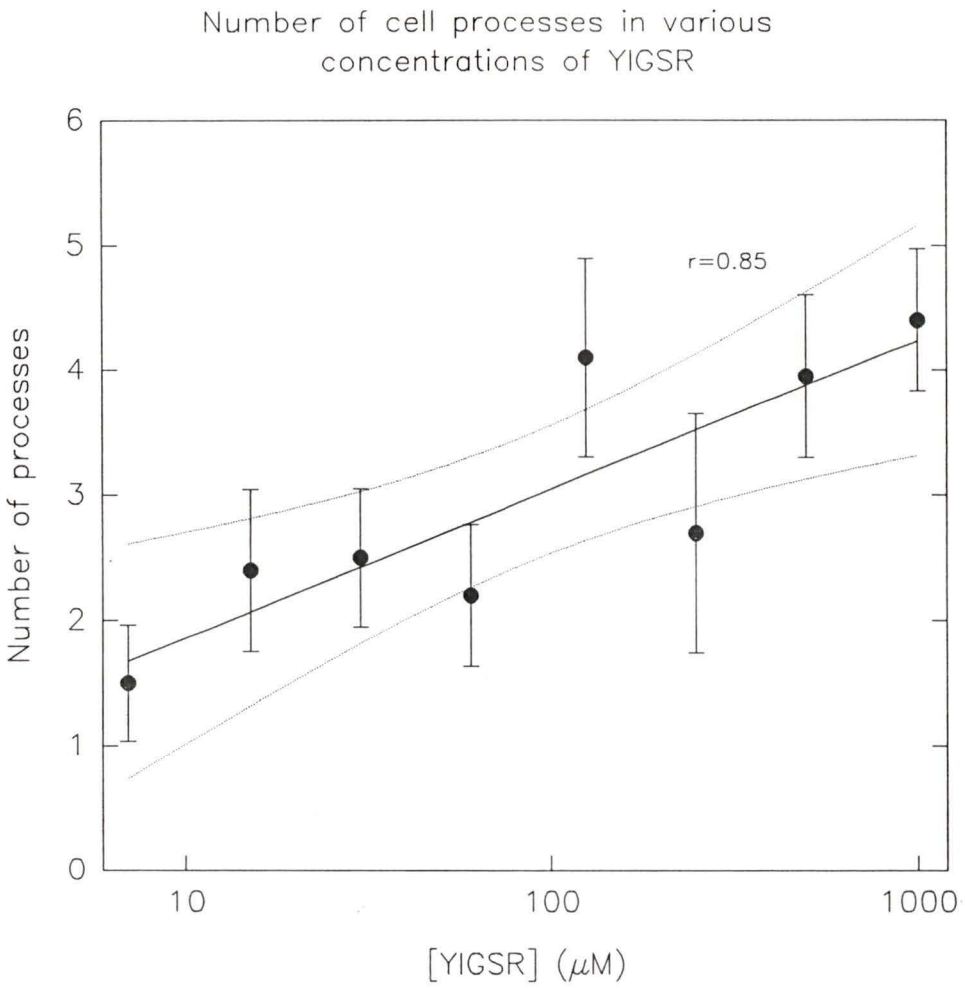
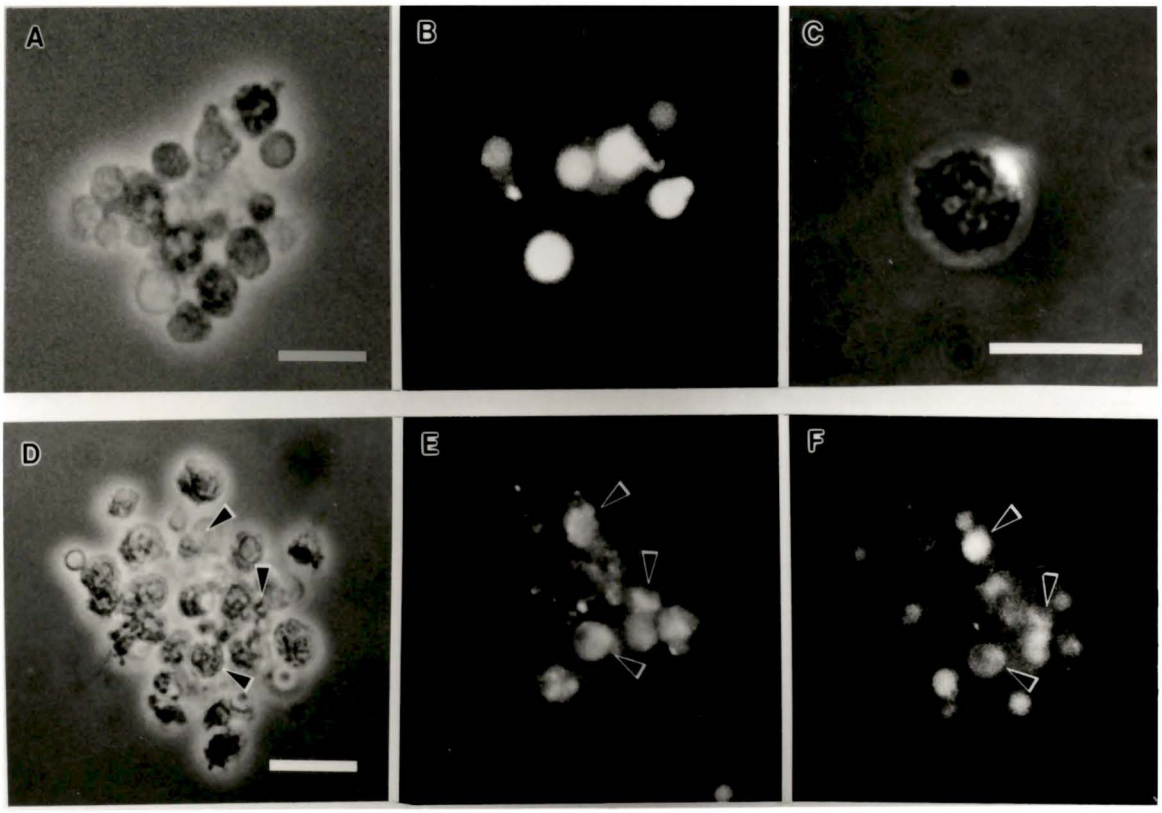

