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A role for regulated secretion of apical extracellular matrix during epithelial invagination in the sea urchin

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SUMMARY

Epithelial invagination, a basic morphogenetic process reiterated throughout embryonic development, generates tubular structures such as the neural tube, or pit-like structures such as the optic cup. The 'purse-string' hypothesis, which proposes that circumferential bands of actin microfilaments at the apical end of epithelial cells constrict to yield a curved epithelial sheet, has been widely invoked to explain invaginations during embryogenesis. We have reevaluated this hypothesis in two species of sea urchin by examining both natural invagination of the vegetal plate at the beginning of gastrulation and invagination induced precociously by Ca^{2+} ionophore. Neither type of invagination is prevented by cytochalasin D. In one species, treatment with A23187 three hours before the initiation of invagination resulted in the deposition of apical extracellular matrix at the vegetal plate, rather than invagination. This apical matrix contains chondroitin sulfate, as does the lumen of the archenteron in normal gastrulae. When the expansion of this secreted matrix was resisted by an agarose gel, the vegetal plate buckled inward, creating an archenteron that appeared 3-4 hours prematurely. Pretreatment with monensin, which blocks secretion, inhibits both Ca^{2+} ionophore-stimulated folding and natural invagination, demonstrating that secretion is probably required for this morphogenetic event. These results indicate that alternatives to the purse-string hypothesis must be considered, and that the directed deposition of extracellular matrix may be a key Ca^{2+}-regulated event in some embryonic invaginations. A bending bilayer model for matrix-driven epithelial invagination is proposed in which the deposition of hygroscopic material into a complex, stratified extracellular matrix results in the folding of an epithelial sheet in a manner analagous to thermal bending in a bimetallic strip.

Abbreviations used: aECM, apical extracellular matrix; CD, cytochalasin D; CSPG, chondroitin sulfate proteoglycan; HL, hyaline layer

Key words: invagination, gastrulation, morphogenesis, proteoglycan, secretion, sea urchin

INTRODUCTION

Epithelial invagination is a basic morphogenetic process reiterated throughout embryogenesis to generate complex tissue architecture. Invagination, or folding, is an especially frequent event during organogenesis and can lead to the formation of tubular structures such as the neural tube or pits such as the optic cup. As this process is ubiquitous in metazoan development and is used repeatedly by an organism during its early ontogeny, there has been much speculation through the years on the mechanical basis of invagination. Hypotheses based on differential cell adhesion, differential cell growth and division, and cytoskeletal-mediated changes in cell shape have been offered but, to date, little experimental evidence supporting one or another of the models has been forthcoming (reviewed in Ettensohn, 1985).

The prevailing viewpoint in the literature is that folding results from the summation of small changes in the shapes of the individual cells comprising the epithelium. In the 'purse-string' hypothesis, bands of microfilaments arranged circumferentially at one end of a columarized epithelial cell constrict (Baker and Schroeder, 1967), decreasing the surface area at that end of the cell, and changing the cell from columnar to wedge-shaped (Gustafson and Wolpert, 1962). When the apical microfilament bands constrict in a group of cells, the result is curvature of the cell sheet and, depending upon the geometry of the group of active cells, tubes or pits are formed.

The evidence supporting the purse-string hypothesis comes from observations and experiments in a number of different systems. Circumapical rings of microfilaments were found in invaginating epithelia by electron microscopy and by staining for assembled actin filaments with phalloidin derivatives. Early studies with the drug cytochalasin B, which was believed to disrupt microfilaments selectively, blocked every embryonic invagination tested (reviewed in

Wessells et al., 1971). Subsequently, circumferential microfilament bands were physically isolated from pigmented retinal epithelium and shown to contract in vitro (Owaribe and Masuda, 1982) in response to ATP.

These arguments provide a less than compelling case for the hypothesis. Ultrastructural studies showed that circumapical microfilament bands are found in non-invaginating epithelia, so their presence does not predict an impending invagination. Cytochalasin B has many side effects (e.g., inhibition of monosaccharide transport across the plasma membrane, Rampal et al., 1980) which confound the interpretation of experiments in which invagination was blocked. Cytochalasin D, which is more specific for microfilaments than cytochalasin B, disrupts the apical microfilament bands in cells of the chick neural plate, but does not block most of the events that shape the neural plate into the neural tube, including elevation of the neural folds (Schoenwolf et al., 1988). The isolated microfilament bands that constricted in vitro were derived from a differentiated tissue (the pigmented retinal epithelium) that is not destined to undergo invagination, and thus may not be an appropriate model for embryonic epithelia. Finally, it should be pointed out that all of the proposed models for invagination begin with the premise that there is a net decrease in concave surface area as invagination proceeds. Although apical constriction has been quantitated in some systems, such as bottle cell formation during amphibian gastrulation (Hardin and Keller, 1988) and amphibian neurulation (Burnside and Jacobsen, 1968), there are no measurements to support this assumption in some of the relatively simple invaginating systems such as the sea urchin and *Drosophila* embryos. One could argue that a more accurate description is that the total apical surface area (concave) increases less than the total basal (convex) surface area during invagination, but both surface areas increase.

Epithelial folding has been induced prematurely in several systems using the calcium ionophore A23187. Precocious neural fold formation in *Xenopus* (Moran and Rice, 1976) and optic cup formation in the chick (Brady and Hilfer, 1982) were observed within 30 minutes of ionophore exposure. The authors in these studies speculated that the major effect of the Ca^{2+} in these morphogenetic movements was activation of microfilament constriction, a process assumed to be somewhat analagous to actin-myosin interactions in skeletal muscle. However, it is now clear that Ca^{2+} regulates many intracellular processes, and direct proof of the purse-string hypothesis is lacking.

Using the sea urchin embryo as a model system, we have reexamined the process of epithelial invagination during gastrulation. In the commonly studied euechinoid species such as *Lytechinus pictus* and *Strongylocentrotus purpuratus*, the second phase of gastrulation is the inpocketing of the vegetal plate, a process known as primary invagination. As is true of most (if not all) invaginating epithelia (Ettensohn, 1985), the first sign of the impending invagination in sea urchins is the lengthening of the vegetal plate cells along their apical-basal axis. In other systems, this process of columnarization is referred to as 'palisading' and the specialized region of the epithelium is called a placode. In euechinoid species of sea urchins, the vegetal plate first thickens prior to primary mesenchyme cell ingression. The thickness of the plate decreases following ingression, but undergoes a second increase in thickness before the vegetal plate folds inward to create a short, stout archenteron. Because the infolding will occur in surgically isolated vegetal plates (Moore and Burt, 1939; Ettensohn, 1984a,b), the forces necessary to buckle this epithelium must be generated by the invaginating cells. However, the nature of these forces is not known.

In the present study, we have investigated the roles of Ca^{2+} and microfilaments during primary invagination in the sea urchin. We present evidence that a major event underlying, and perhaps driving, the initial steps of primary invagination in the sea urchin is regulated, apically directed secretion of extracellular matrix. The matrix deposited during natural and precocious invagination is specific to the invaginating region and includes a chondroitin sulfate-containing molecule or molecules. Treatments that perturb the synthesis or secretion of sulfated extracellular matrix also perturb invagination, suggesting that deposition of this specialized matrix is instrumental in driving the invagination process.

MATERIALS AND METHODS

Embryo culture

Gametes from *Lytechinus pictus* and *Strongylocentrotus purpuratus* were obtained by intracoelomic injection of 0.55 M KCl. The eggs were washed, fertilized with a dilute sperm suspension and cultured at 15°C in millipore-filtered sea water (MFSW). Only highly synchronous cultures of embryos (>95% at the same stage) were used in these experiments, and the two species were utilized during their natural, seasonal reproductive cycles. In typical cultures, under these conditions, hours of development correspond to embryonic stages as summarized in Table 1.

