RhoA regulates initiation of invagination, but not convergent extension, during sea urchin gastrulation

Wendy S. Beana,⁎, Jeffrey M. Grossb, David R. McClaya

a Department of Biology, Developmental, Cell and Molecular Group, Duke University, PO Box 91000, Durham, NC 27708, USA
b Molecular Cell and Developmental Biology, University of Texas at Austin, 1 University Station A6700, Austin, TX 78712, USA

Received for publication 6 October 2005; revised 6 December 2005; accepted 14 December 2005
Available online 3 February 2006

Abstract

During gastrulation, the archenteron is formed using cell shape changes, cell rearrangements, filopodial extensions, and convergent extension movements to elongate and shape the nascent gut tube. How these events are coordinated remains unknown, although much has been learned from careful morphological examinations and molecular perturbations. This study reports that RhoA is necessary to trigger archenteron invagination in the sea urchin embryo. Inhibition of RhoA results in a failure to initiate invagination movements, while constitutively active RhoA induces precocious invagination of the archenteron, complete with the actin rearrangements and extracellular matrix secretions that normally accompany the onset of invagination. Although RhoA activity has been reported to control convergent extension movements in vertebrate embryos, experiments herein show that RhoA activity does not regulate convergent extension movements during sea urchin gastrulation. Instead, the results support the hypothesis that RhoA serves as a trigger to initiate invagination, and once initiation occurs, RhoA activity is no longer involved in subsequent gastrulation movements. © 2005 Elsevier Inc. All rights reserved.

Keywords: Sea urchin; RhoA; Gastrulation; Invagination; Convergent extension; Brachyury

Introduction

During gastrulation, embryonic cells rearrange dynamically to organize the basic body plan. A prominent feature of gastrulation is the formation of the archenteron, which begins with an inpocketing of the epithelial cell sheet at the vegetal pole of the sea urchin embryo. This epithelial bending is then transformed into a tube by cell migration, convergence, and extension movements. In deuterostomes, the archenteron later connects to a distinct invagination called the stomodaeum, the site of the future mouth. For the embryonic gut to form correctly, many cellular and molecular components must be carefully coordinated. The morphogenetic movements associated with gastrulation have received close scrutiny, most notably in Xenopus, Drosophila, and sea urchin embryos, and current efforts are directed toward detailed molecular explanations for the phenomena observed (Keller et al., 2003; Leptin, 2005; McClay et al., 2004; Solnica-Krezel, 2005). Among the important outstanding questions are the mechanisms by which the morphogenetic events during gastrulation are regulated. Recent studies have highlighted an important role for RhoA in gastrulation movements.

RhoA is a member of the Rho family of small, monomeric, GTPases. Like all GTPases, RhoA acts as a molecular switch, cycling between active GTP-bound and inactive GDP-bound states (Etienne-Manneville and Hall, 2002). Several classes of proteins regulate the nucleotide exchange: guanine nucleotide exchange factors (GEFs) which promote the active state, GTPase-activating proteins (GAPs) which promote the inactive state, and guanine nucleotide dissociation inhibitors (GDIs) which inactivate Rho by sequestering protein in the cytoplasm (Ridley, 2001). RhoA was initially recognized for its role in cytokinesis, cell cycle progression, microtubule stabilization, and gene expression (Etienne-Manneville and Hall, 2002; Manser, 2002; Wennerberg and Der, 2004). In particular, a role for RhoA in gastrulation has been identified.
through studies of the planar cell polarity pathway in *Drosophila*, *Xenopus*, and zebrafish embryos (Mlodzik, 2002; Wallingford et al., 2002). These studies outline a pathway by which extracellular signals, acting through RhoGEFs, activate RhoA, which in turn activates its downstream effector Rho kinase (ROCK), to regulate gastrulation movements (Habas et al., 2001; Hacker and Perrimon, 1998; Marlow et al., 2002; Winter et al., 2001; Zhu et al., 2006).

Gastrulation in the sea urchin is often divided into four stages, although each stage has its own complex cell biology.