Number of cell processes in various concentrations of YIGSR
r=0.85
Number of processes
0
1
2
3
4
5
6
10
100
1000
[YIGSR] (μM)

Figure 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. **6A)** phase contrast image of cell aggregate **6B)** laminin labelling of same aggregate **6C)** capping of laminin labelling on dissociated cell **6D)** phase contrast image of cell aggregate **6E)** Sp12 labelling of same aggregate **6F)** CDPGYIGSR labelling of same aggregate (arrows indicate cells which are labelled by both Sp12 and CDPGYIGSR). Scale bar = 20 μm


A
B
C
D
E
F

small round Sp12 negative cells, and was also frequently capped.

Neither label is bound exclusively by Sp12 positive cells, or by Sp12 negative cells.

Cell iodination and laminin affinity chromatography

Cell surface labelling resulted in 50% of radioactivity being incorporated. After lysis, 90% of incorporated activity was found in the water insoluble, octylglucoside soluble extract.

After the labelled extract was applied to the column and it was rinsed with FSW until all unbound radioactivity was eluted, a single peak of radiolabelled material was eluted by 1.5 mM YIGSR in FSW. A subsequent rinse with 2 mM EDTA in CMFSW failed to elute radiolabelled material, but 50 mM diethylamine (pH 11.5) eluted a large peak (Fig. 7). SDS-PAGE of YIGSR eluted material failed to produce a convincing band. The material eluted by diethylamine runs at 150, 130, 105, 80, and 70k (Fig. 8).

CDPGYIGSR affinity chromatography

Silver staining reveals a heterogeneous population of proteins in the membrane extract of 2/3 gastrulae (Fig. 9, lane 1). A single component of the membrane extract was bound by the column. This material could be eluted using 1.5 mM YIGSR. SDS-PAGE reveals this material as a single band at 70 k (Fig. 9, lane 2). The strong staining at the bottom of the gel is due to myoglobin added to the sample as a carrier

Figure 7: Laminin affinity chromatography

^{125}I-labelled cell surface components were run over a laminin affinity column. Unbound radioactivity was eluted with FSW. A single peak of radioactivity was eluted with 1.5 mM YIGSR. No detectable peak was eluted with 1 mM EDTA in CMFSW. Remaining bound radioactivity was eluted with pH 11.5 diethylamine.