To generate sulfate-deprived embryos, eggs were shed into sulfate-free sea water (SFSW, Anstrom et al., 1987), washed 2× in SFSW and fertilized in SFSW containing 3 mM p-aminobenzoic acid to prevent hardening of the fertilization membrane. After the softened fertilization membranes were removed by filtration through Nitex, the embryos were washed and cultured in SFSW. During the 1-2 hours in which the parallel cultures of control embryos were hatching, sulfate-deprived embryos were washed 3 times with SFSW and cultured at 1% (v/v) in SFSW. Changing the SFSW several times during and after hatching is crucial for the appearance of the sulfate-deprived phenotype we show in Fig. 7A. Cameron and Holland (1985) reported that prior to hatching, the hyaline layer of *Lytechinus variegatus* is surrounded by a granular layer that disappears after hatching. The staining properties of this granular layer suggests it may contain proteoglycans, and the layer's disappearance may serve as a source of sulfate for subsequent development. Changing the SFSW several times seems to reduce the availability of sulfate and results in the phenotype that we report.

For experiments in which embryos were embedded in agarose, ultra-low temperature agarose (Sigma, A-5030) was mixed in MFSW to 0.8%, dissolved at 50°C, and adjusted to 16°C. Three volumes of dilute embryo culture, either controls or embryos treated with A23187 (see below), were mixed with one volume of the agarose/sea water stock, transferred to a microchamber and kept on a 15°C stage until viewed in a Nikon Diaphot inverted microscope equipped with DIC optics. The embryos were optically sectioned and the images made using a Dage MTI CCD video camera and an Image One video image processor (Univer-

Table 1. Descriptive landmarks in *L. pictus* and *S. purpuratus* as a function of time at 15°C

Hours of development	Stage	*L. pictus*	*S. purpuratus*
19	early mesenchyme blastula	PMCs ingressed and sitting on vegetal plate. embryos hatched.	PMCs ingressed and sitting on vegetal plate. embryos hatching.
20	mesenchyme blastula	PMCs migrating away from vegetal plate. plate thinned out.	PMCs migrating away from vegetal plate. plate thinned out.
21	mesenchyme blastula	as above.	cells in vegetal plate beginning to columnarize again.
23	mesenchyme blastula	PMCs well away from vegetal plate. plate beginning to columnarize again.	PMCs well away from vegetal plate. pronounced, columnarized plate.
24	late mesenchyme blastula	vegetal plate flattened. first signs of invagination.	vegetal plate flattened. first signs of invagination.
26	early gastrula	primary invagination completed.	primary invagination completed.

sal Imaging, Media, PA), and recorded on a Panasonic TQ-2028F optical memory disk recorder. Micrographs for publication were photographed from the monitor.

Ionophore and cytochalasin D treatment

Stocks and working solutions of pharmacological reagents were prepared as follows. A23187, Br-A23187 and monensin (Calbiochem, La Jolla, CA) stocks were 100 mM in DMSO, 4 mM in DMSO and 10 mM in EtOH, respectively, and stored at −20°C. Cytochalasin D (Sigma) was 1 mg/ml in EtOH and stored at 4°C. All working solutions were made fresh before an experiment by mixing the appropriate volume of the stock solution (or for controls, DMSO or EtOH) with MFSW (or SFSW, as required) to 2× the final concentration. 1 ml of embryo culture was then added to 1 ml of the double strength solution in a 35 mm plastic Petri dish to produce the final concentration (20 μM A23187 or Br-A23187, 4 μM monensin, and 3 μg/ml cytochalasin D) and an embryo concentration of about 2000/ml. The embryos were kept at 15°C until viewed with DIC optics in the microscope. Early and late in the reproductive season, embryos of both species were especially sensitive to the pharmacological reagents. In particular, A23187 caused cells in the vegetal plate to be extruded to the outside of the embryo. This was a rare event during most of the reproductive season, and we report results for healthy cultures in season. To score and photograph phenotypes, a drop of embryos was transferred to a well made on a slide using Scotch double sticky tape (no. 665) and covered with a coverslip. The coverslip presses down on the embryos, trapping them and giving them a slightly expanded appearance. All of the embryos in the chamber (except those along the edges), or 100 total, were scored.

Immunofluorescence

For whole-mount staining, embryos were fixed on ice with 3.7% formalin/90% sea water/0.05 M Tris buffer, pH 8.1, for 2 hours. The formaldehyde was washed away with three changes of sea water, and the embryos dehydrated to 70% EtOH and stored at 4°C. Embryos to be sectioned were fixed on ice for 1 hour with cold 99% EtOH/1% HOAc, washed 2× in 100% EtOH, resuspended in 50% Histosol/50% EtOH, and cleared overnight in 100% Histosol. The specimens were infiltrated with 50% Histosol/50% paraffin for 3 hours, followed by four changes (of at least 1 hour per cycle) of 100% paraffin. 10 μm sections were cut. Prior to immunostaining, sections were blocked in 2% goat serum in PBS (0.02 M, pH 7.4) for 2 hours.

The monoclonal antibody CS-56 was purchased from ICN Immunobiologicals (Irvine, CA) or Sigma (St Louis, MO). Fixed embryos were rehydrated, then blocked with 2% goat serum in PBS overnight at 4°C. The embryos were incubated in a 1:100 dilution of CS-56 in 2% goat serum in PBS for 1 hour, washed 4 times with PBS and incubated in 1:200 fluorescein isothiocyanate-conjugated goat anti-mouse IgM (Zymed, So. San Francisco, CA) for 1 hour. After 4 washes with PBS, the embryos were viewed on a Nikon Diaphot inverted microscope equipped with epifluorescence.

RESULTS

Ca^{2+}-ionophore stimulates precocious invagination of the vegetal plate

Treatment of cells with the Ca^{2+} ionophore A23187 stimulates an increase in the intracellular [Ca^{2+}]. When late mesenchyme blastulae of *Lytechinus pictus* (23 hour, Fig. 1A) or *Strongylocentrotus purpuratus* (not shown) are treated with 20 μM A23187, the embryos are paralyzed for approximately 5 minutes, but then resume swimming. Soon after, the vegetal plate begins folding inward, creating within 30 minutes an incipient archenteron (Fig. 1B, Table 2) that is virtually indistinguishable from the primary invagination that will have formed in controls 3 hours later. In *L. pictus*, which does not possess a pronounced vegetal plate until immediately before invagination, this response is elicited only at late mesenchyme blastula stage (23 hours); younger embryos exposed to the Ca^{2+} ionophore at 20-22 hours do not invaginate (Fig. 1C, Table 3). Thus, the vegetal plate epithelium at late mesenchyme blastulae stage is prepared to invaginate well in advance of gastrulation, and the machinery required for the morphogenetic movement can be triggered by an increase in intracellular Ca^{2+}.

To test whether dynamic actin filaments play the critical role during invagination proposed by the purse-string hypothesis, late mesenchyme-blastula stage embryos were

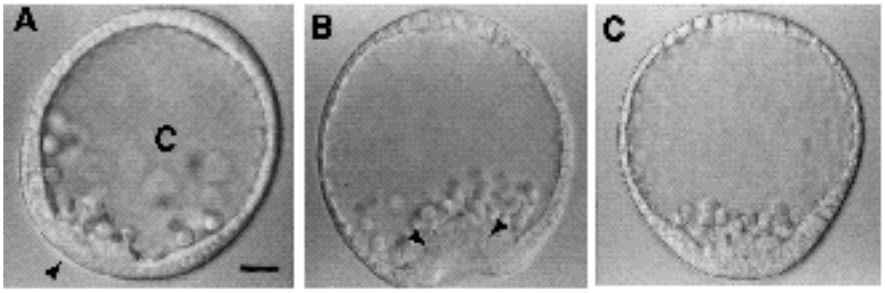

Fig. 1. Precocious invagination of the vegetal plate in response to the calcium ionophore A23187. (A) A control *L. pictus* embryo at 23.5 hour (late mesenchyme blastula). The cells of the vegetal plate (arrowhead) have lengthened along their apical-basal axis, but the plate has not yet flattened. (B) A sibling embryo, also 23.5 hour old, after 30 minutes in 20 μM A23187. The vegetal plate has folded inward to yield a precocious archenteron (arrowheads). This response by the vegetal plate epithelium is specific to late mesenchyme-blastula stage. (C) An early mesenchyme-blastula stage embryo (19 hours) in which the primary mesenchyme cells recently ingressed into the blastocoel and the cells of the vegetal plate have columnarized, does not invaginate when treated with A23187. Scale bar, 20 μm.