1. Ingression of skeletogenic primary mesenchyme cells (PMCs) from the vegetal pole occurs several hours before invagination of the archenteron is initiated (Kominami and Takata, 2004; McClay et al., 2004). (2) The blastopore forms by an inward bending of the vegetal plate. This is marked by the appearance of bottle cells and apical deposits of new extracellular matrix (ECM) and results in invagination of the archenteron (Kimberly and Hardin, 1998; Lane et al., 1993; Nakajima and Burke, 1996). (3) The archenteron elongates and forms a tube that extends across the blastocoel in a process that is driven by both convergent extension movements and recruitment of additional cells (Ettensohn, 1985; Hardin, 1989; Logan and McClay, 1997). (4) Finally, filopodia are extended by secondary mesenchyme cells (SMCs) at the tip of the archenteron toward the animal pole, and these provide a pulling force to complete elongation and help guide the gut toward the stomodeum, placing the archenteron near the site where the mouth will form (Hardin, 1996; Hardin and Cheng, 1986; Hardin and McClay, 1990).

Specification of the endomesoderm is a prerequisite for morphogenesis, as perturbation of many genes in the endomesodermal gene regulatory network (GRN) shows that failure to specify endomesoderm eliminates competence to invaginate (Davidson et al., 2002a,b). That network directs cellular alterations necessary for invagination: several ECM components (Berg et al., 1996; Hardin, 1996; Lane et al., 1993), as well as components of the actomyosin contractility machinery (Schatten et al., 1986), are required for invagination. Five cytoskeletal actin genes and a number of myosins have been identified in the sea urchin, many of which are either upregulated just prior to invagination or differentially expressed in the vegetal plate (Cox et al., 1986; Lee et al., 1986; Miller et al., 1996; Sirotkin et al., 2000). An important remaining step is to understand the molecular apparatus that controls initiation and execution of this sequence of morphogenetic events.

This study investigates the role of RhoA during sea urchin gastrulation. Our results show that RhoA activity is important for initiating invagination of the archenteron, but RhoA does not regulate convergent extension movements during elongation of the archenteron tube.

**Materials and methods**

**Animals**

Adult *Lytechinus variegatus* were obtained from the Duke University Marine Laboratory, Susan Decker (Davie, FL), or Sea Life, Inc. (Tavernier, FL).

Gametes were collected following intracoelomic injection of 0.5 M KCl. Eggs were fertilized and raised in artificial sea water (ASW) at 23°C as described (Hardin et al., 1992).

**Cloning of *LvRhoA***

Degeneratively primers were designed to the amino acids VIVGDGA (forward)/MKQEVPVKP (reverse) and used in the PCR of cDNA prepared from midgastrula poly(A)+ mRNA. The amplified product was gel purified, cloned into pGEMT-Easy vector (Promega), and sequenced with T7 and SP6 primers (Duke Sequencing Core). Clones were identified as LvRho PCR products by BLAST search. LvRho fragment was used to probe a *L. variegatus* CDNA library as described (Gross et al., 2003). After reSCREEN, clones were bidirectionally sequenced and confirmed by BLAST search to be *LvRhoA*.

**Generation of Rho constructs**

Constitutively active (actRhoA) and dominant negative (dnRhoA) constructs were generated by designing PCR primers surrounding aa 14 (incorporating a G to V mutation for actRhoA) or aa 19 (incorporating a T to N mutation for dnRhoA) using the Transformer Site-directed Mutagenesis Kit (Clontech). PCS-2 constructs expressing wild-type *LvRhoA*, actRhoA, and dnRhoA were produced by standard molecular biology techniques, and all clones were verified by sequence analysis.

**mRNA preparation and injection**

Full-length *LvRhoA*, *dnRhoA*, or actRhoA were linearized with *NotI*. Each was used as template to transcribe in vitro 5’ capped mRNA using the SP6 mMessage Machine Kit (Ambion). Injections were carried out as described (Sherwood and McClay, 1999). For injections at the 2-cell and 4-cell stages, rhodamine-conjugated dextran was co-injected to label the injected cell.

**Pharmacological treatments**

*Clostridium butulatum* toxin, C3 transferase, was obtained from Cytoskeletons, resuspended in sterile water, and used at 10 μg/ml. The ROCK inhibitor, Y-27632, was obtained from Calbiochem, resuspended in DMSO, and used at 100 μM. Treatment with DMSO vehicle had no effect (not shown).

**Immunolocalization and image analysis**

For whole-mount staining, embryos were fixed in 3% paraformaldehyde, followed by 100% cold MeOH, then incubated and imaged using a Zeiss 410 laser-scanning confocal microscope, all as described (Range et al., 2005), with the exception of mounting in 70% glycerol. For chondroitin sulfate (CS) staining, embryos were fixed in 10% formaldehyde with 0.05 M Tris pH 8.0 for 20 min, instead of paraformaldehyde; then incubated and imaged as before. 1,000 dilutions of RhoA/B/C antibody (Upstate), 1:100 dilutions of Notch antibody (*Sherwood and McClay, 1999*), 1:100 dilutions of CS antibody (CS-56, Sigma) were used. Secondary antibodies included Cy3-conjugated goat anti-rabbit for RhoA, Cy5-conjugated donkey anti-guinea pig for Notch, and Cy2-conjugated anti-mouse IgM for CS (Jackson Immunoresearch Laboratories). For Hoechst staining, embryos were incubated for 5 min at 1:1000 (Hoechst 33342, Molecular Probes) then washed 3× and imaged as above.