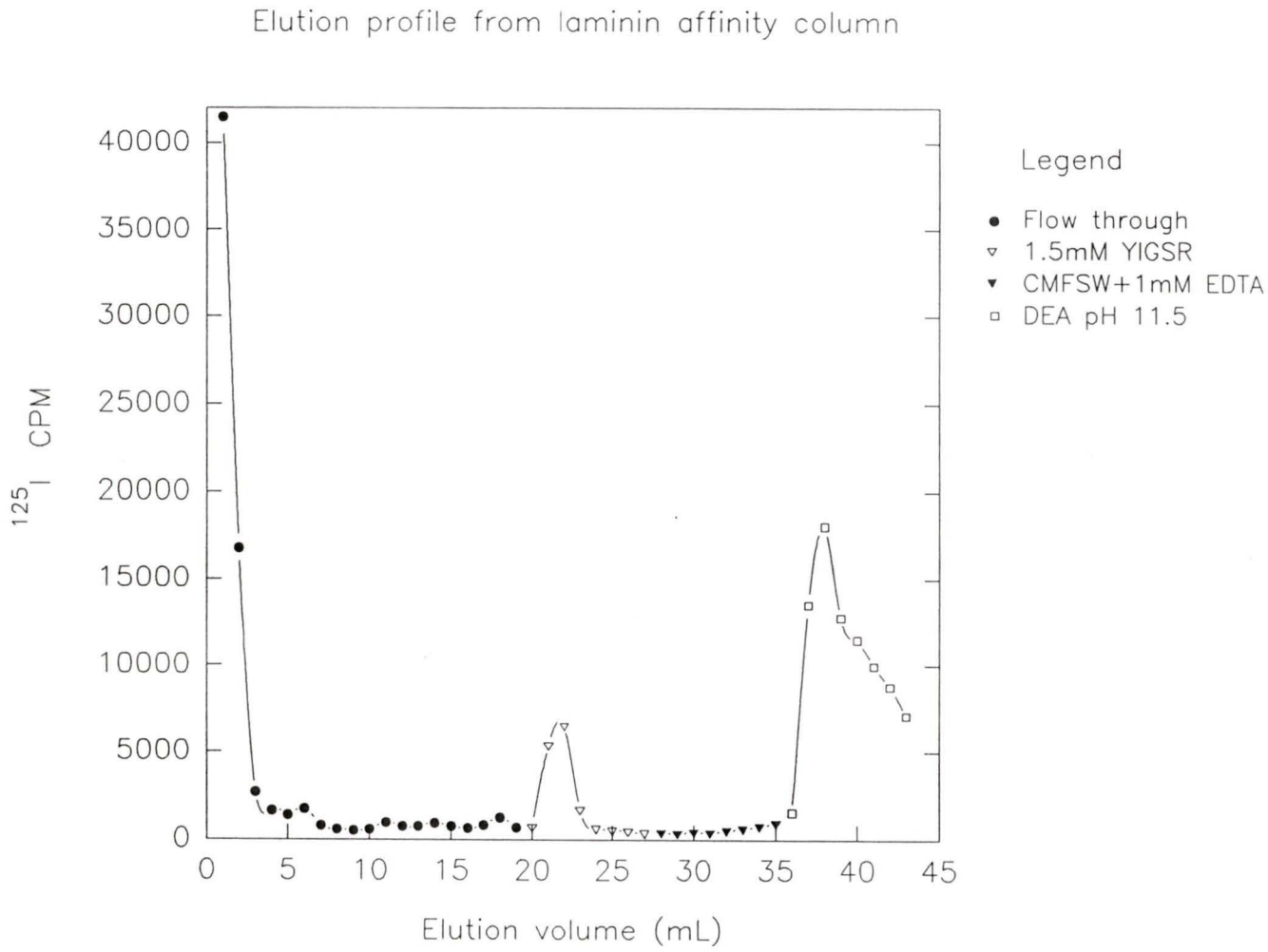

Elution profile from laminin affinity column
40000
35000
30000
25000
20000
15000
10000
5000
0
^{125}I CPM
0
5
10
15
20
25
30
35
40
45
Elution volume (mL)
Legend
Flow through
1.5mM YIGSR
CMFSW+1mM EDTA
DEA pH 11.5