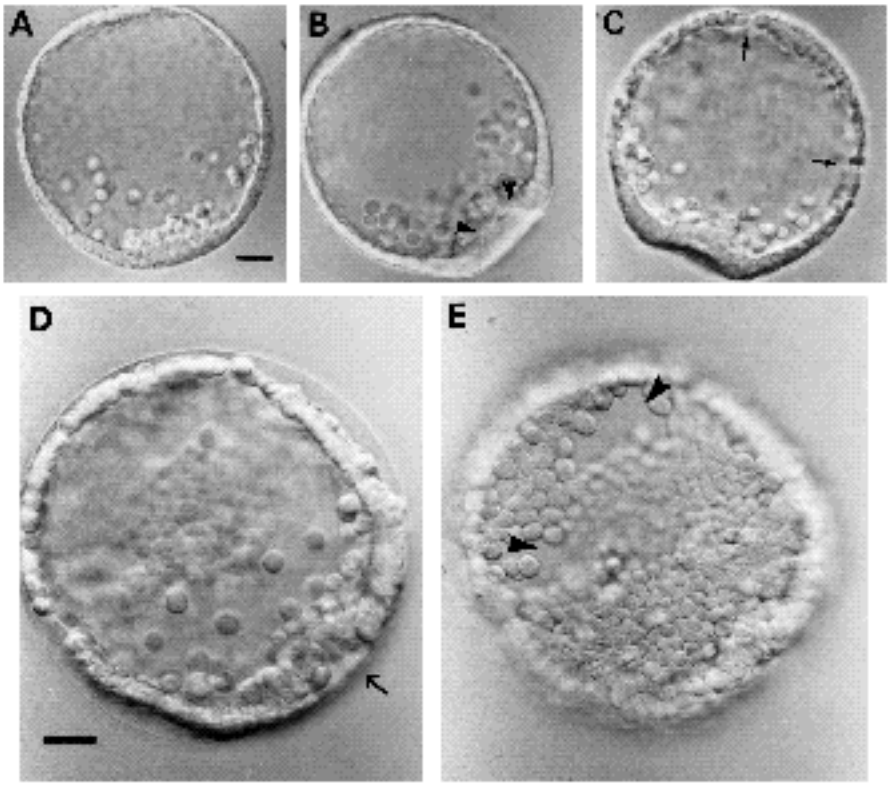

Fig. 2. The effect of cytochalasin D (CD) on precocious and natural invagination. (A) A control *L. pictus* (23.5 hour) embryo after 50 minutes in 3 μg/ml CD. The epithelial wall of the blastocoel thins out, there are no filopodial processes on the mesenchyme cells and the embryos are slightly enlarged. (B) A sibling embryo (23.5 hour) that has been in CD for 50 minutes and A23187 for 20 minutes concurrently. The vegetal plate has invaginated precociously in response to the calcium ionophore (arrowheads). Natural invagination is not blocked by CD treatment. (C) CD added to embryos just before the start of invagination does not prevent inward folding of the vegetal plate. This embryo has been in CD for approximately 70 minutes and is dissociating (arrows). In both B and C, the shape of the archenteron is much broader than normal, suggesting that microfilaments control the shape of the archenteron. (D) An *L. pictus* embryo after 1 hour in CD and 30 minutes in A23187 concurrently, optically sectioned through the archenteron. The vegetal plate has invaginated (arrow) and holes are present in the epithelial wall. (E) The extent of the damage caused by CD can be seen in the same embyo as depicted in Fig. 2D. The focal plane now passes through the epithelium pressed against the coverslip. Gaping holes can be seen in the epithelial wall (arrowheads). Scale bar, 20 μm.

incubated in 3 μg/ml cytochalasin D (CD) 30 minutes prior to A23187 exposure. In a dose-response determination, a concentration of 2 μg/ml CD is sufficient to block completely two sequential rounds of cytokinesis in 94% of embryos when added 45 minutes after fertilization, while 3 μg/ml prevented cytokinesis in 100% of the embryos and caused a visible disruption of the cortex (not shown). Embryos pretreated with 3 μg/ml CD (Fig. 2A, Table 2) still invaginated following exposure to the Ca^{2+} ionophore (Fig. 2B). The shape of the archenteron is generally much broader (i.e., the radius of curvature is increased) than in normal gastrulating controls, which suggests that microfilaments may play a role in controlling the exact shape of the archenteron. Indeed, the overall shape of the embryo is also modified after 1 hour in CD (compare Figs 2A and 1A), and between 60 and 70 minutes of exposure to CD, gaping holes are apparent in the epithelial wall, indicating that CD affects cell adhesion, as previously reported (Ettensohn, 1984b). The severity of this effect on cell adhesion can be seen in the embryo optically sectioned in Fig. 2D and E. The focal plane in Fig. 2D goes through the invaginated vegetal plate (arrow) and in Fig. 2E the focal plane shows the wall of the epithelium pressed against the coverslip. Huge gaps are present (arrowheads) where cells have dissociated from one another. These gaps are more pronounced in the animal hemisphere, but dissociation is also occurring in the vegetal half of the embryo.

The sequence of events involved in natural invagination normally occurs over a two hour period. Given the catastrophic effects CD has on cell adhesion by 60 to 70 min-

Table 2. Precocious invagination in *L. pictus*: the effects of cytochalasin D, monensin and sulfate deprivation

	Invagination (number of embryos)	No invagination (number of embryos)
Trial 1*		
control	1	99
control + A23187	89	11
cyto D	7	93
cyto D + A23187	84	16
monensin	2	98
monensin + A23187	62†	37
sulfate-deprived	0	100
sulfate-deprived + A23187	15	74
Trial 2		
control	6	94
control + A23187	92	8
cyto D	11	89
cyto D + A23187	89	11
monensin	8	92
monensin + A23187	57	43
sulfate-deprived	0	110
sulfate-deprived + A23187	3	69

*Trials were performed on separate days with full sibling embryos. Regardless of the treatment regime, A23187 was added to embryos at 23 hours of age and scoring began thirty minutes later. One hundred embryos in each treatment class, except in the case of sulfate-deprived trials, were scored within 10 minutes of preparation for microscopy.

†The invaginations in monensin-treated embryos were much smaller than the invaginations in control embryos. An example is shown in Fig. 6C.

utes, it is difficult to ascertain whether CD prevents natural invagination. However, CD added about 30 minutes before the onset of natural invagination does not prevent infolding (Fig. 2C), even as the embryo dissociates (arrows in Fig. 2C). The inability of CD to prevent both precocious and natural invagination argues against a central and unique role for microfilament-mediated constriction during vegetal plate invagination.

Secretion of extracellular matrix at the vegetal plate

The cellular process affected by the intracellular [Ca^{2+}] was revealed by exposing developmentally younger *S. purpuratus* embryos, which have a pronounced vegetal plate at 21.5 hour, to A23187. While 20- to 22-hour-old *L. pictus* embryos, which will not form a thickened vegetal plate until 23 hours, displayed no obvious response to A23187 (Fig. 1C, Table 3), treatment of *S. purpuratus* mesenchyme blastulae (19.5-22 hours, Fig. 3A, Table 2) results in the progressive appearance of a bulging apical extracellular matrix (aECM) at the vegetal plate over a 30 minute period (Fig. 3B), which we will show represents newly secreted material. This response is stage-specific, as such protuberances are not observed in younger *S. purpuratus* embryos treated with the ionophore (18-19 hours; Table 3).

