Bottle Cells Are Required for the Initiation of Primary Invagination in the Sea Urchin Embryo

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Invagination of epithelial tissue occurs during gastrulation, neurulation, and organogenesis in many organisms. However, the underlying morphogenetic mechanisms of invagination are not understood. To elucidate these mechanisms, we have analyzed the initial invagination of the vegetal plate in the sea urchin embryo, a process termed primary invagination. At the onset of invagination, a ring of cells with highly constricted apices (bottle cells) encircles a group of two to eight round, central cells. To investigate the morphogenetic role of the bottle cells in the process of primary invagination, we have undertaken a series of laser ablation studies in which different proportions of various cell types were ablated and the effects were recorded using 4-D microscopy. Elimination of a 90°–180° arc of bottle cells markedly retards invagination, but only within the ablated region. Ablation of other cell types does not result in a statistically significant effect on primary invagination. These studies indicate that the number and arrangement of the bottle cells are critical factors for proper initiation of invagination. In addition, we have used the perturbing anti-hyalin antibody mAb183 to show that cell attachment to the hyaline layer is necessary for bottle cell formation and the initiation of primary invagination.© 1998 Academic Press

Key Words: epithelial morphogenesis; invagination; bottle cell; apical constriction.

INTRODUCTION

During embryonic development dramatic, highly organized morphogenetic movements completely reorganize the embryo. Invagination is one kind of tissue movement used during these extensive embryonic reorganizations. It is an efficient and deceptively simple-looking mechanism for folding tissue and internalizing it from the surface. Epithelial invaginations play a critical role in the development of both vertebrates and invertebrates during gastrulation, neurulation, and organogenesis. The process has been studied extensively during Drosophila ventral furrow formation (Costa et al., 1994), sea urchin primary invagination (Lane et al., 1993; Davidson et al., 1995; Nakajima and Burke, 1996), Xenopus dorsal blastopore lip development (Hardin and Keller, 1988), chick neurulation (Schoenwolf and Smith, 1990), and optic and otic cup development in the chick (Brady and Hilfer, 1982; Hilfer et al., 1989).

Numerous theories have been proposed to explain the mechanics of invaginations, including differential cell adhesion, differential cell growth and division, cell shape changes, and secretion of extracellular matrix (Bard, 1990; Fristrom, 1988; Ettensohn, 1985). However, it has been difficult to definitively prove whether one or another of these mechanisms actually operates during an invagination.

During invagination some of the epithelial cells involved in the bending process undergo a characteristic cell shape change. Their apical ends decrease in surface area and their basal surfaces expand, thereby producing wedge-shaped cells. The function of such wedge-shaped cells, or bottle cells, in producing invaginations has been heavily debated.

The sea urchin embryo has been used as a model for studying the mechanisms of epithelial invagination for over a century. The appeal of the system lies in its simple organization and its transparency, which make it ideal for detailed in vivo microscopy studies. Prior to gastrulation the sea urchin embryo is an epithelial monolayer. Following the ingress of the skeletogenic mesenchyme into the blastocoel, the thickened vegetal plate buckles into the blastocoel, forming a stout cylindrical archenteron in a process known as primary invagination. It is known that the forces responsible for primary invagination are localized...
within the vegetal plate, since vegetal plates isolated prior to the onset of primary invagination invaginate normally (Ettensohn, 1984a,b). However, the nature of these localized forces remains elusive.

Davidson et al. (1995) used continuum mechanical modeling to evaluate five models of primary invagination, which included (1) apical constriction of vegetal plate cells, (2) tractoring of cells lateral to the vegetal plate, (3) contraction of a cytoskeletal ring running through a circle of cells at the periphery of the vegetal plate, (4) apicalobasal contraction of the cytoskeletal cortex of the vegetal plate cells, and (5) the swelling of extracellular matrix secreted apically by the vegetal plate cells. Each of the five models could generate an invagination within specific numerical ranges of cellular and extracellular matrix stiffness and elasticity and defined parameters to consider when examining potential mechanisms operating during primary invagination.

In addition to such mechanical modeling, several experimental analyses of primary invagination have been performed. Ettensohn (1984a) found that cells in the vegetal plate of Lytechinus pictus embryos move 10–15 μm toward the vegetal pole during primary invagination. Based on these findings, he proposed that convergence of cells toward the vegetal pole creates lateral pressure which buckles the vegetal plate. Burke et al. (1991) provided similar evidence for convergence toward the vegetal plate in Strongylocentrotus purpuratus. Lane et al. (1993), studying L. pictus and S. purpuratus embryos, suggested an alternative mechanism of primary invagination involving swelling of chondroitin sulfate proteoglycans, which are secreted at the vegetal plate concomitant with invagination.

Although a potential role for bottle cells in primary invagination was proposed long ago (Rhumbler, 1902; reviewed in Gustafson and Wolpert, 1963, 1967), the theory had largely been discounted for lack of solid support. It appeared that insufficient numbers of apically constricted cells existed at the vegetal plate to be involved in primary invagination (Ettensohn, 1984a,b). Recently, Nakajima and Burke (1996) provided the first evidence for the presence of bottle cells. Coincident with the onset of primary invagination, they found a ring of bottle cells at the vegetal plate of S. purpuratus embryos. In addition, embryos treated with the A11 antibody, which is directed against the apical lamina proteins known as fibropellins, fail to invaginate and fail to form bottle cells (Nakajima and Burke, 1996). As Nakajima and Burke (1996) stated, these experiments do not prove that the bottle cells initiate primary invagination; however, they argue for a reexamination of the functional role of bottle cells in primary invagination.