For actin/phalloidin staining, embryos were fixed in 3% paraformaldehyde with 0.1 M HEPES for 20 min, followed by 1 min in PBS and 1 min in cold EtOH. Embryos were transferred through 50% EtOH/Blok [PBS with 4% normal goat serum (Blok and 20% EtOH/30% Block, then washed 2× in PBS. Embryos were blocked for 10 min, treated with hyaluronidase [750 U/ml in PBS (Sigma)] for 10 min, 2× wash in PBS and 1 h in block. Embryos were treated overnight with 1:20 phalloidin (Alexa
Flour 488/Molecular Probes), then washed 3× in PBS, and blocked for 20 min. Embryos were imaged as above.

**Embryo manipulations**

Embryos to be manipulated were fertilized in ASW with 0.2mM paraminobenzoic acid and injected either with dye-labeled constructs or control buffer. Animal/Vegetal chimeras were produced by surgical separation of embryos perpendicular to the animal/vegetal axis at the 60-cell stage along the Veg1-An1 border (in Ca²⁺-free ASW), followed by recombination of a control half with the opposite dnRhoA-injected half.

**DiI labeling**

Embryos were injected with actRhoA, then cultured to the hatched blastula stage. 5 mg/ml DiIC₁₆ (Molecular Probes) in 100% EtOH was applied to a pulled glass needle (as per communication with Dr. Gregory Wray). Embryos were immobilized between a protamine sulfate-coated glass slide and coverslip. DiI-labeled needles were held against the embryo for 5–8 min until precocious invaginations were labeled, as checked by fluorescence.

**Results**

**Cloning and sequence analysis of LvRhoA**

A single *L. variegatus* Rho isoform (LvRhoA) was isolated (GenBank accession no. DQ225184). Sequence analysis revealed a predicted open reading frame for LvRhoA of 192 amino acids, and protein alignment with known homologues shows the Rho GTPase is highly conserved between species (not shown). LvRhoA shares 94% identity with HpRho1, a sea urchin orthologue cloned from *Hemicentrotus pulcherrimus* (Nishimura et al., 1998), as well as 83% identity with the human RhoA isoform.

![Fig. 1. LvRhoA regulates the onset of invagination.](image)

Fig. 1. LvRhoA regulates the onset of invagination. (A–E) Controls. (F–J) Embryos expressing dominant negative LvRhoA (T19N; dnRhoA). (K–O) Embryos expressing constitutively active LvRhoA (G14V; actRhoA). Arrows denote precocious invaginations. Number of precocious invaginations in actRhoA-injected embryos: \( n = 99/345 \) (28.69%). Number of dnRhoA-injected embryos which failed to invaginate \( n = 173/236 \) (73.31%).
Phylogenetic analysis supports LvRhoA homology to the RhoA isoform (not shown).

**LvRhoA regulates initiation of archenteron invagination**

Microinjection of wild-type LvRhoA mRNA had no effect (not shown), as expected for a protein expressed ubiquitously and activated post-translationally. To determine the role of LvRhoA during sea urchin embryogenesis, constitutively active (G14V) and dominant negative (T19N) constructs were designed based on well-established mutations (Schmitz et al., 2000). Constitutively active LvRhoA (actRhoA) is unable to hydrolyze GTP and is therefore locked in the active conformation, while dominant negative LvRhoA (dnRhoA) out-competes endogenous RhoA for binding to activating GEFs (Bishop and Hall, 2000; Feig, 1999). mRNAs encoded by these constructs were microinjected, and embryos were assessed throughout development (Fig. 1). Embryos expressing dnRhoA (0.5–1.0 pg/pl) developed normally through mesenchyme blastula stage, but 73% of the embryos scored failed to initiate archenteron invagination at a time when controls had reached late gastrula stage (Figs. 1F–J). Conversely, embryos expressing actRhoA (0.05–0.08 pg/pl) developed normally until hatched blastula stage, at which time precocious invagination was observed in 29% of embryos (arrows in Figs. 1K–M), about 3 h prior to PMC ingression and 5 h prior to initiation of invagination in control embryos. Thus, manipulation of RhoA results in perturbation of the onset of invagination. PMC ingression occurred at the normal time in both actRhoA- and dnRhoA-expressing embryos. Thus, only the timing of archenteron invagination was altered when RhoA was perturbed, indicating that LvRhoA specifically regulates initiation of invagination but has no effect on normal ingression movements.