To demonstrate that the observed protuberance is the manifestation of a newly secreted, swelling matrix and not a delamination of the hyaline layer from the vegetal plate epithelium, 21.5-hour-old embryos were treated with A23187 and immediately embedded in 0.2% agarose. If the protuberance represents newly deposited, swelling matrix, the mechanical resistance of the gel to the expanding matrix should result in the formation of a precocious invagination, as the expanding matrix pushes the vegetal plate inward. In contrast, if the protuberance is caused by delamination of

Table 3. Species differences in response by the vegetal plate to A23187

	Hours						
	18	19	20	21	22	23	24
L. pictus	–	–	–	–	–	i	i
S. purpuratus	–	s	s	s	s	i	i
S. purpuratus (in agarose)		nd	nd	i	i		

(−) = no observed response
(s) = secreted matrix at vegetal plate
(i) = invagination
(nd) = not determined

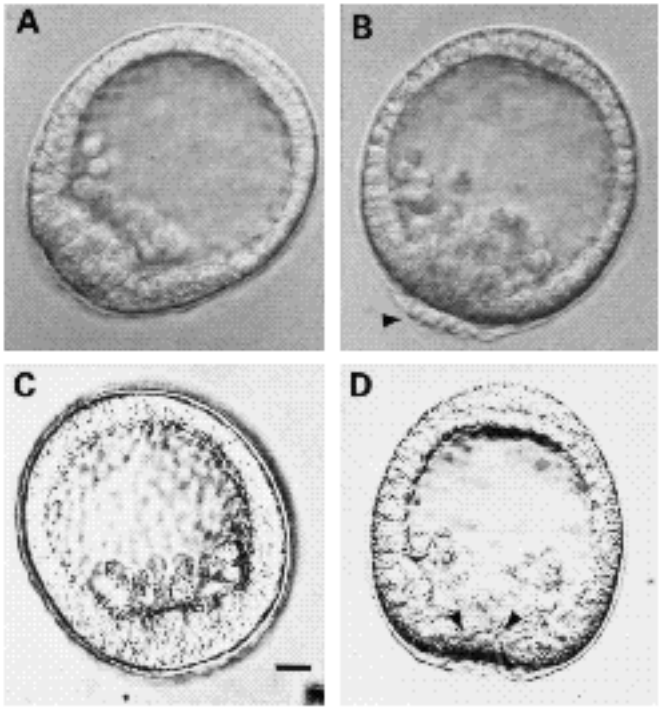

Fig. 3. The calcium ionophore A23187 stimulates secretion of apical extracellular matrix (aECM) in 22-hour-old *S. purpuratus* embryos. (A) A control embryo in which the primary mesenchyme cells have begun migrating from the the vegetal plate. (B) A sibling embryo after 30 minutes in A23187. The hyaline layer over the apical surface of the vegetal plate is markedly thickened. The protrusion is first visible several minutes after addition of the ionophore and enlarges over 20-30 minutes. (C) A control embryo embedded in 0.2% agarose. (D) An embryo treated with A23187 and immediately embedded in agarose. In the presence of the mechanical resistance provided by the gel, the expanding matrix forces the vegetal plate inward and generates an archenteron that is premature by 3-4 hours. C and D were optically sectioned using DIC optics and an image processor (see Materials and Methods). Scale bar, 15 μm.

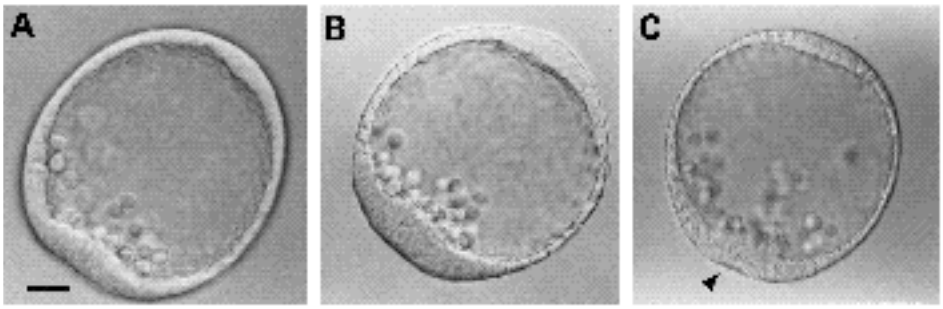

Fig. 4. Pretreatment with 4 μM monensin inhibits precocious invagination stimulated with A23187. (A) A 23.5-hour-old *L. pictus* embryo after 1.5 hours in monensin. (B) A sibling 30 minutes after exposure to A23187 which does not show signs of invagination. (C) A sibling treated with A23187 that shows a very small invagination at the vegetal plate (arrowhead). Scale bar, 25 μm.

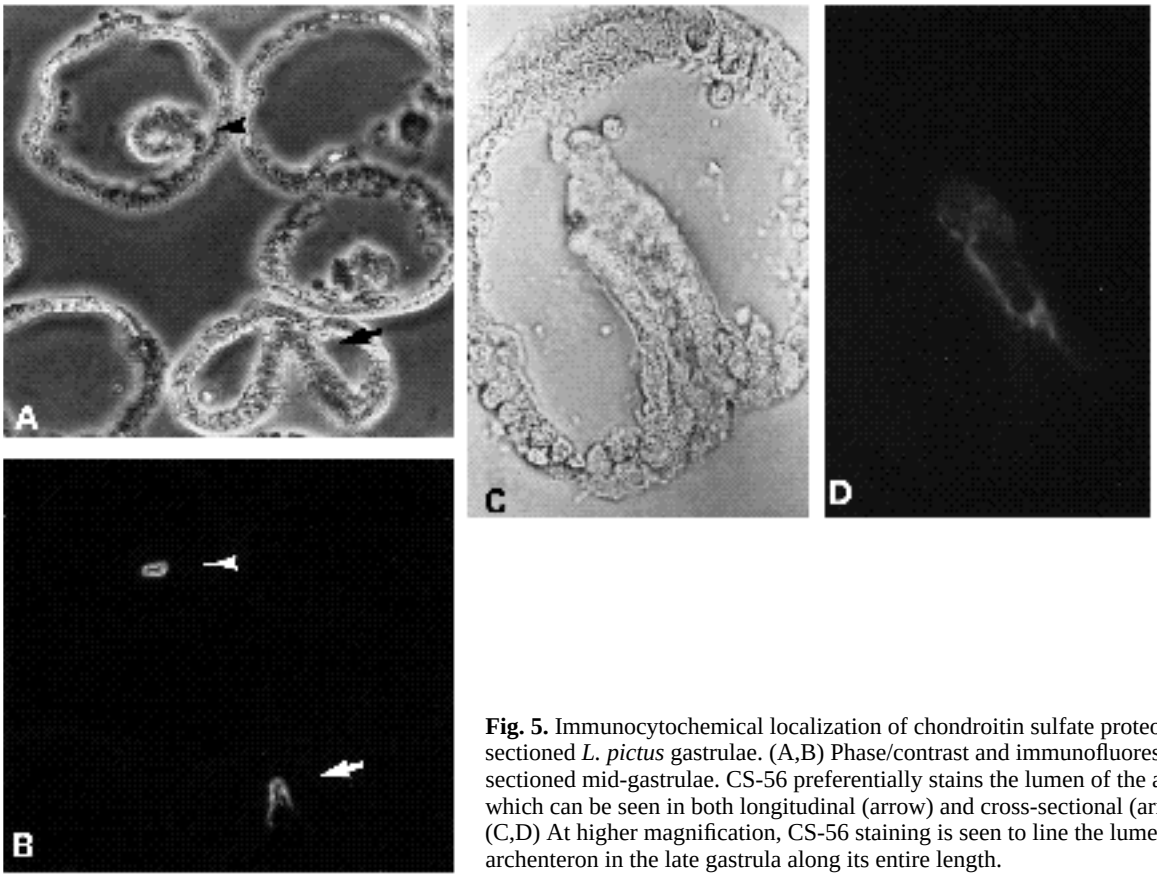

Fig. 5. Immunocytochemical localization of chondroitin sulfate proteoglycan in sectioned *L. pictus* gastrulae. (A,B) Phase/contrast and immunofluoresence views of sectioned mid-gastrulae. CS-56 preferentially stains the lumen of the archenteron, which can be seen in both longitudinal (arrow) and cross-sectional (arrowhead) views. (C,D) At higher magnification, CS-56 staining is seen to line the lumen of the archenteron in the late gastrula along its entire length.

the ECM, the agarose gel should have no effect on the morphology of the embryo. When embryos are treated with A23187 and immediately embedded in agarose, precocious invagination is observed (Fig. 3C,D). These results show that the stage-dependent, apically directed secretion of ECM may be a critical event regulated by Ca^{2+} during epithelial invagination.