Figure 8: Autoradiography of high pH eluent from laminin affinity column

^{125}I-labelled material which was eluted with pH 11.5 diethylamine from a laminin affinity column was dialysed against distilled water and concentrated by vacuum centrifugation, and then run on a 8% poly-acrylamide gel under reducing conditions. Arrows indicate mobility of molecular weight standards. Eluted material runs at 150, 130, 105, 80 and 70 k.

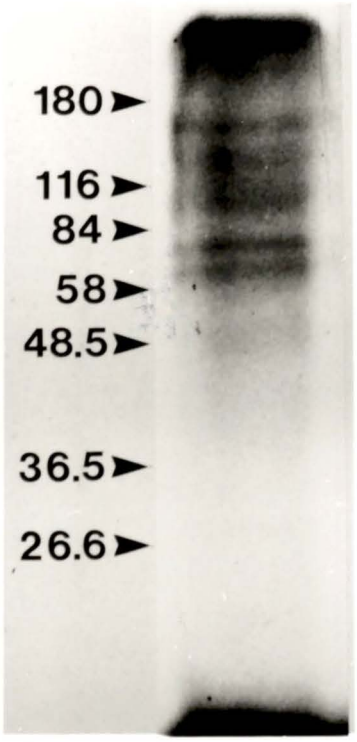
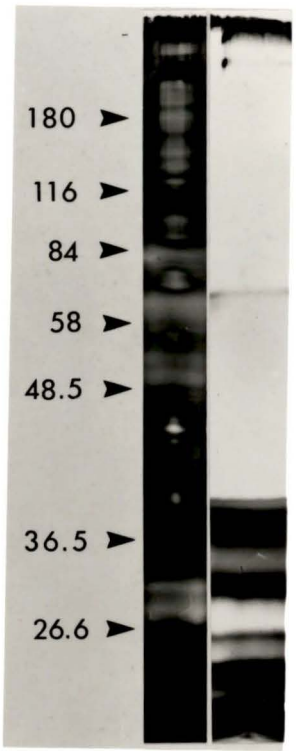

180
116
84
58
48.5
36.5
26.6

Figure 9: CDPGYIGSR affinity chromatography

Octylglucoside extract of $1x10^9$ cells from 2/3 gastrulae was passed over immobilized CDPGYIGSR. Unbound material was washed off with 20 ml of FSW. Material eluted with 1.5 mM YIGSR was collected, desalted and concentrated with 1 mg/ml horse heart myoglobin as a blocking agent. Fractions were then run on a 10% gel under reducing conditions. Lane 1: starting material, lane 2: YIGSR eluent, heavy staining below 30 k is due to myoglobin contamination. Arrows indicate mobility of molecular weight standards.


180
116
84
58
48.5
36.5
26.6

protein. Although the 30 kDa cut off of the microcon 30 concentrators should allow myoglobin (19 kDa) to pass through, extensive myoglobin contamination of samples was unavoidable.

DISCUSSION

Collapse of BECM preparations reveals a force exerted during gastrulation

Archenteron elongation is thought to proceed in two phases. The initial phase of elongation is the result of cell rearrangement in the archenteron walls, resulting in a longer, more slender tube (reviewed in McClay, *et al.*, 1991). The last 1/3 of archenteron elongation will not occur without the attachments made by the cells at the tip of the archenteron to the blastocoel roof (Hardin, 1988). Early investigators suggested that these processes exerted a contractile force on the roof of the blastocoel, pulling the archenteron to its destination (Dan and Okazaki, 1956; Gustafson and Kinnander, 1956). However, the counter hypothesis, that the cells at the tip of the archenteron are providing guidance to an extensive force intrinsic to the archenteron, has not been refuted.