In L. pictus embryos, we have found that a distinct pattern of cell shapes arises at the vegetal plate at the onset of primary invagination, including the formation of a ring of apically constricted cells. To investigate the morphogenetic role of bottle cells and the other cell populations during the initiation of primary invagination, we have undertaken a series of laser ablation studies. In addition, we have examined the role of the hyaline layer in the initiation of primary invagination. Our experiments provide the first direct in vivo analysis of apical constriction during epithelial invagination at the level of single cells.

**MATERIALS AND METHODS**

**Embryo Culture**

Adult L. pictus sea urchins were obtained from Marinus, Inc. (Long Beach, CA) and intracoelomic injection of 0.5 M KCl was used to induce gamete shedding. The eggs were filtered through cheesecloth to remove the jelly coats, fertilized with dilute sperm, and raised in stirring cultures in artificial seawater (ASW). Their developmental rate was controlled by placing the cultures in incubators ranging in temperature from 14° to 19°C.

**Nomarski Four-Dimensional Videomicroscopy**

Four dots of silicon vacuum grease (Dow Corning) were placed at the corners of coverslips precoated with 0.1% poly-L-lysine (Sigma). Approximately 50 ml of ASW containing late mesenchyme blastulae was mouth-pipetted onto the coverslip. While observing the embryos under a dissecting microscope (Wild, Heerbrug, Switzerland), they were oriented so that their vegetal plates were parallel to the coverslip using a glass microneedle made by hand-pulling a 5-ml micropipet (Fisher) in a Bunsen burner flame. A ring of mineral oil (Sigma) was pipetted around the ASW and a slide was placed over the coverslip and compressed slightly. More mineral oil was pipetted under the coverslip to minimize evaporation. Primary invagination was filmed using four-dimensional (4-D) microscopy as described in Williams-Masson et al. (1997).

**Laser Ablations**

Embryos were mounted as described above. Laser ablations of individual cells were performed using a VSL-337ND nitrogen laser (Laser Science, Inc.) which was used to pump a tunable dye laser (Bull’s Eye, Fryer Co.) mounted on the stand of a Nikon Optiphot-II via a fiber optic conduit. Ablations were performed on the vegetal plates of manually oriented embryos which had not invaginated more than 3 μm. Immediately following the ablations, the embryos and their subsequent development were filmed by 4-D videomicroscopy. The rate at which invagination occurred following bottle cell ablation varied widely from embryo to embryo, ranging from a 5- to 25-μm increase in depth over 30 min. This variation in rate was true for normal embryos and embryos which had been ablated. Embryos were filmed for 30 min to 2 h. The health of the embryos was monitored using two or more of the following criteria: (1) ciliary beating, (2) the integrity of the epithelium, (3) health of surrounding embryos, and (4) migration of primary mesenchyme cells.

In the case of the bottle cell ablations, the dead matter produced after ablating the cells was deposited in the blastocoel. However, in the elongated cell ablations, the debris was extruded to the exterior of the embryo where it could accumulate in a localized position between the epithelium and the extracellular matrix (ECM). If this mass persisted, it affected the shape of the invagination; such cases were excluded from the results. In some of the central cell ablations the dead matter moved to the interior of the embryo; in other cases it was extruded to the exterior of the embryo, but it did not interfere with the invagination process because of its central location.
Three-Dimensional Graphs

Using a modified version of NIH Image in conjunction with an image marking macro written in our laboratory, the x, y, and z coordinates of vegetal plate cell apices were marked and recorded. These coordinates were then imported into Transform 2D, v. 3.3 (Fortner, Inc., Sterling, VA) which was used to generate the three-dimensional graphs of the invaginations. NIH Image is a public-domain image processing program available via anonymous ftp from codon.nih.gov/pub/nih-image/.

Quantitative Evaluation of Ablation Results

To eliminate measurement biases, the following method of determining the depths of invagination of ablated embryos was used: (1) The center of the blastopore was determined; (2) the deepest part of the archenteron was located and the radius from the center of the blastopore to the deepest point was measured; (3) using the radial length from (2), the depths of three additional points at 90° intervals from the deepest point were determined; (4) the ablated region was superimposed on the 4-point depth measurements; (5) the interpolated depth at the midpoint of the ablated arc was determined and defined as the depth of the ablated region. The interpolated depth 180° opposite from the midpoint of the ablated arc was determined and defined as the depth of the unablated region. Two sets of these measurements were performed for each embryo: (a) at the time point just prior to the ablation and (b) at the final time point of the 4-D recording; (6) the initial depths were subtracted from the final depths, yielding the increase in the depth of invagination for the ablated and unablated side of each embryo. For central cell ablations and normal embryos the same 4-point depth sample was generated and then the measurements were averaged to obtain the depth of invagination. Statistical analyses were performed using StatView II (Abacus Concepts, Inc., Berkeley, CA).