Precocious invaginations occurred as early as prehatched blastula stage (Fig. 1K) and at any time until the normal onset of invagination, although they were most frequently first seen at hatched blastula stage (Fig. 1L). The precocious invaginations stalled after the initial invagination and only proceeded through archenteron extension later, concurrently with controls. By late gastrula stage, actRhoA-injected embryos resembled controls (Fig. 1O). Injection of actRhoA at high levels (>0.1 pg/pl) gave rise to embryos in which the entire blastoderm was buckled, giving it a folded appearance (not shown). But these “folded” embryos still developed a single precocious invagination internal to the “buckled” blastoderm and by 72 h had recovered into normally patterned pluteus larvae (not shown).

dnRhoA functions by competition for GEFs which can interact with multiple GTPases (Feig, 1999). To address the specificity of the dnRhoA phenotype, the effect of the *C. botulinum* toxin C3 transferase (C3) was tested. C3 inhibits Rho activity directly by ADP-ribosylation of Rho, leaving the GTPase biologically inactive without affecting nucleotide binding (Aktories et al., 2004; Sekine et al., 1989). A dose-response experiment showed that treatment of embryos with the optimal dose of C3 (10 μg/ml) perturbed invagination, phenocopying dnRhoA (Fig. 2A2). Treatment with C3 completely blocked invagination without recovery as long as the drug was present. Removal of the drug reversed this effect. These results show that RhoA is specifically required for invagination.

![Fig. 2](image)

**Fig. 2.** LvRhoA activity is required just prior to invagination. (A) Pharmacological inhibition phenocopies dnRhoA. (A1) Late gastrula-stage control. (A2) Embryo treated with C3-transferase [10 μg/ml], an inhibitor of Rho activity. (A3) Embryo treated with Y-27632 [100 μM], an inhibitor of the downstream Rho-effector, Rho kinase (ROCK). (B) Window of sensitivity to C3 treatment. Embryos were treated for the times indicated (filled bars) then assessed for invagination at late gastrula stage. Partial gut refers to archenterons less than 2/3 of normal length by late gastrula stage. (B1) Time course. (B2) Wash out.
The reversibility of C3 treatment allowed for assessment of the timing of LvRhoA activity requirement (Wilde and Aktories, 2001). Embryos were treated with and washed from C3 at various time points throughout embryonic development (Fig. 2B). The results show that a short 1 h treatment just prior to blastopore formation was sufficient to disrupt normal archenteron formation (Fig. 2B2). C3 inhibited invagination movements when added prior to onset, but once invagination had begun, C3 was no longer inhibitory (Fig. 2B1). Wash out experiments show that invagination was not affected when C3 was added earlier in cleavage but washed out 1 h prior to blastopore formation, further indicating that RhoA activity is necessary for gastrulation at the time that invagination begins (Fig. 2B2). At a minimum, these experiments suggest that LvRhoA activity is necessary proximal to early invagination movements.

The downstream RhoA effector Rho kinase (ROCK) has been implicated in RhoA signaling during gastrulation movements in vertebrate embryos (Kim and Han, 2005; Lai et al., 2005), and therefore, the possibility that ROCK participates in sea urchin gastrulation was examined. ROCK activity was inhibited using the well-established compound Y-27632 (Davies et al., 2000; Riento and Ridley, 2003). Treatment with Y-27632 at the optimal dose (100 μM) delayed invagination, although embryos recovered by pluteus larvae stage (Fig. 2A3). These data provide at least some support for the hypothesis that LvRhoA signals through ROCK to regulate invagination of the archenteron, and they further corroborate the observation that LvRhoA is necessary early in this process.

LvRhoA is required in the vegetal hemisphere to regulate invagination during gastrulation

The next group of experiments examined the localization and fate of the precocious invaginations induced by actRhoA expression. Only a single precocious invagination was observed in actRhoA-expressing embryos. To assess the spatial requirements for RhoA to initiate invagination, actRhoA was expressed only in either one half or one quarter of the embryo (Fig. 3). When actRhoA was injected in one blastomere at the 2-cell (Fig.