The collapse of BECM preparations provides evidence that a contractile, rather than extensive force is being exerted. In these preparations the cells at the tip of the archenteron attach to the basal lamina of the blastocoelar ECM and pull on it, causing it to collapse inward. In intact embryos, this collapse would be prevented by the structural integrity of the overlying ectoderm, and perhaps by additional ECM which is lost in these preparations. BECM collapse is not observed in preparations made from mesenchyme blastulae, even though the primary mesenchyme cells in these preparations can be seen to migrate within the BECM preparations. This illustrates that the cells at the tip of the archenteron are necessary for BECM collapse. The fact

that this collapse is inhibited by cytochalasin B and not cholchicine suggest that this process is actin, and not microtubule mediated. Early work with cytochalasin and colchicine suggested that the last phase of archenteron elongation involves both actin and microtubules (Tilney and Gibbins, 1969). However, more recent investigation has shown that the effects of colchicine on archenteron extension are also caused by ß-lumicolchicine, a colchicine analog which does not bind tubulin, and that the microtubule inhibitor nocodazol, while effectively blocking microtubule formation, does not inhibit archenteron elongation (Hardin, 1987). Thus, it would appear that microtubules are not necessary for archenteron elongation.

These results do not rule out the possibility that the cells at the tip of the archenteron extend processes through the basal lamina and attach to cells of the ectoderm in intact embryos. However, the absence of ectodermal cells in BECM preparations ensures that that is not happening in this case. Therefore it is reasonable to conclude that the last phase of archenteron elongation involves the attachment of processes extending from the tip of the archenteron to the basal lamina of the blastocoelar ECM, and the exertion of a contractile force upon it.

YIGSR and laminin affect cell motility

In gastrulating embryos, 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 BECM preparations incubated in YIGSR, the normal sequence of events leading to collapse of the ECM takes place, however, there is an increase in

migratory activity in these preparations. This migratory behaviour, as quantified by the number of cell processes protruding from the preparations, varies in a dosage dependant way with the concentration of YIGSR. Curiously, incubation of BECM preparations with whole 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 apparently opposite effects of vertebrate laminin and YIGSR on our BECM preparations may reflect a non-linear dose/response curve, or consequences of presentation context. It should be noted that due to its high molecular weight and limited solubility, the concentrations of laminin used (0.085 mg/ml = 0.1 pM) were 8 orders of magnitude lower than the concentrations of YIGSR which had an observable stimulating effect (8 μM). Thus, it may be that at extremely low concentrations YIGSR has an effect on cell migration that is opposite to its effect at high concentrations.

YIGSR and laminin bind to the surfaces of cells within the blastocoel

Populations of cells within the blastocoels of 2/3 gastrulae bind biotinylated laminin and CDPGYIGSR. These populations appear to include mesenchyme cells identified in double labelling experiments with the mesenchyme specific mAb, Sp12. Laminin labelling is very strong and labels both small round Sp12 negative cells, and fewer large irregular Sp12 positive cells. It is worth noting that the intensity of laminin labelling varies between cells, from very strong, to weak, to none at all