The Na^{+}-H^{+} ionophore monensin blocks secretion by perturbing Golgi function (Tartakoff, 1983), and has been used to block membrane trafficking and secretion in the sea urchin embryo (Anstrom and Raff, 1988; Roe and Lennarz, 1990). Pretreatment of embryos with 4 μM monensin for 1 hour before stimulation with A23187 (at 23 hour) reduced precocious invagination (Fig. 4A,B; Table 2), as well as aECM deposition in *S. purpuratus* mesenchyme blastulae (not shown). The number of embryos with invaginations decreased, as did the size of the invaginations that formed (Fig. 4C). The small invaginations may represent the contributions of microfilament constriction, residual secretion (discussed further below), or other unknown processes. Natural invagination was also inhibited by monensin. When the drug was added 2 hours before the onset of gastrulation, invagination was blocked reversibly. Removal of monensin within 4 hours delayed, but did not prevent, subsequent morphogenesis. These results indicate that secretion may be required for vegetal plate invagination in the sea urchin.

Sulfated ECM appears in the hyaline layer at the vegetal plate during invagination

During invagination, the hyaline layer (HL) at the vegetal plate maintains its association with the apical surface of the invaginating cells, and comes to line the luminal surface of the forming archenteron. In embryos fixed by a variety of protocols, the HL within the lumen of the archenteron of the gastrula appears morphologically distinct from the rest of the HL (e.g., see fig. 2, Kawabe et al., 1981; fig. 1,

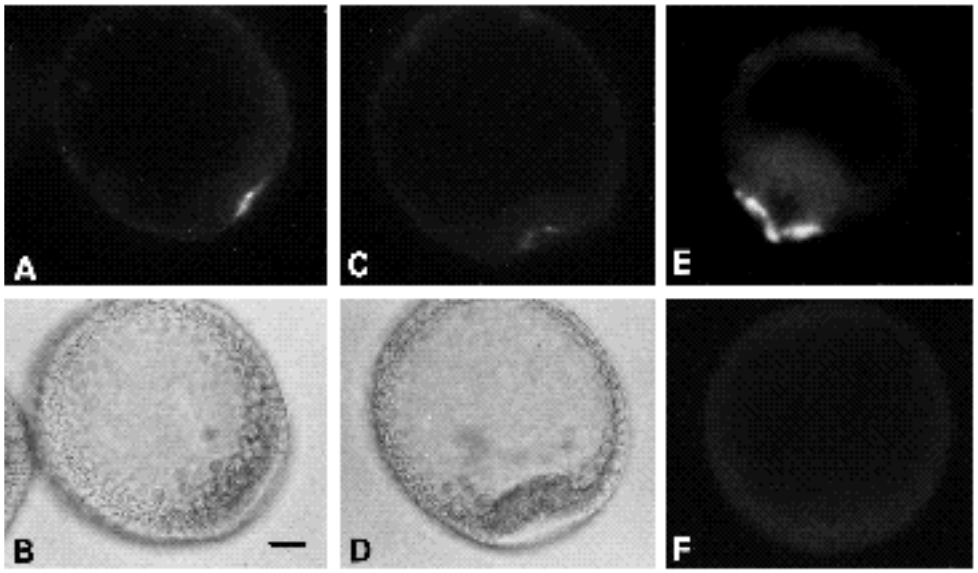

Fig. 6. Whole-mount immunocytochemical localization of chondroitin sulfate proteoglycan in normal and Bromo-A23187-stimulated *L. pictus* (A-D and F) and *S. purpuratus* (E) embryos. The monoclonal antibody CS-56 was used to localize CSPG in formalin-fixed, whole-mount embryos. (A,B) At early gastrula, when the plate has flattened and is just beginning to press inward, chondroitin sulfate is present in the hyaline layer at the vegetal plate. (C,D) In precocious gastrulae, chondroitin sulfate lines the lumen of the precocious archenteron. (E) The protruding aECM that forms when 22-hour-old *S. purpuratus* are stimulated with Bromo-A23187 also contains chondroitin sulfate. (F) The CS-56 epitope is not present in the HL of the vegetal plate at mesenchyme-blastula stage. Scale bar, 20 μm.

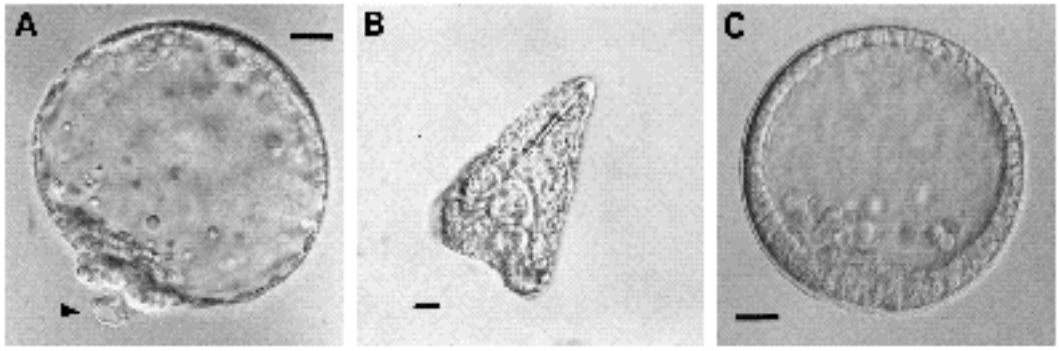

Fig. 7. The effects of severe sulfate deprivation on gastrulation in *S. purpuratus*. (A) A 68-hour-old embryo cultured as described in Materials and Methods. A large bolus of apparently disorganized apical matrix protrudes from the vegetal plate (arrowhead). The cells of the vegetal plate may have columnarized, although much of the apparent thickening of the plate is due to the primary mesenchyme cells, which have remained on the plate. (B) A control embryo at 68 hours. Sphincters have formed, splitting the gut into three parts. (C) A 30-hour-old, sulfate-deprived embryo, 30 minutes after treatment with A23187. There is little increase in the thickness of the HL in response to the calcium ionophore. All scale bars, 20 μm.

Akasaka and Terayama, 1983; fig. 7, Ettensohn, 1984a; figs 6A and 7D, Morrill and Santos, 1985; figs 5-7, Amemiya, 1989); this luminal matrix appears to be either thicker than the rest of the HL, or possibly less adherent to the adjacent epithelium than other regions of the HL. Several lines of evidence described below indicates that the apical extracellular matrix of the archenteron is also a biochemically distinct region of the HL.