Antibody Treatment

To examine the role of the hyaline layer in primary invagination, living embryos at the hatched blastula stage were transferred to ASW containing 45 μg/ml of the monoclonal antibody 183 (mAb183) purified from ascites fluid (kindly provided by T. Humphreys; Adelson and Humphreys, 1987). mAb183 binds the protein hyalin and blocks primary invagination at this concentration (Adelson and Humphreys, 1987). When control embryos incubated at an equivalent concentration with the nonrelevant Caenorhabditis elegans MH27 antibody (kindly provided by Dr. R. Waterston, Washington University, St. Louis, MO) initiated primary invagination, mAb183 treated embryos were mounted as for the 4-D films and observed using Nomarski microscopy.

Immunostaining

To visualize the morphology of the hyaline layer and apical lamina before and during primary invagination, living embryos at the early mesenchyme blastula stage were transferred into ASW containing 0.4 μg/ml mAb183, or 0.1 μg/ml of the monoclonal antibody AL1 affinity-purified from ascites fluid, an anti-fibropellin antibody which labels the apical lamina (kindly provided by R. Burke; Burke et al., 1991). At these low concentrations neither of these antibodies perturbs development (E.L.K, unpublished observations). When the embryos reached the desired developmental

FIG. 1. Apical surface views of living Lytechinus pictus vegetal plates during the initiation of primary invagination. (A) Nomarski image of the vegetal plate at the onset of primary invagination. (B) Same image as A with the three cell populations outlined: central cells (blue), bottle cells (red), and elongated cells (yellow). (C) Two-photon image of an FM4-64-labeled embryo showing the relative sizes of the three cell populations at the vegetal plate. Bars, 10 μm.
stage, they were washed twice in ASW and then transferred to a 1:25 dilution of lissamine rhodamine goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 5 min, washed twice in ASW, and immediately mounted as for time-lapse videomicroscopy. In the experiments where the effects of the laser on the ECM were assessed, the embryos were treated with mAb183 and AL1 as described above but a Cy5-conjugated goat anti-mouse IgGs secondary antibody (Jackson ImmunoResearch) was used because the excitation wavelength of Cy5 (550–680 nm) was outside of the wavelength at which the laser operates (420–475 nm). The embryos were mounted as for the 4-D microscopy and observed with both Nomarski and epifluorescence microscopy on a Zeiss Axioskop microscope using either a 40× Plan apo dry objective (NA = 1.3) or a 63× Plan apo oil immersion objective (NA = 1.4). Images were acquired using a MicroMax cooled CCD camera (Princeton Instruments, Princeton, NJ) and Metamorph software (Universal Imaging, Westchester, PA).

Two Photon Microscopy

Late mesenchyme blastulae were incubated in ASW + 1 μg/ml of the lipophilic membrane dye FM 4-64 (Molecular Probes Inc., Eugene, OR) for 1 min. After two washes in ASW, the embryos were mounted as described above and imaged using two-photon microscopy. The two-photon system consists of a Nikon Quantum inverted microscope equipped with an Nd:YLF laser and Bio-Rad MRC-600 configured as described by Wokosin et al. (1996). No pinhole was inserted in the optical path. The signal generated by two photon excitation was descanned into the MRC-600 scanhead that had been manually positioned so that their vegetal plate were parallel to the surface of the coverslip. By orienting the embryos in this way the entire vegetal plate and the apical surfaces of all of the vegetal plate cells could be observed (Fig. 1A). Using 4-D Nomarski microscopy to film the embryos, we were able to observe the formation and initial inpocketing of the vegetal plate which mark the initiation of primary invagination. During this initial stage the vegetal plate invaginates approximately 5–10 μm.

The onset of primary invagination in L. pictus embryos is preceded by the dispersal of primary mesenchyme cells (PMCs). When embryos at this stage are oriented as described above, three distinct populations of cells are consistently observed. A ring of cells with highly constricted apices ("bottle cells"; Fig. 1B, red outlines) encircles a group of two to eight larger, roughly isodiametric cells (Fig. 1B, blue outlines) which we refer to as "central cells." Surrounding the ring of bottle cells are one to two tiers of cells elongated along their radial axis (Fig. 1B, yellow outlines) which we will refer to as “elongated cells.” Outside of the elongated cells are cells which are roughly hexagonal in shape which we refer to as “surrounding cells.” The three cell populations are readily apparent in both Nomarski images as well as two photon images of embryos stained with the fluorescent lipophilic membrane dye FM 4-64 (Fig. 1C). The shape of the cells in each of the three cell populations is quantitatively different (Table 1). The apical surface area of both the central cells and elongated cells is much greater than that of the bottle cells. Additionally, the length width ratio of cells elongated at the onset of primary invagination is much greater than that of the central cells and bottle cells.

Prior to the onset of primary invagination the PMCs ingress. As they do so a depression forms at the apex of the vegetal plate (Katow and Solursh, 1980) which frequently disappears prior to the initiation of primary invagination (E.L.K., unpublished observation). Therefore, at the onset of primary invagination, the surface of the vegetal plate is essentially flat. At the time the pattern of cell populations begins to appear, a new indentation in the vegetal plate becomes apparent. Sagittal views of embryos reveal a marked bend in the vegetal plate at sites of bottle cell formation and the shallower position of the central cells (Figs. 2A and 2B). In surface views, the indentation appears as a ring. This indented ring corresponds to the position of the ring of bottle cells which are the first cells to invaginate. In the middle of the indented ring lie the central cells, which are often shallower than the bottle cells throughout the initiation of primary invagination. Ultimately, the central cells invaginate as deeply as the bottle cells, but not until the invagination is deeper than the initial 5-10 μm invagination which was the focus of this study. The first tier of elongated cells forms the early blastopore lip and the second tier lies peripheral to the lip.