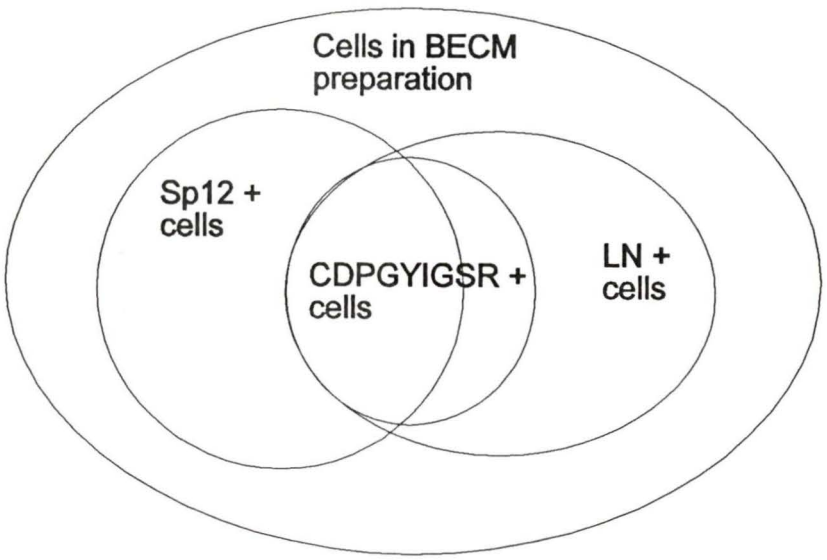
(Fig. 5b). This may be the result of qualitatively different binding sites with differing affinities on different cells, or variation in the quantity of a single class of binding sites from cell to cell. Capping, which is characteristic of transmembrane receptors which interact with the cytoskeleton, 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 labels a more restricted population than whole laminin. This population consists of fewer small round Sp12 negative cells than laminin, and similar numbers of large irregular Sp12 positive cells. Intensity of labelling was fairly homogeneous from cell to cell, suggesting a similar number of binding sites on each positive cell. Again, there are both Sp12 positive and Sp12 negative cells which do not bind CDPGYIGSR. These observations suggest that the population of cells which bind CDPGYIGSR is a subset of the population which binds whole laminin, and that both of these populations include mesenchyme cells (Fig. 10).

Affinity chromatography extracts cell surface laminin receptors

A number of cell surface components bind to a laminin affinity column. Some of this material can be specifically eluted with 1.5 mM YIGSR. Due to the small amounts of this material eluted from the column, and unavoidable losses during sample preparation and gel electrophoresis, autoradiographs reveal only a faint band around 70 k. High pH eluted more 70 k material, as well as 150, 130, 105 and 80 k bands.

Figure 10: Inferred distribution of labels used in cell labelling experiments.



By preparing a column using CDPGYIGSR instead of laminin, several orders of magnitude more sites for receptor binding are made available. Using this column nanogram quantities of a 70 k membrane protein can be isolated. No 150, 130, 105 or 80 k bands are observed.

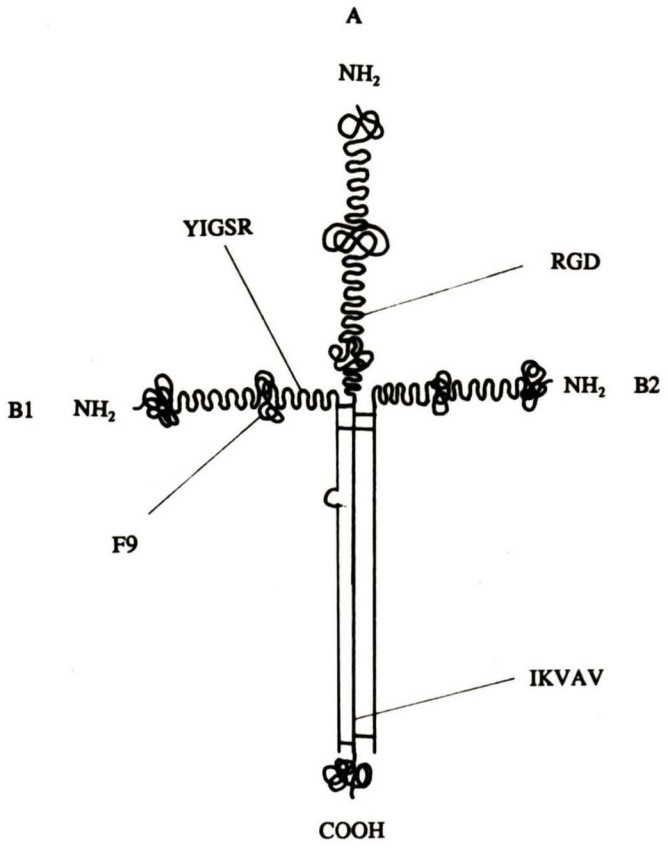
The presence of a 70 k membrane component which binds both laminin and CDPGYIGSR, is eluted by 1.5 mM YIGSR, and is iodinated by surface iodination methods is evidence that this component is a cell surface laminin binding protein which binds laminin via the YIGSR domain. Since no other material was bound by the CDPGYIGSR column, it is reasonable to speculate that the other bands in the high pH eluent from the laminin column represent other laminin binding proteins. This interpretation also offers an explanation for the differences in cell labelling patterns observed using biotinylated laminin and biotinylated CDPGYIGSR. Cells expressing a YIGSR-binding receptor would be labelled by both CDPGYIGSR and laminin, whereas cells expressing a non-YIGSR-binding laminin receptor would be labelled by only laminin. Similarly, cells which were strongly labelled by laminin may have been expressing two or more different laminin receptors, whereas weakly labelled cells may have been expressing only one type. Many examples of cells expressing more than one type of cell surface laminin binding protein have been observed in vertebrates (Tomaselli, *et al.*, 1987; Clément, *et al.*, 1990; Davis, *et al.*, 1991; Tashiro, *et al.*, 1991; Moutsita, *et al.*, 1991).