![Fig. 3. LvRhoA directly initiates precocious invagination. Partial embryo microinjections (as indicated in fluorescence panels). Embryos injected in a single blastomere at the 2-cell (A–B) or 4-cell stage (C–D). (A, C) Control embryos. (B, D) actRhoA-injected embryos. Arrows denote precocious invaginations.](image)

![Fig. 4. LvRhoA is required in the endomesoderm. Animal/Vegetal chimeras. (A) Diagram of experimental design. (B–D) Chimeric embryos at gastrula stage; control injection in green, dnRhoA injection in red. (B) Surgery control with two control halves recombined. (C) dnRhoA expressed only in the animal half; n = 6/6. (D) dnRhoA expressed only in the vegetal half; n = 5/5.](image)
3B) or the 4-cell stage (Fig. 3D), precocious invaginations were observed, but only in sectors of the embryo that expressed actRhoA, as shown by the co-injected florescent marker (Figs. 3B, D). These data indicate that RhoA functions in an autonomous manner, suggesting that precocious invaginations require actRhoA directly, rather than being downstream of a RhoA-activated non-autonomous signal.

To further define the territory of RhoA function, chimeric embryos were produced at the 60-cell stage by combining animal and vegetal halves from control (green) and dnRhoA-injected (red) embryos (Fig. 4). When dnRhoA was expressed only in the animal half, invagination of the chimeras was similar to surgical controls (Figs. 4B, C). However, embryos expressing dnRhoA only in the vegetal half of the embryo failed to invaginate (Fig. 4D). These data suggest that LvRhoA activity regulates invagination of endomesodermal cells and, thus, only in tissue that normally invaginates. These data further support the hypothesis that LvRhoA activity is necessary for localized activation of endomesoderm invagination.

Because RhoA is known to participate in cytokinesis (Glotzer, 2005), the actRhoA-induced precocious invaginations could include an increased number of cell divisions. To assess the effects of RhoA on cell cycle, hatched blastula stage embryos were treated with Hoechst nucleic acid stain, and nuclei counts were performed on whole embryos (Fig. 5A). Nuclei were counted in individual z-sections across an entire embryo, and the numbers complied to generate counts for a whole embryo. The results demonstrate that actRhoA-injected embryos expressing precocious invaginations contain comparable numbers of cells to control embryos. These data rule out additional cell divisions as having a mechanistic role in early invaginations.

The site of the precocious invagination was important to determine. Was it a temporary ectopic invagination, or the early
appearance of the true embryonic archenteron? To assess the fate of actRhoA-induced precocious invaginations, the early invaginations were labeled with the lineage tracer Dil at hatched blastula stage, then the same embryos were examined at late gastrula stage (Fig. 5B). In every case, the precocious invagination persisted to become the embryonic archenteron. Because actRhoA was expressed across the entire embryo, yet the invagination occurred at a single endogenous site, this strongly suggests that RhoA is not involved in site selection for archenteron formation, but rather with the timing for onset of invagination at that predefined site.

LvRhoA regulates the cytoskeletal and ECM changes associated with invagination

RhoA expression was assessed next, to determine whether endogenous LvRhoA expression is spatially and temporally compatible with a role in initiating invagination. Rho is ubiquitously expressed throughout embryonic development in other organisms (Wennerberg and Der, 2004), and quantitative PCR analysis confirms that LvRhoA is expressed both maternally and zygotically throughout sea urchin development (not shown). Although Rho protein is present in every cell, it localizes to the plasma membrane when GTP bound, while most GDP-bound Rho is cytoplasmic (Adamson et al., 1992; Fleming et al., 1996; Kranenburg et al., 1997). Thus immunofluorescence staining was used to assess the subcellular distribution of LvRhoA protein as an indicator of activity (Fig. 6A). RhoA was stained using an antibody directed against a peptide region of human Rho that is identical to L. variegatus RhoA, except for a S to N substitution at aa 88. Significant levels of LvRhoA-specific staining were found at the apical membrane throughout early development, particularly in actin-rich areas such as adherens junctions (arrows in Fig. 6A1).

During early cleavage, LvRhoA protein expression was ubiquitous, localizing to cleavage furrows during mitosis (not shown) as was observed with H. pulcherrimus Rho1 (Nishimura et al., 1998). At hatched blastula stage, LvRhoA was still ubiquitous and mostly localized to the apical membrane (Fig. 6A2). During gastrula stages, apical LvRhoA was eliminated from the elongating gut as those cells progressed internally past the site of the blastopore (Figs. 6A3, 4). Detailed examination of the blastopore region revealed that membrane-localized LvRhoA was heavily concentrated at the apical surface of the blastopore but absent just inside that opening (Fig. 6A5). Along with previous data, this implies that LvRhoA signaling is crucial at the site of invagination, then is inactivated as soon as the endomesodermal cells have moved to the interior of the embryo.