One matrix molecule that appears de novo in the aECM of the vegetal plate during gastrulation bears a chondroitin sulfate epitope. We used the monoclonal antibody CS-56, which recognizes native chondroitin sulfate A and C glycosaminoglycan chains (Avnur and Geiger, 1984), to determine the chondroitin sulfate proteoglycan (CSPG) distribution in the embryo. In sectioned *L. pictus* embryos, there is no staining in mid-mesenchyme blastulae (not shown, but see Fig. 6F for whole-mount staining); by gastrula stage there is intense staining in the lumen of the archenteron (Fig. 5B,D). Analysis of staining in *L. pictus* whole-mount embryos shows that CS-56 immunoreactivity appears on the apical surface of the plate as the vegetal plate flattens (Fig. 6A,B). In embryos stimulated to invaginate with Bromo-A23187 (a nonfluorescent analogue of A23187), the CS-56 epitope is found in the lumen of the precociously formed archenteron (Fig. 6C,D), as well as in the aECM protrusion seen in 22-hour *S. purpuratus* (Fig. 6E). These observations demonstrate that both precocious and natural invagination involve the deposition of a molecule (or molecules) that bear the CS-56 epitope (hereafter referred to as CSPG) into the aECM of the vegetal plate/archenteron.

It has been shown previously that prior to gastrulation, the vegetal end of the sea urchin embryo incorporates greater levels of [^{35}S]sulfate ions than the animal end (Immers, 1961; Sugiyama, 1972). When *S. purpuratus* embryos are cultured under a stringent sulfate-deprivation regime, they develop a unique vegetal plate morphology. The cells columnarize more slowly than in controls, there is no invagination and, by 30-36 hours, a small nubbin of

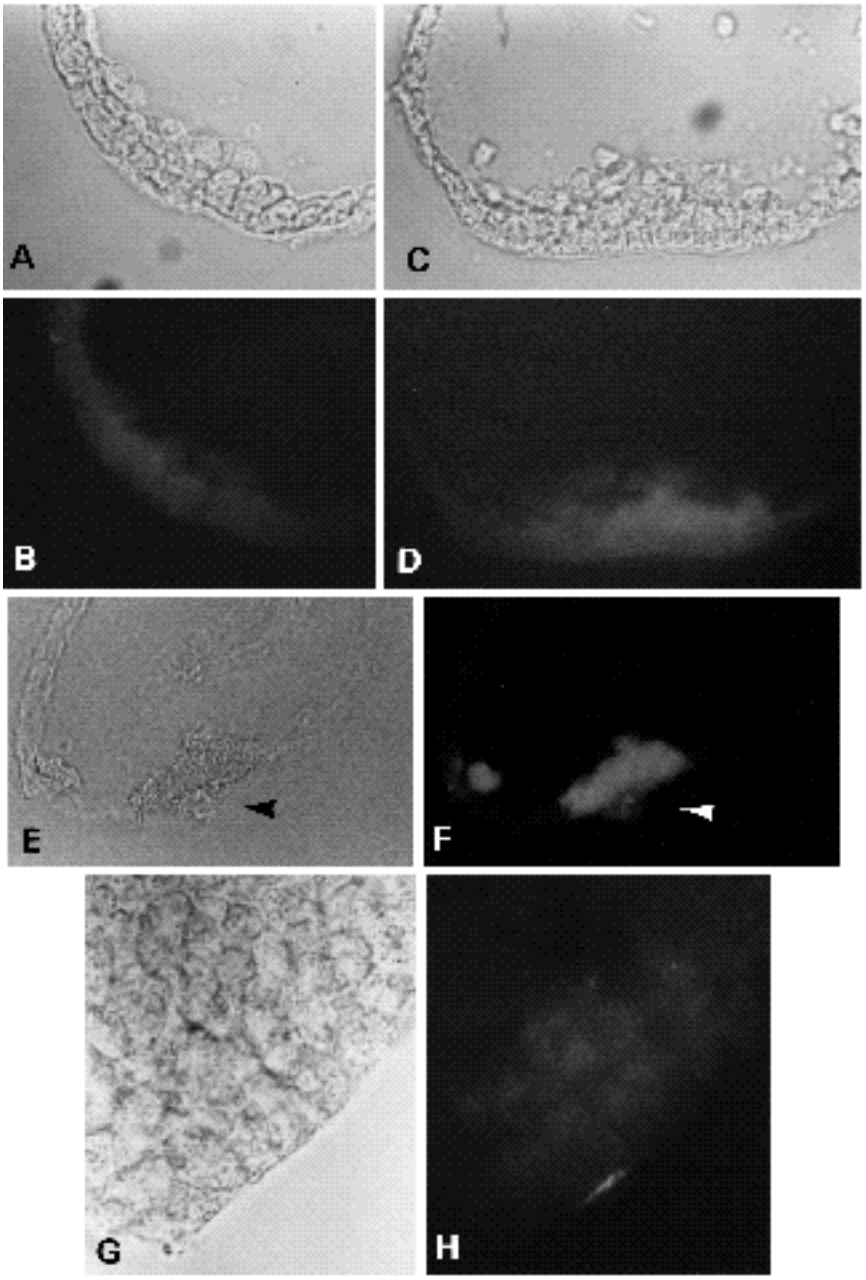

Fig. 8. Immunolocalization of CSPG in sectioned, sulfate-deprived embryos and whole-mount, monensin-treated embryos. (A,B) A 25-hour-old *S. purpuratus* embryo by phase contrast and immunofluorescence. No CSPG is localized to the vegetal plate. (C,D) A 36-hour-old *L. pictus* embryo by phase contrast and immunofluoresence. There is no staining of CSPG at the vegetal plate. Autofluorescence of the vegetal plate is increasing. (E,F) A 68-hour-old *S. purpuratus* embryo. The protruding aECM (arrowhead) does not contain CSPG. Autofluorescence of the vegetal plate and primary mesenchyme cells has markedly increased. (G,H) A 24-hour-old *L. pictus* treated with 4 μM monensin for 2 hours and Br-A23187 for 30 minutes concurrently. The small invaginations seen in about 60% of the embryos contain CSPG.

apical matrix appears where the blastopore normally forms. With time, this nubbin enlarges into a bolus of highly disorganized ECM (Fig. 7A), and no gastrulation movements are detected. If 22- to 30-hour-old, sulfate-deprived embryos are exposed to A23187, little or no swollen aECM is deposited, nor do the embryos invaginate as frequently (Fig. 7C; Table 2). Thus, buckling of the vegetal plate is apparently contingent on the deposition of sulfated aECM.

When sulfate-deprived embryos are immunostained with CS-56 antibody, the epitope is not present in 25-hour-old (Fig. 8A,B) or in 36-hour-old embryos (Fig. 8C,D). For unknown reasons, autofluorescence of the vegetal plate and the primary mesenchyme cells increases with time as embryos are cultured under sulfate-free conditions. The large bolus of extruded matrix seen on older sulfate-deprived embryos (e.g., Fig. 7A) also does not contain the CSPG, as judged by immunostaining (Fig. 8E,F). Thus, as expected, the sulfate deprivation regime inhibits synthesis of the CSPG.

Monensin-treated embryos were also analyzed for the presence of the CS-56 epitope, and two pertinent observations were made in these embryos. First, following Br-A23187-stimulation, many of the embryos are negative for the CS-56 epitope, but those with small invaginations (seen in 57-62% of the embryos in Table 2) contained CSPG by immunocytochemistry (Fig. 8G,H), indicating that treatment with 4 μM monensin for 1 hour does not completely block secretion of the CSPG. Second, in such monensin-treated embryos, we have observed faint intracellular stain-

ing of cells in the vegetal plate (not shown), which we have not observed in control embryos.

DISCUSSION

Summary: calcium ionophore stimulates regulated, apically directed secretion of extracellular matrix

The increase in intracellular Ca^{2+} induced by A23187 triggers premature folding of the vegetal plate prior to gastrulation in the sea urchin on a time scale similar to precocious invaginations in the amphibian neural tube and the chick optic cup (Moran and Rice, 1976; Brady and Hilfer, 1982). In *S. purpuratus* embryos, which form a pronounced vegetal plate hours before gastrulation, early exposure to A23187 stimulates the exocytosis of apical extracellular matrix (aECM), presumably by the cells of the vegetal plate. When the swelling of the matrix deposited during this exocytotic event is mechanically resisted by an agarose gel, the epithelium of the vegetal plate folds inward, as it does during normal primary invagination.