A 67 k laminin binding protein has been found in vertebrates

Laminin is a well characterized component of both vertebrate and invertebrate ECMs. In vertebrates it consists of three chains connected by disulphide bridges which form a ridged cruciform structure (Fig. 11) and has several functional domains (Paulsson, *et al.*, 1985; Sasaki and Yamada, 1987; Sasaki, *et al.*, 1987; Sasaki, *et al.*, 1988; Durkin, *et al.*, 1988; reviewed in Timpl, 1989). Although primary sequence information is not available for sea urchin laminin, ultrastructural examination and immunological data show that laminin is structurally conserved in urchins and several other invertebrates (McCarthy, *et al.*, 1987; Fessler, *et al.*, 1987; Chiquet, *et al.*, 1988; Miller and Hadley 1990; reviewed in Pedersen, 1991). Cell interactions with laminin are thought to occur through a variety of cell surface receptors which interact with various domains of this large glycoprotein. The A chain of laminin contains an Arg-Gly-Asp (RGD) sequence which may be bound by integrins (Tomaselli, *et al.*, 1987; Davis, *et al.*, 1991; Tashiro, *et al.*, 1991; reviewed in Mecham, 1991) as well as the sequence Ile-Lys-Val-Ala-Val (IKVAV) which is bound by a 110 kDa receptor, and is thought to stimulate neurite outgrowth (Kleinman, *et al.*, 1991). The B1 chain of laminin is known to contain a sequence (F9) in the innermost globular domain which specifically binds heparin, but not other glycosaminoglycans (Charonis, *et al.*, 1988). The B1 chain also contains the YIGSR motif, which computer modelling places just proximal to the globular domain containing the heparin-binding sequence (Graf, *et al.*, 1987).

Laminin affinity chromatography has been used to purify a 67 k laminin

Figure 11: A schematic drawing of the structure of laminin showing domains known to be active in cell adhesion.


A
NH2
YIGSR
RGD
B1
NH2
NH2
B2
F9
IKVAV
COOH

binding protein from cell surface extracts of a variety of neoplastic and normal vertebrate tissues (Malinoff and Wicha, 1983; Rao, *et al.*, 1983; Wewer, *et al.*, 1986; Sugrue, 1988; Davis, *et al.*, 1991; reviewed in Mecham, 1991). This protein appears to bind laminin with a high affinity via the YIGSR domain (Graf, *et al.*, 1987). This cell surface protein is of great interest because it appears to play a role in several important processes.

A variety of neoplastic cells have been observed to up-regulate the expression of the 67 k laminin receptor (Malinof and Wicha, 1983; Terranova, *et al.*, 1983; Hand, *et al.*, 1985; Wewer, *et al.*, 1986; Wewer, *et al.*, 1987; Martigonone, *et al.*, 1992). YIGSR has been found to interfere strongly with metastasis formation by melanoma cells in mice (Nakai, *et al.*, 1992), and antibodies which recognize the carboxyl terminus of the 67 k receptor block haptotaxis of melanoma cells on laminin (Wewer, *et al.*, 1987). It is interesting to note, in light of the dichotomy between the effects of YIGSR and whole laminin on cell motility presented here, that whole laminin is observed to have the opposite effect to YIGSR on metastasis formation as well (Nakai, *et al.*, 1992).