Dense accumulations of filamentous actin have previously been reported to form at the apical surface of the blastopore lip of urchin embryos (Kimberly and Hardin, 1998; Nakajima and Burke, 1996). Actin also accumulated precociously at the blastopore in blastula stage actRhoA-injected embryos, as seen by phalloidin labeling (Fig. 6B2). Control blastula stage embryos lacked any such accumulation (Fig. 6B3). The actin accumulations associated with actRhoA-induced precocious invaginations resembled those seen in much later gastrula stage control invaginations (Fig. 6B1), suggesting that RhoA

Fig. 6. LvRhoA regulates gastrulation-associated molecular rearrangements. (A) Antibody staining of endogenous LvRhoA. (A1) LvRhoA is concentrated at the apical membrane, particularly in actin-rich areas such as adherens junctions (arrows). Whole embryo staining at blastula (A2), midgastrula (A3), and late gastrula stages (A4). Panel (A4) is co-stained for Notch (in green), which at late gastrula is localized to the apical membrane of archenteron cells. (A5) View of the blastopore. (B) Actin staining as measured by phalloidin binding. Arrowheads indicate actin accumulation in phenotypic gastrula stage control (B1) and precocious blastula stage (B2) invaginations that are lacking in blastula stage controls (B3). (C) Chondroitin sulfate antibody staining (CSPG). CSPG accumulation in gastrula stage control (C1) and actRhoA-injected precocious blastula stage (C2) invaginations not seen in blastula stage controls (C3).
regulates the actin rearrangements associated with the initiation of invagination in the sea urchin, in keeping with Rho’s well-known function as a cytoskeletal regulator.

Previous studies have also shown that initiation of invagination is accompanied by the secretion of apical extracellular matrix containing chondroitin sulfate (Lane et al., 1993). To determine if actRhoA-induced invaginations also displayed precocious deposition of chondroitin sulfate (CS) at the site of the blastopore, actRhoA-injected embryos were stained with an antibody recognizing chondroitin sulfate proteoglycans (Fig. 6C). Control gastrula-stage embryos displayed concentrated staining of CS at the blastopore (Fig. 6C1), as previously described. actRhoA-induced blastula stage invaginations also displayed such concentrated staining (Fig. 6C2), which was lacking in blastula stage controls (Fig. 6C3). These data indicate that LvRhoA induces the secretion of apical extracellular matrix associated with invagination. Together, these data suggest that LvRhoA induces the known cytoskeletal and extracellular rearrangements that accompany invagination of the archenteron.

LvRhoA is not involved in convergent extension during gastrulation

Previous studies have indicated that RhoA is required for the regulation of convergent extension (CE) events during gastrulation in other species (Marlow et al., 2002; Tahinci and Symes, 2003). Sea urchin embryos also utilize CE movements to extend the nascent gut across the blastocoel. These CE movements elongate the gut tube by rearranging the invaginated cells within the plane of the epithelium in such a way that cells move laterally and insert themselves in between two other cells, thereby extending the length of the tube and at the same time narrowing its diameter (Ettensohn, 1985; Hardin, 1989; Hardin and Cheng, 1986).

To determine whether LvRhoA is necessary for convergent extension in the sea urchin, Dil labeling experiments were performed in which labeled precocious invaginations were tracked throughout gastrulation (Fig. 7). Convergent extension events involving intercalation of cells can be visualized by the insertion of non-labeled cells between patches of labeled cells (Hardin, 1989). Although intercalation was observed in actRhoA-expressing embryos (Fig. 7I), it did not occur precociously (Figs. 7F–H). Instead, after the early invagination occurred, elongation of the gut tube was halted. CE-associated intercalations only appeared later, concurrently with controls. Thus, actRhoA did not induce precocious convergent extension, despite the early formation of the blastopore. This is consistent with data above indicating that RhoA is not active in cells post-invagination, as suggested by the loss of apical RhoA staining from invaginated tissue. This is also consistent with the above C3 results, which suggest there is no further requirement for RhoA function after blastopore formation and initial invagination. Thus, unlike vertebrate RhoA, LvRhoA does not appear to regulate later CE movements but instead regulates initiation of invagination during sea urchin gastrulation.
LvRhoA rescues invagination in Brachyury-inhibited embryos