Three-dimensional representations of developing archenterons were generated using 4-D movies and a computer marking program. The x-y coordinates and depth of each cell apex in the vegetal plate were marked and recorded.
Using these measurements, topographical maps of the developing archenteron were generated to visualize the change in depth and shape of the entire invagination over time (Figs. 3A–3C). In Figs. 3A and 3A', the invagination begins as a shallow indented ring which corresponds to the ring of bottle cells (light purple) surrounding the central cells (purple). The invagination proceeds symmetrically (Figs. 3B and 3B') reaching a final depth of approximately 25 μm at the end of primary invagination (Figs. 3C and 3C'). The rate of invagination varied widely from embryo to embryo. Additionally, the rate of invagination of an individual embryo can change markedly as primary invagination proceeds (Fig. 3).

The high-resolution of the 4-D recordings allowed dynamic changes in the apical shapes of the cells at the vegetal plate to be followed and characterized as they underwent primary invagination. The apical surface area of bottle cells gradually decreases with increasing depth of invagination (red cells, Figs. 4A–4C). Concurrent with bottle cell constriction, the elongated cells become more highly elongated as the invagination deepens; a gradual increase in the length width ratios of these cells confirms this (yellow cells, Figs. 4A–4C). Measurements of cell movements during primary invagination show that the distance of the bottle cells and elongated cells from the center of the vegetal plate decreases by 3.25 ± 0.52 μm (mean ± SEM) as the invagination deepens. This finding is consistent with previous measurements of the movement of beads attached to the embryo near the blastopore lip during primary invagination (Ettenson, 1984a).

### TABLE 1

<table>
<thead>
<tr>
<th>Shapes of the Three Cell Populations at the Vegetal Plate</th>
<th>Mean apical surface area (μm²) ± SEM</th>
<th>Length width ratio ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bottle cells</td>
<td>26</td>
<td>6.5 ± 0.7</td>
</tr>
<tr>
<td>Elongated cells</td>
<td>35</td>
<td>35.6 ± 1.6</td>
</tr>
<tr>
<td>Central cells</td>
<td>18</td>
<td>38.9 ± 2.7</td>
</tr>
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Alterations in Morphology of the Hyaline Layer and Apical Lamina during Primary Invagination

In addition to the cell shape changes which occur during primary invagination within the vegetal plate, the apical extracellular matrices change during this process as well. The sea urchin embryo is wrapped in a two-layered extracellular matrix (ECM). The outermost layer, the hyaline layer, is a thick (~2 μm), fibrous matrix consisting largely of the molecule hyalin, which is initially secreted at fertilization (Dan, 1960; McClay and Fink, 1982; Wessel et al., 1998). The internal layer, the apical lamina, lies just inside the hyaline layer and directly apposes the apical surface of the cells. Three related proteins encoded by two genes, termed fibropellin Ia, Ib, and III, make up this layer (Bisgrove and Raff, 1993; Bisgrove et al., 1991).

Outside of the vegetal plate the hyaline layer and apical lamina consist of fibrous meshworks which lie closely opposed to the ectoderm cells (Adelson and Humphreys, 1988; Bisgrove et al., 1991; E.L.K., unpublished observations). Dan and Inaba (1968) and Katow and Solursh (1980) noted that the hyaline layer develops blisters and wrinkles over the vegetal plate coincident with the ingestion of the PMCs, a process which precedes the initiation of primary invagination by several hours. At low concentrations (0.4 μg/ml) the anti-hyalin monoclonal antibody, mAb183, can be used to label the hyaline layer without perturbing it. We found that the hyaline layer remains wrinkled and blistered over the entire vegetal plate from the time of PMC ingestion through the initiation of primary invagination (Figs. 5A and 5B). Once invagination has begun, the wrinkles become oriented radially along the same axis as the elongated cells (Fig. 5C). It appears that the hyaline layer...
remains attached to the vegetal plate throughout primary invagination (Citkowitz, 1971; McClay and Fink, 1982). The morphology of the apical lamina was examined using the anti-fibropellin monoclonal antibody AL1 at a nonperturbing concentration (0.1 μg/ml). We found that, like the hyaline layer, the apical lamina is wrinkled from the time of PMC ingestion through the initiation of primary invagination (Figs. 5D and 5E). The apical lamina also appears to invaginate along with the vegetal plate; however, it is not thrown into highly oriented folds like the hyaline layer after the onset of invagination (Fig. 5F). Thus, the morphology of the hyaline layer and apical lamina over the vegetal plate region is different from the rest of the embryo. However, this change takes place prior to the onset of primary invagination. Following the initiation of invagination, additional alterations occur as both matrices invaginate with the vegetal plate.

**Laser Ablation of Subpopulations of Cells within the Vegetal Plate**

Given the conspicuous appearance of bottle cells as primary invagination begins, a reasonable hypothesis regarding the mechanism of primary invagination is that the gradual and continuous contraction of the apical ends of the bottle cells buckles the vegetal plate and deepens the invagination. If such active contraction were to occur, the constriction would be expected to exert tension on the surrounding cells, stretching them to generate the elongated tiers of cells encircling the bottle cells. The central cells would also experience tension from the constriction of the bottle cells. However, the tension would be exerted equally on all sides by the bottle cells, and therefore the central cells would retain an isodiamic shape.