Invasive, but non-neoplastic tissue, such as trophoblast cells during placenta formation, are also observed to express high levels of the 67 k laminin binding protein (Wewer, *et al.*, 1987). Furthermore, YIGSR has been used to block solid, but not ascitic, tumour growth and angiogenesis by inhibiting endothelial cell migration (Sakamoto, *et al.*, 1991). This evidence has suggested that the 67 k YIGSR receptor plays a role in metastasis and other invasive cell behaviours. This receptor

has also been implicated in cell differentiation (Grant, *et al.*, 1989; Vukicevic, *et al.*, 1990), membrane polarity establishment (Salas, *et al.*, 1992), and cell attachment to elastin (Mecham, *et al.*, 1989).

Investigations aimed at cloning the gene for this receptor have yielded a multi-copy gene which codes for a 33 kDa (295 amino acid) YIGSR-binding polypeptide which has exact sequence identity with the 135 amino acids sequenced previously from the 67 k receptor (Wewer, *et al.*, 1986; Yow, *et al.*, 1988; Rao, *et al.*, 1989; Fernández, *et al.*, 1991; Grosso, *et al.*, 1991). Apart from the obvious size discrepancy, this clone exhibits several other unusual characteristics. The derived amino acid sequence from the open reading frame of the complete cDNA sequence yields a peptide which has a trypsin-resistant, negatively charged C-terminal region with several repeating motifs, but no N-terminal leader for incorporation into the endoplasmic reticulum, and although it contains a strongly hydrophobic region, this region shows little homology to traditional transmembrane domains (Yow, *et al.*, 1988). The initial interpretation of these data was that this was a cytoplasmic, rather than cell surface protein (Grosso, *et al.*, 1991), however pulse-chase and immunological analyses have elucidated a precursor-product relationship between the 33 kDa YIGSR-binding peptide and the mature 67 k laminin binding domain (Rao, *et al.*, 1989; Castronovo, *et al*, 1991a). Current thinking views the 67 k laminin receptor as a chimeric protein consisting of a 33 kDa YIGSR-binding domain, covalently linked to an undescribed polypeptide, possibly with carbohydrate-binding activity (Castronovo, et al., 1991b).

YIGSR and laminin affect cell motility in other organisms

YIGSR and laminin have both been shown to be chemotactic for both normal and neoplastic cells in vertebrates (Albini, *et al.*, 1986; Grotendorst, 1987; Graf, *et al.*, 1987; Kleinman, *et al.*, 1989; Armstrong, *et al.*, 1990). Laminin has also been characterized as an important substrate for cell motility in various invertebrates, including arthropods, annelids, cnidarians, and molluscs (Fessler, *et al.*, 1987; Chiquet, *et al.*, 1988; Agosti and Stidwill, 1991; Miller and Hadley, 1991). Blastocoelar ECM components have also been demonstrated to be chemotactic for sea urchin cells (Solursh and Lane, 1988). However, to date, no work has been published on the interactions of sea urchin cells with laminin.

A laminin binding ECM receptor may play a role in morphogenesis in the sea urchin

In embryos of the sea urchin *Sphaerechinus granularis* laminin immunoreactivity is concentrated in the basal lamina of the blastocoel, with maximal concentrations in the animal and vegetal poles (McCarthy and Burger, 1987). During archenteron elongation, immunoreactivity is observed at the tip of the extending archenteron. It has been demonstrated that the subunit composition of laminin is not necessarily consistent over time or between tissues during embryogenesis (Cooper and MacQueen, 1983). It may be that the laminin on the lateral walls of the blastocoel, and especially the basal surface of the extending archenteron, contains two B2 chains, and therefore no YIGSR motif. If this is the case, then the cells at the tip of the

archenteron will only be exposed to domains of laminin other than YIGSR until they reach the roof of the blastocoel. At this time the 70 k YIGSR receptor on the surface of these cells would be exposed to its ligand, stimulating their release from the tip of the archenteron and subsequent migratory behaviour. Receptors for other domains of laminin may be involved in repressing premature release and migration during archenteron elongation. If this hypothesis is correct, the 70 k laminin binding protein may provide a valuable invertebrate model for investigations into the evolution and behaviour of this idiosyncratic and potentially important extracellular matrix receptor.

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