Preincubation of embryos in cytochalasin D (CD) did not prevent primary invagination nor precocious invagination in response to A23187. These results are contrary to all the previous reports in which cytochalasin B was found to inhibit embryonic invaginations (Karfunkel, 1971; Wrenn, 1971; Spooner and Wessells, 1972; Morriss-Kay, 1981; and other instances, including the sea urchin, as recounted in Wessells et al., 1971), but confirms the findings of Schoenwolf et al. (1988). These latter investigators reported that, although the circumapical microfilament bands in the chick neural plate are disrupted by CD, invagination is not prevented by the drug. Cytochalasin B has many deleterious side effects (Rampal et al., 1980) which confound the interpretation of the early experiments cited above. Since CD is now considered the drug of choice for disrupting microfilaments, the experiments that utilized cytochalasin B, which constitute the bulk of the empirical support for the purse-string hypothesis, should be repeated using CD. In light of our knowledge that many cell adhesion molecules are coupled to the cortical microfilament network in cells, it should be kept in mind that the results of the experiments cited above may be a reflection of disrupted cell adhesion.

While the insensitivity of invagination to CD that we observed forces us to question the universality of the purse-string hypothesis, the appearance of the aECM in 19- to 22-hour-old *S. purpuratus* embryos exposed to A23187 leads us to consider whether some invaginations may be guided or driven by well-disguised, regulated secretory events. We found that blocking secretion by preincubation with monensin reversibly blocked primary invagination and greatly reduced precocious invaginations stimulated by A23187 (fewer embryos invaginated and, in those that did, the invaginations were much smaller). We suggest that higher concentrations of monensin might prove more effective at blocking invagination. Roe and Lennarz (1990) were unable to stop secretion of the hatching enzyme in the sea urchin when treating embryos for 2 hours with 10 μM monensin. We used a lower concentration and shorter exposure time because it was easily reversible and the embryos appeared more viable. Higher monensin concentrations, especially when combined with A23187 stimulation, made it difficult to score vegetal plate morphology.

We have identified one matrix molecule secreted just before invagination as a CSPG on the basis of CS-56 immunoreactivity. In normal development, CSPG appears in the aECM at the vegetal plate as it flattens, and immunoreactivity persists in the lumen of the archenteron of the gastrula. The CSPG is present in the precocious archenteron stimulated by A23187, as well as in the aECM secreted by 19- to 22-hour-old *S. purpuratus*. Thus, the CSPG is stored between the time of its synthesis and deposition, and it is transported to the extracellular compartment in response to an increase in intracellular Ca^{2+}. It remains to be established whether the CSPG is the critical sulfated molecule at the vegetal plate. Since we have a marker only for chondroitin sulfate, we do not know whether other sulfated matrix molecules are involved in the secretory event. We are presently testing whether the CSPG is a critical participant in invagination by exposing live embryos to chondrotinases during gastrulation.

In the course of observing both sectioned and whole-mount, normal embryos from many cultures, we have never seen intracellular staining with CS-56. This result is surprising since we propose that the CSPG is stored between its synthesis and extracellular deposition. One possible explanation concerns the accessibility of the antibody to the epitope. The only published, high resolution (i.e., single cell) staining of which we are aware is the original report on CS-56 by Avnur and Geiger (1985). These authors show no intracellular staining in either fibroblasts or chondrocytes, and state that detergent permeabilization does not increase staining. This suggests that there are accessibilty problems in intracellular compartments, since chondrocytes are known to be synthesizing and constitutively transporting CSPG through the Golgi and post-Golgi compartments. Problems with antibody accessibility would be compounded in the regulated secretory pathway, where material stored in vesicles awaiting export is highly condensed (reviewed in Burgess and Kelly, 1987). This process could render an epitope inaccessible to antibody staining, particularly to an IgM (M_r=820×10^3) such as CS-56. We have seen faint, punctate staining in cells of the vegetal plate in embryos treated with monensin. It is known that one effect of monensin is dilation of the Golgi cisternae (Tartakoff, 1983) and that material in the trans Golgi does not subsequently condense (reviewed in Mollenhauer et al., 1990). Glycosaminoglycan side chains are added and sulfated in the Golgi, so the CS-56 epitope would first appear in the Golgi compartment. Thus, intracellular chondroitin sulfate may be unmasked and accessible to the CS-56 antibody only in monensin-treated embryos.

We have no data that explains the differences in timing seen in the response of the two species to A23187 (summarized in Table 3). One hypothesis is that *S. purpuratus* begins synthesizing and storing the CSPG (and any other secreted molecules) around 19 hours, while *L. pictus* does not begin to synthesize and store material until 22-23 hours. This could underlie the difference seen between the two species in the appearance of the plate in the hours before invagination. An understanding of these differences awaits

a molecular characterization of the secreted material, and experiments to determine when these molecules are synthesized in the two species.

The reduction in the size of the archenteron in the presence of monensin is somewhat reminiscent of the effect that microtubule disruption is reported to have on invagination, and our results may help to explain that observation. The distribution of microtubules in placodes destined to invaginate was originally described in the neural plate of newts by Burnside (1973). Microtubules are arrayed lengthwise along the apical-basal axis (an arrangement since observed in polarized epithelia, Bacallao et al., 1989), and it has been surmised that the increase in the height of the placode, an event observed before all invaginations, is due to an increase in the length of the apical-basal microtubules. Ettensohn (1984b) reported that disruption of microtubules with colchicine prior to gastrulation in the sea urchin resulted in small primary invaginations. It has since been shown that regulated secretion towards the apical surface of epithelial cells (Caco-2 cells, Eilers et al., 1989; MDCK cells, Parczyk et al., 1989; intestinal brush border cells, Achler et al., 1989) is dependent on microtubules, and disruption of the microtubules decreases apical secretion by 50% or more. The small archenterons observed by Ettensohn may be a reflection of a decrease in regulated, apical secretion in the embryo when the apical-basal microtubules have been depolymerized.

Invagination events are complex and may involve multiple contributing forces (Hilfer et al., 1977; Schoenwolf and Smith, 1990). It is not clear why 23-hour-old *S. purpuratus* respond to A23187 by invaginating, while 22-hour-old embryos secrete but do not invaginate unless provided with the external mechanical resistance of an agarose gel. This observation suggests that a second event occurring between 22 and 23 hours is required, and that an external resistance may be able to substitute for the unknown event. It has been suggested that vegetally directed tractoring of the epithelial cells lateral to the vegetal plate occurs at this stage in *S. purpuratus* (Burke et al., 1991). If the cells lateral to the vegetal plate were pulling the hyaline layer towards the equator of the embryo, this might stretch the hyaline layer taut, and somehow facilitate inward bending of the epithelial sheet. Such hypotheses await further experimentation.