To investigate the morphogenetic function of the three
cell populations in primary invagination and to elucidate the mechanical forces operating in the vegetal plate, we performed a series of laser ablations: (1) ablation of a 90°–120° arc of bottle cells, (2) a 90°–120° arc of surrounding cells, and (3) all central cells (Table 2). The effects of the missing cells on primary invagination were assessed using 4-D Nomarski microscopy combined with topographical mapping of the invagination.

To determine the effect of the laser on the ECM, fluorescent images of the hyaline layer and apical lamina were compared before and after an ablation in living embryos. These revealed that the laser produced no observable structural lesion in the hyaline layer or the apical lamina under the conditions used throughout our ablation experiments (data not shown). Thus we believe the laser microbeam only caused damage to irradiated cells within the vegetal plate, and could be used to investigate their role during primary invagination.

**Ablation of a 90°–120° Arc of Bottle Cells**

Ablation of a 90°–120° arc of bottle cells inhibits the normal invagination of that region of the vegetal plate. There is a marked, statistically significant difference between the mean invagination depth of the ablated region (3.5 ± 2.2 μm) and the unablated region (9.8 ± 2.2 μm; Table 2). In these embryos, 75–100% of the bottle cells were successfully ablated within the 90°–120° arc. Following the ablation, the dead cells were extruded into the blastocoel, and the hole left by the ablated cells healed, typically within 5 min, bringing elongated cells and central cells in contact with one another (Figs. 6A and 6A'). During and after the healing process, the embryo had to be monitored for the reappearance of bottle cells within the ablated region. If bottle cells only partially disabled by the ablation reinitiated constriction, and/or bottle cells adjacent to the ablation site reconstituted the ring, then these bottle cells were subsequently ablated. In the ablated embryos, the least invaginated region consistently corresponded to the region where the bottle cells were ablated. In each case the unablated side served as an internal control for the general health of the embryo during the experiment.

In three additional cases 180°–360° arcs of bottle cells were ablated. In the first case, a 180° arc of the bottle cells was ablated, and the remaining nine bottle cells coalesced into a tight group forming a localized pit in the epithelium on the unablated side which deepened (Fig. 7A). In the second case an attempt to ablate all of the bottle cells was made. However, four remaining bottle cells were later found and these had coalesced into a localized pit as well (Fig. 7B). In both of these cases the rest of the vegetal plate stopped invaginating. In the final case all of the bottle cells were ablated and further invagination of the vegetal plate ceased (data not shown). However, we did not attempt further 360° bottle cell ablations because no unablated portion of the ring remained to serve as an internal control. Without this control it was difficult to determine if the lack of invagination was due to the loss of all the bottle cells or an unhealthy embryo.

**Ablation of a 90°–120° Arc of Surrounding Cells**

Embryos in which a 90°–120° arc of surrounding cells was ablated (Figs. 6B and 6B') have a mean increase in invagination depth on the ablated side of 6.1 ± 2.1 μm versus 7.8 ± 2.4 μm on the unablated side (Table 2). Although it is not statistically significant, there is a moderate difference between these two means. We also attempted elongated cell ablations. However, in the majority of cases the results were uninterpretable due to the extrusion of the dead cells.
cellular material to the exterior of the embryo which deformed the shape of the invagination. In addition, the wound created by these ablations was large compared to the other ablations, and it was difficult to evaluate the health of the embryo following the ablation.

Ablation of All Central Cells

Ablation of all central cells does not affect the initiation of primary invagination (Figs. 6C and 6C'). Comparison of the increase in mean invagination depth between normal, unablated embryos (8.7 ± 1.3 m) and embryos in which all of the central cells have been ablated (7.8 ± 2.4 μm) is not significantly different (Table 2).

Following ablation of the central cells, it appeared that ≥75% of the bottle cells in the ring partially relaxed and moved toward the wound to fill it. The bottle cells that did not relax coalesced in the center of the vegetal plate. Most of the original bottle cells remained relaxed, with <10% of them reconstricting; as few as three centrally located bottle cells can remain and invagination will occur. These results demonstrate that the central cells are not necessary for the initiation of primary invagination and that their ablation, which produces a relatively large wound, does not generally disable the embryo and prevent it from invaginating.

Long-Term Recovery of Ablated Embryos

If following the ablation, the embryos remain in their microscope mounts and are stored in a humid chamber, they can survive for as long as 18 h and be monitored for further development. Ultimately, embryos in which a 120° arc of bottle cells have been ablated are able to complete primary and secondary invagination (Fig. 8). These findings provide further evidence that the ablations do not generally disable the embryos. Unfortunately, it is impossible to monitor the state of the vegetal plate throughout the recovery process because invariably the embryos turn and detach from the coverslip after approximately 1.5 h.