Signaling by the endodermal T-box transcriptional activator Brachyury (Bra) is required for archenteron formation in the sea urchin (Croce et al., 2001; Gross and McClay, 2001). Bra is first expressed just prior to hatching in a vegetal ring, and its expression is dynamic so that a ring of Bra is maintained surrounding the future site of the blastopore (Croce et al., 2001; Gross and McClay, 2001). Inhibition of Bra, through the use of a Brachyury-Engrailed (Bra-Eng) construct that changes Bra into a transcriptional repressor, results in embryos which fail to invaginate (Gross and McClay, 2001). As the dnRhoA-injected and C3-treated phenotypes were very reminiscent of the Bra-Eng phenotype, the relationship between LvRhoA and LvBra was assessed.

Co-injections of actRhoA and Bra-Eng mRNA were performed to determine whether LvRhoA was capable of rescuing invagination in Bra-inhibited embryos (Fig. 8). At late gastrula stage, both control and actRhoA-injected embryos had fully invaginated (Figs. 8A–B), while Bra-Eng embryos lacked an archenteron (Fig. 8C). Co-injection of actRhoA rescued the invagination movements inhibited by Bra-Eng (Fig. 8D). Furthermore, QPCR analysis of the endodermal marker Endo16 showed that while Endo16 levels were decreased in Bra-Eng-injected embryos, co-injection with actRhoA was sufficient to rescue Endo16 to control levels (Fig. 8E). These data reveal that actRhoA can rescue archenteron invagination and subsequent endoderm differentiation in the absence of Bra transcriptional regulation.

QPCR analysis of Bra expression in actRhoA-expressing, dnRhoA-expressing, and C3-treated embryos revealed that perturbation of RhoA had no effect on Bra transcript levels (not shown). This suggests that RhoA functions downstream of Bra activity. Conversely, RhoA transcript levels are not altered in Bra-Eng-injected embryos (not shown). This result is not surprising as RhoA activity is primarily regulated not at the transcript level but instead it is the protein’s GTPase activity that is regulated. Together, these data are consistent with RhoA being at least functionally downstream of Bra transcriptional regulation to trigger archenteron invagination in the sea urchin.

Discussion

Gastrulation is a complex, multi-stage process requiring the coordination of both gene expression and cytoskeletal rearrangements in order to execute the patterned movement and rearrangement of endomesodermal cells to form a digestive tube. In this study, the role of the small GTPase RhoA during sea urchin gastrulation was examined. The results indicate that LvRhoA activity is required for initiation of archenteron invagination in sea urchin embryos. Inhibition of RhoA, by either expression of dominant negative RhoA or treatment with the Rho-specific inhibitor C3 transferase, resulted in a failure to initiate archenteron invagination. Conversely, expression of constitutively activated LvRhoA initiated single, precocious invaginations, on average 5 h prior to the normal onset of invagination. Antibody staining showed that RhoA is localized to the apical membrane, particularly at the blastopore, and thus in the correct location to drive invagination movements, while chimeric expression of dominant negative RhoA demonstrated that RhoA activity is required only in the endomesoderm to regulate invagination. C3 treatments also revealed that the minimum temporal requirement for RhoA activity is just prior to blastopore formation. Thus, the results are spatially and temporally consistent with RhoA functioning as a trigger that initiates invagination in sea urchin embryos.

Previous investigations of Rho function in the sea urchin were confined to the initial events in cleavage (Castellano et al., 1997; Covian-Nares et al., 2004; Manzo et al., 2003; Nishimura et al., 1998). These studies found that sea urchin embryos treated with C3 prior to fertilization fail to complete the first cleavage (Manzo et al., 2003). This is consistent with findings...
RhoA localizes to the cleavage furrow during mitosis (Nishimura et al., 1998), reinforcing a role for RhoA in cytokinesis. However, such cleavage defects were not observed in this study. In the present study, the earliest C3 was added was following fertilization envelope formation, although C3 was also effective when added 1 h prior to blastopore formation. Given the lower membrane permeability of C3 (Aktories et al., 2004), post-envelope delivery may have delayed the onset of effectiveness for the inhibitor and thereby masked a requirement for RhoA during the first cleavage. This argument would also apply to the injection of mRNA encoding dominant negative RhoA, in the sense that protein concentrations necessary for alteration of function accumulate after some delay. The ubiquitous endogenous expression of LvRhoA, combined with the myriad known roles for RhoA in such diverse processes as dendritic outgrowth, cell polarity and cell cycle progression (Etienne-Manneville and Hall, 2002; Modzik, 2002), makes it highly unlikely that LvRhoA serves only as a single function during embryonic development. The identification of sea urchin RhoA effectors will provide the tools necessary to discriminate potential additional and/or redundant functions for RhoA, both during early cleavage as well as later during morphogenesis by providing unambiguous measures of RhoA signaling.