The newly deposited apical extracllular matrix is required to buckle the vegetal plate

It has long been contended that the hyaline layer plays a critical role in early development (Gustafson and Wolpert, 1962), in particular at gastrulation (Citkowitz, 1971; Adelson and Humphreys, 1988), and our results support this conclusion. It is known that the HL is a complex, multi-laminated extracellular matrix (Hall and Vacquier, 1982; Cameron and Holland, 1985; Speigel et al., 1989) that undergoes changes throughout development. The HL is originally constructed by a series of post-fertilization exocytotic events. The most distal lamina of the HL is deposited in the the first wave of exocytosis, and subsequent waves deposit successively more proximal laminae (Alliegro and McClay, 1988; McClay et al., 1990), thereby establishing the concentric layered structure of this matrix. Although it has long been known that gastrulation in the sea urchin is sensitive to a number of agents or treatments that perturb ECM synthesis (e.g., tunicamycin, Schneider et al., 1978; xylosides, Kinoshita and Saiga, 1979; and sulfate-deprivation, Herbst, 1904), it has never been clear whether these were direct or indirect effects. The localization of CSPG to the aECM of the vegetal plate/archenteron in both normal and precocious gastrulae (Fig. 6A,C) argues that the effects are direct, particularly in the case of sulfate deprivation and exogenous xyloside exposure, treatments that would certainly affect synthesis of the CSPG. The morphology of sulfate-deprived embryos suggests that the sulfated molecules in the aECM of the vegetal plate may play two critical roles. (1) The disorganized bolus of matrix in sulfate-deprived embryos suggests that a sulfated molecule (or molecules) is required to organize the assembly of this matrix. Although heparan sulfate proteoglycans play this role in other tissues (Brauer et al., 1990), in this case a CSPG may fulfill this function. (2) The fact that the plate does not invaginate even though aECM is being deposited in sulfate-deprived *S. purpuratus* embryos may reveal a second role of the aECM. An under-sulfated CSPG should be secreted to the extracellular compartment, but it would not be expected to have the same physical properties as a fully sulfated CSPG. In particular, it would not hydrate to the same extent and, in addition, it might disrupt proper assembly of the matrix (reviewed in Hascall and Hascall, 1981). These may be critical factors in producing the necessary forces to deform the vegetal plate from its original convex shape to a concave one. In emphasizing hydration of the newly deposited matrix, we do not wish to imply that the CSPG simply acts as a space-generating molecule (reviewed in Bard, 1990), or as a 'propellant' for epithelial movement, as has been proposed for hyaluronic acid during organogenesis (Haddon and Lewis, 1991). We believe that, under certain conditions, the deposition of a hygroscopic matrix may generate forces that could bend an epithelial sheet (described below).

Speculation: a model for epithelial invagination based on extracellular matrix deposition and hydration

At the onset of gastrulation, the sea urchin embryo is arranged as an epithelial sphere with an entrapped mesenchymal cell population. There are two extracellular matrices, an internal blastocoelar matrix, limited by a basal lamina, and the external hyaline layer lying along the apical surface of the epithelial cells. The hyaline layer (HL) consists of six or more layers by ultrastructural analysis, and the innermost layer is designated as the apical lamina (Hall and Vacquier, 1982). Microvilli from the apical plasma membrane of the cells extend across the HL and embed their tips in an outer layer of this complex extracellular matrix (Spiegel et al., 1989), as diagrammed in Fig. 9B.

We envision that the hydration of newly deposited, hygroscopic matrix in an internal lamina of the HL could cause bending of an epithelial sheet in a mannner somewhat analagous to the bending of a bimetallic strip in a thermostat. In a bimetallic strip (Fig. 9A), strips of two metals (M_1 and M_2) with different coefficients of thermal expansion are bonded together (Fig. 9A). As the tempera-

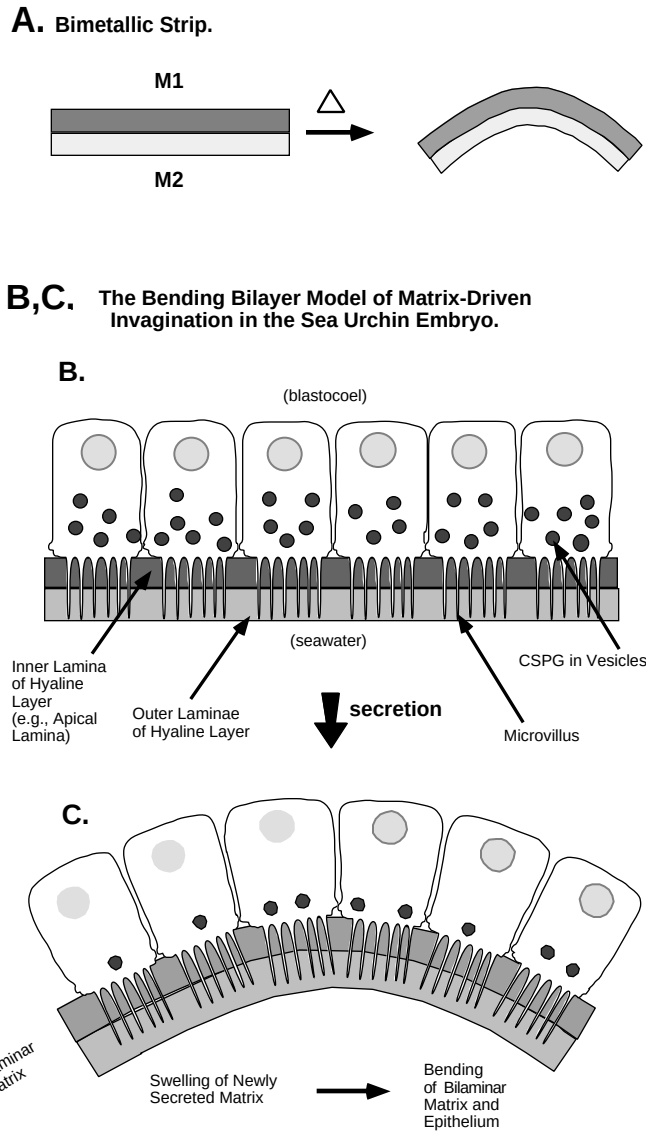

Fig. 9. A model for epithelial invagination driven by the secretion of a hygroscopic molecule into a complex, multilayered ECM. (A) The physical principle of the bimetallic strip. (B,C) The bending bilayer model of matrix-driven epithelial invagination during sea uchin gastrulation. See text for explanation. This diagram is intended to be schematic; no features (e.g., cell heights or volumes) are drawn to scale. The outer laminae have been combined into a single layer for illustrative purposes.

ture is increased, M_1 (with the higher coefficient of expansion) expands more than M_2. The result is bending of the bimetallic strip with M_1 on the convex side and M_2 on the concave side. In the embryo, the multilaminate HL (including the inner apical lamina and the outer layers) is analogous to the bimetallic strip, with the microvilli integrating the various layers (Fig. 9B). As the condensed matrix (in this situation including CSPG) is secreted to the extracellular compartment, it attracts water and swells an internal lamina of the HL, analagous to M_1 in the bimetallic strip. The outer laminae (analagous to M_2), which do not contain newly deposited, hygroscopic matrix, do not swell, and hence the hydration of an inner lamina bends the HL and attached epithelium inwards (Fig. 9C).

In the case of the sea urchin, the model allows us to make a number of predictions about the localization of the CSPG with respect to other identified components of the HL (e.g., hyalin, fibropellins), which we are currently testing. If we are correct about the physical function of the CSPG within the aECM, its continued presence along the length of the archenteron throughout gastrulation may hold the archenteron lumen open against the internal pressure of the blastocoel. Thus, this model could be extended to explain some instances of lumen formation by epithelial cells.

Treatments that disrupt ECM synthesis (e.g., tunicamycin, sulfate-deprivation, and β-D-xyloside) affect not only sea urchin gastrulation, but also neural tube, optic cup, and otic pit morphogenesis (Morriss-Kay and Crutch, 1982; Yang and Hilfer, 1981; Rausch and Hilfer, 1988). Indeed, the secretion of aECM may accompany many invaginations during organogenesis: substantial amounts of apical matrix are found along the luminal surface of the optic cup (Hilfer and Yang, 1980), lens vesicle (Van Rybroek and Olson, 1981), neural tube (Morriss-Kay and Crutch, 1982) and otic vesicle (Sinning and Olson, 1988). Such invaginations are generally preceded by placode formation and explanations for placode formation based on cytoskeletal rearrangements have been offered (reviewed in Ettensohn, 1985). Our experiments suggest that the vegetal plate may be the morphological manifestation of a regulated secretory event stored within an epithelium and other embryonic placodes should be reexamined with this in mind.

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