Role of the Hyaline Layer in Bottle Cell Formation

When embryos are treated prior to the onset of gastrulation with perturbational concentrations of either mAb183, which disrupts the hyaline layer (Adelson and Humphreys, 1988) or AL1, which disrupts the apical lamina (Burke et al.,...
1991; Nakajima and Burke, 1996) primary invagination is inhibited. In embryos incubated with high concentrations (45 μg/ml) of mAb183, the hyaline layer detaches from the epithelium and primary invagination does not occur. This indicates that cell adhesion to the hyaline layer is required for successful primary invagination (Adelson and Humphreys, 1988). We examined the effects of mAb183 treatment on bottle cell formation and primary invagination and found different results depending on the time of antibody application. If mAb183 was applied at the hatched blastula stage, the embryos failed to form bottle cells and did not invaginate (Fig. 9A). However, if mAb183 was added at the mesenchyme blastula stage, all of the embryos were able to form bottle cells but to varying degrees. About 25% of the embryos developed isolated bottle cells but no invagination occurred. The remaining 75% of the embryos developed a full ring of bottle cells with two possible phenotypes. In half of these embryos, regions of the ring of bottle cells initiated invagination, generating localized pits and troughs in the vegetal plate occasionally as deep as 5 μm (Fig. 9B). The other embryos with a full ring of bottle cells initiated primary invagination normally, invaginating approximately 3 μm (Fig. 9C). However, in both cases the embryos did not invaginate further. Therefore, cellular attachment to the hyaline layer prior to invagination is critical for the initiation of bottle cell formation. In addition, after bottle cell formation, attachment to the hyaline layer is necessary for the successful progression of primary invagination.

**DISCUSSION**

**Primary Invagination as a Model for Epithelial Invagination**

Apically constricted cells are a characteristic feature of epithelial invaginations; however, it has been difficult to assess their role in the process experimentally. Much of the mechanistic study of apical constriction has focused on generating a precocious invagination using chemical treatments which stimulate apical contraction (Owaribe et al., 1981; Burgess and Prum, 1982; Hilfer et al., 1977, 1981). These studies demonstrate that apical constriction can produce an invagination and provide valuable information regarding potential molecular players in the process. However, they do not address the role apical constriction plays in naturally occurring invaginations.

In Drosophila embryos, apically constricted groups of cells develop at the positions of the posterior midgut invagination and ventral furrow. The zygotic lethal mutation folded gastrulation (Costa et al., 1994; Morize et al., 1998) and the maternal-effect mutation concertina (Costa et al., 1994; Parks and Wieschaus, 1991) result in aberrant timing and spatial patterns of apical constriction and abnormal invaginations. Folded gastrulation and Concertina are believed to be signaling molecules involved in coordinating apical constriction. Despite these important findings, the actual role of apical constriction in the formation of these two invaginations has not been tested but assumed. Corre-
lation of apical constriction with invagination does not directly demonstrate a cause-effect relationship between the two.

Careful analysis of the bottle cells located at the dorsal blastopore lip of *Xenopus* indicates they undergo active apical constriction during formation of the blastopore lip (Hardin and Keller, 1988). However, it was not possible to examine the function of these bottle cells at the individual cell level within the intact embryo.

In the present study, we were able to directly assess the role of apically constricted cells in the formation of an invagination within the context of the living embryo. Four-dimensional Nomarski microscopy provided live documentation of primary invagination in both normal and ablated embryos. Additionally, the high resolution of these time-lapse recordings allowed us to characterize the process at the level of single cells. It has not been previously possible to perform this direct and detailed analysis of the function of apically constricted cells in an epithelial invagination.

**The Formation of Bottle Cells Initiates Primary Invagination**

The formation of bottle cells at the vegetal plate, which appears to occur via active apical constriction, is critical for the initiation of primary invagination. Establishing this direct cause-effect relationship between bottle cell formation and the initiation of primary invagination required demonstrating the following: (1) bottle cells must be present at the vegetal plate at the onset of primary invagination, (2) the forces that generate the bottle cell shape must come from the bottle cells themselves and (3) the invagination must depend on the presence of the bottle cells.
(1) Bottle cells are present at the vegetal plate. We consistently found a group of apically constricted cells arranged in a ring at the onset of primary invagination. These cells are always the first region of the vegetal plate to invaginate and do so prior to the center of the plate. Nakajima and Burke (1996) had found similar results in fixed, phalloidin-stained _S. purpuratus_ embryos: a ring of cells with constricted apices and more intense phalloidin staining at the vegetal plate. Thus apically constricted cells are present at the right time and place to initiate primary invagination. However, such correlative data say little about the functional activity of the bottle cells in the process.

Katow and Solursh (1980) showed in _L. pictus_ that prior to primary invagination during PMC ingresson a ring of apically constricted cells forms around a group of eight

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**FIG. 7.** Nomarski images of embryos with ≥180° arc of bottle cells ablated. (A) 180° arc of bottle cells was ablated (between arrowheads) when the invagination was already ~5 μm. The remaining bottle cells coalesced (arrow) on one side of the vegetal plate, forming a pit which continued to deepen. (B) 240° arc of bottle cells was ablated at the onset of invagination (between arrowheads), and the remaining four bottle cells coalesced and formed a shallow pit (arrow). Bars, 10 μm.

**FIG. 8.** Recovery of an embryo from 180° bottle cell ablation. (A) Nomarski image of the vegetal plate following the ablation (between arrowheads). The unablated side has invaginated 3.5 μm (arrow). (B) 5 h after the ablation, the embryo has completed primary invagination and initiated secondary invagination, i.e., the archenteron has begun elongating across the blastocoel. Bars, 10 μm.
central cells and a corresponding depression forms in the vegetal plate. We have noticed this depression during PMC ingression as well. Presumably this depression largely disappears after PMC ingression because the vegetal plate is frequently flat or only slightly invaginated (≈3 μm) when we begin our experiments. In addition, we believe the ring of bottle cells we observe at the initiation of primary invagination is distinct from the ring observed during PMC ingression. All the PMCs have ingressed and dispersed from the vegetal plate at the time of our ablations, and we have observed the ring of bottle cells involved in primary invagination after PMC ingression is complete. Neither the ingression of PMCs nor their presence in the blastocoel is necessary for primary invagination to occur. Hörstadius (1928) and Ransick and Davidson (1995) showed that primary invagination is delayed but occurs normally in embryos in which the micromeres, the PMC precursors, have been removed. In addition, Ettensohn and McClay (1988) removed the PMCs from the blastocoel following their ingression and primary invagination occurred normally.

We believe the eight central cells at the center of the ring of bottle cells during primary invagination are the same eight central cells at the center of the ring of ingressing PMCs seen by Katow and Solursh (1980). A detailed fate map of the vegetal plate is not available for L. pictus. However, based on those produced in L. variegatus (Ruffins and Ettensohn, 1996) it is thought that these eight central cells are small micromere derivatives which become part of the coelomic sacs which ultimately give rise to much of the adult sea urchin (Endo, 1966; Pehson and Cohen, 1986; Tanaka and Dan, 1990).

(2) Bottle cells appear to form actively. Several findings indicate that the force for apical constriction of the bottle cells is generated by the bottle cells themselves, i.e., it is an active process. When an ≈180° arc of bottle cells was ablated, the remaining bottle cells coalesced to form a localized pit which deepened, demonstrating the autonomous ability of the bottle cells to constrict. In addition, the shape of the elongated cells suggests they are being actively pulled on as a result of the constriction of the bottle cells. Finally, from a mechanistic stance, there must be a progressive decrease in the apical surface area of the bottle cells in order for them to initially bend the vegetal plate and then gradually deepen the invagination. Measurements of bottle cell surface areas during the invagination process demonstrate that they do decrease with time and concurrently the elongated cells increase in length.

(3) Bottle cells are required for primary invagination. The results of the bottle cell ablations establish the direct relationship between the presence of bottle cells and the local progression of primary invagination. Ablation of a 90°–120° arc of bottle cells impairs the ability of that side of the vegetal plate to invaginate. This finding, combined with the evidence for the autonomy of bottle cell formation, suggests that apical constriction is critical for the initiation of primary invagination.

In the ≈180° bottle cell ablations, the remaining bottle cells coalesced to form a pit on the unablated side. This result is consistent with the central cell ablations in which a small number of constricted bottle cells remain in the center of the vegetal plate, and the invagination continues to deepen uniformly. These results indicate that a very
small number of centrally located bottle cells is sufficient for invagination and that the normal, symmetric arrangement of bottle cells at the vegetal plate is important for the success of the invagination. Such conclusions from direct evidence cannot currently be drawn about the mechanistic role of bottle cells in invagination in any other system.

**The Potential Role of the Surrounding Cells in Primary Invagination**

Although not statistically significant, the difference between the depth of invagination on the ablated versus unablated side for embryos in which an arc of surrounding cells was ablated leaves open the possibility that these cells may be involved in mechanisms which transform the initial 5- to 10-μm-deep invagination into the 25-μm-deep, hat-shaped cylinder at the end of primary invagination. Although such mechanisms are currently uncharacterized, they may include active convergence of material toward the vegetal plate (Ettensohn, 1984a; Burke et al., 1991), rearrangement of cells within the initial invagination (Keller, 1981), or other processes. It is possible that these processes normally show temporal overlap with apical constriction during the initial stage of invagination. Ablation of a 90°–120° arc of surrounding cells may affect the onset of these processes.

It is also possible that the mechanical integration within the vegetal plate is slightly disrupted by the ablation of the arc of surrounding cells. Volume measurements (Ettensohn, 1984a) and cell marking experiments (Burke et al., 1991) as well as our tracking of individual cell movements (Fig. 4) established that a small but noticeable amount of involution occurs over the blastopore lip during the initial stage of primary invagination corresponding to at least the first tier of elongated cells. In sagittal views, elongated cells are apparent near the vegetal pole several cell diameters away from the vegetal plate. However, it is difficult to assess how many tiers of such cells exist. Cells that would initially have been classified as “surrounding cells” may be pulled around to the vegetal surface over the course of invagination, and appear elongated. It is unclear whether the involution is a passive process resulting from the apical constriction of the bottle cells or an active process whereby the cells lateral to the blastopore converge toward the blastopore. Regardless, such events may require integration and coordination among participating cells. Ablation of the 90°–120° arc of surrounding cells may slightly disrupt this integration.

Analogous ablations of arcs of the elongated cells presented experimental difficulties which made interpretation of the results impossible. The wound created by the elongated cell ablations was large compared to the other ablations. In the majority of cases the ablated material was extruded from the embryo and caught between the embryo and coverslip which distorted the shape of the embryo and the developing invagination. In the few cases in which this did not occur, it was impossible to determine whether the results reflected unhealthy embryos or a requirement for elongated cell function. For these reasons we were unable to assess the role of the elongated cells during primary invagination.

**Attachment to the Hyaline Layer Is Required for Bottle Cell Formation and the Progression of Primary Invagination**

Attachment to the apical lamina and the hyaline layer is critical for the process of primary invagination (Adelson and Humphreys, 1988; Burke et al., 1991). Nakajima and Burke (1996) found that S. purpuratus embryos treated with the AL1 antibody at the mesenchyme blastula stage were unable to form bottle cells. mAb183 must be applied at the hatched blastula stage to L. pictus embryos to entirely prevent bottle cell formation. Application of the antibody to mesenchyme blastulae produces a range of effects including reducing the number of bottle cells which form and the ability of the cells to constrict. Primary invagination initiates to varying degrees in these treated embryos but never progresses beyond the initial stage.

It is clear that a critical interaction occurs between the invaginating cells and the apical ECM. However, it is not currently possible to distinguish whether this interaction primarily involves signaling or mechanical interactions between the vegetal plate and the apical ECM, or a combination of both (McClay et al., 1990; Wessel, 1993). The computer-simulated mechanical analysis of Davidson et al. (1995) suggested that apical constriction can produce a life-like invagination. In addition, the simulation suggested that the stiffness of the ECM is crucial for apical constriction to produce an invagination. Realistic invaginations occur only if both the apical lamina and hyaline layer are of equal or lesser stiffness than the cell layer. While we have not been able to measure the actual biomechanical stiffness of the two ECM layers, the folding patterns we have observed in both the hyaline layer and apical lamina before and during primary invagination indicate that both layers are deformable.

**A Model for the Initiation of Primary Invagination**

The process of primary invagination, like all morphogenetic movements, is accomplished through mechanical interactions between force-producing cells and surrounding tissue. The bottle cell ablations revealed that the ring of apically constricted cells is crucial for the initiation of primary invagination. The surrounding cells may play a role during invagination, but currently it is unclear whether they are actively or passively involved. We propose a model in which apical constriction of the bottle cells initiates primary invagination (Fig. 10). Because the epithelial cells of the vegetal plate are bound tightly to one another via adherens junctions, the gradual decrease in the apical surface area of the ring of bottle cells forces the vegetal plate to bend. The bending accommodates the cell shape change...
caused by the apical constriction and maintains the integrity of the epithelium. Attachment of cells to both the apical lamina and hyaline layer is required for apical constriction of the bottle cells to occur. We hypothesize that one role for the apical ECM is to correctly distribute the forces generated by apical constriction.

Embryos Can Compensate for the Loss of Many Bottle Cells

The ultimate successful gastrulation of embryos in which a 120° arc of bottle cells was ablated indicates that the embryos have the capacity to compensate for the lost bottle cells. There are several potential recovery mechanisms. It is possible that the remaining bottle cells produce a sufficient, passive indentation of the ablated side for subsequent events to occur successfully. That fewer bottle cells may have the mechanical strength to do so is suggested by the central cell ablations, in which as few as three centrally located bottle cells were capable of initiating primary invagination. Another possibility is that the remaining bottle cells were able to rearrange in order to reconstitute the bottle cell ring with fewer cells, producing an invagination. Alternatively, the embryos may regulate after a delay to reform bottle cells in the ablated region and then complete gastrulation. The sea urchin embryo, and the vegetal plate territory in particular, has an incredible capacity to regulate its development following microsurgical operations. A particularly dramatic example is provided by the work of McClay and Logan (1996), who found that they could microsurgically remove the entire archenteron during secondary invagination, and the embryo could recover and regenerate a complete, new archenteron. The regulative capabilities of the sea urchin vegetal plate make the reformation of bottle cells in the ablated embryos a distinct possibility. Finally, if other morphogenetic forces operate during primary invagination, they may compensate for the missing bottle cells and be responsible for the recovery and gastrulation of the embryos (see below). Currently, we are not able to distinguish between these possible recovery mechanisms.

Apical Constriction and Other Processes during Primary Invagination

Although the laser ablation experiments demonstrated that bottle cells play a critical role in the initiation of primary invagination, they do not rule out other concurrent mechanistic events that contribute to an invagination. Indeed, the concurrent operation of multiple morphogenetic mechanisms during primary invagination is likely, given findings in other systems. In chick neurulation and Xenopus gastrulation, bottle cells appear to function within the context of several concurrently operating morphogenetic mechanisms (reviewed in Schoenwolf, 1994; Keller, 1981; Hardin and Keller, 1988). The involution process discussed above may reflect the operation of such mechanisms.

Lane et al. (1993) examined the potential role of apically secreted ECM in primary invagination. They proposed that swelling of proteoglycans secreted beneath the vegetal plate, in conjunction with a stiff hyaline layer to hold the bolus of secreted ECM in place, provides the force which buckles the vegetal plate. Computer simulations by Davidson et al. (1995) indicated that the hyaline layer must be stiffer than the apical lamina, and both layers must be stiffer than the cell layer for this mechanism to produce an invagination. In addition, the cell shapes at the vegetal plate remain cuboidal in such simulations. As discussed above, these mechanical parameters contrast with those necessary for the apical constriction model (Davidson et al., 1995). While it appears that ECM is secreted at the vegetal plate at the onset of primary invagination, Burke and Nakajima (1996) found that bottle cells are also present at this time. The seemingly opposite mechanical requirements of the apical constriction and gel swelling models of primary invagination make it unclear how to unify them into a single, coordinated model. Experimental examination of these other morphogenetic mechanisms and how they are integrated with apical constriction of bottle cells to produce an invagination are important questions for future study.

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