Activated RhoA caused precocious invagination beginning at hatched blastula stage (approximately 7.5 h post-fertilization) and thus is the earliest known invagination initiator. RhoA-induced early invaginations were accompanied by actin accumulation, as well as apical secretion of extracellular matrix at the blastopore, molecular changes previously shown to be associated with normal blastopore formation (Kimberly and Hardin, 1998; Lane et al., 1993; Nakajima and Burke, 1996). This points toward a role for LvRhoA in directing the entire cassette of changes associated with the onset of invagination. Precocious invaginations have also been observed after treatment with the calcium ionophore A23187, which functions by a mechanism proposed to involve the secretion of extracellular matrix (Lane et al., 1993). However, A23187-treated embryos only invaginated at late mesenchyme blastula stage, after PMC ingestion had occurred, while invaginations induced by activated RhoA occur several hours prior to PMC ingestion. Moreover, the data herein show that LvRhoA is upstream of the extracellular matrix secretion as reported by Lane et al. These observations are consistent with RhoA activation serving as the initiating trigger that induces primary invagination.

Activated RhoA, when expressed experimentally, was expressed throughout the embryo; however, only a single precocious invagination was ever observed in each embryo. Dil labeling experiments demonstrated that these early invaginations persisted to become the embryonic archenteron. Thus, the precocious invaginations caused by activated RhoA are in fact the early appearance of the true archenteron. This implies that LvRhoA does not function in selecting the site for blastopore formation, as ectopic expression of activated RhoA never resulted in multiple invaginations. Prior specification of the endomesoderm therefore appears to be a prerequisite for LvRhoA function at the blastopore. Activated LvRhoA was not able to induce invagination earlier than one-half hour prior to hatching, suggesting that prior this time the vegetal plate is not competent to respond to RhoA. Perturbation experiments support this hypothesis, since many early molecular events are required for blastopore formation. For example, if any of a number of endomesodermal specification genes are inhibited, invagination fails or is greatly delayed (Davidson et al., 2002a, b). A temporal view of the endodermal gene regulatory network (GRN) supports the contention that endodermal specification is incomplete at hatched blastula stage (Davidson et al., 2002b) but, for the purposes of invagination, is complete enough to support initiation of invagination if supplied with activated RhoA. Further implied is that while specification is a prerequisite competence to respond to RhoA, the actual trigger that normally activates RhoA occurs only after a considerable delay. Frizzled5/8 signaling may be responsible for this delay since recent experiments suggest this planar cell polarity pathway functions to control invagination (Croce et al., in press). The identification of modulators of RhoA activity in sea urchin will provide insight into the mechanism underlying RhoA activation and its connection to prior specification events.

Determining the exact placement of RhoA activity within the context of the entire endomesodermal GRN is beyond the scope of this investigation, as the network outlines more than 20 genes required for endoderm specification alone (Davidson et al., 2002a,b). However, the data herein show that RhoA can rescue invagination when signaling by the endodermal transcriptional activator Brachyury is lost. Thus, it appears that RhoA is, in effect, downstream of Brachyury. However, while our data satisfy the definition of a rescue, much remains to be learned. Since Brachyury is first expressed at hatching and in a ring that marks the future site of the blastopore (Croce et al., 2001; Gross and McClay, 2001), it is in the right place and time to function upstream of RhoA. It is also intriguing to note that a target screen identified Kakapo (a cytoskeletal linker) and Gelsolin (an actin capping protein) as direct targets of Brachyury signaling (Rast et al., 2002), indicating the possibility that Brachyury is upstream of cytoskeletal remodeling. However, since Bra does not regulate the level of expression of RhoA mRNA but is upstream of RhoA activity, this connection must be indirect. Nonetheless, these data point to the importance of RhoA in regulating invagination, suggesting that RhoA must play a central role in the onset of cell movements if it is capable of rescuing invagination in the absence of an important transcriptional activator upstream of those events.

The RhoA regulation of gastrulation movements is limited to early invagination events in the sea urchin embryo. Neither positive nor negative perturbation of RhoA had any effect on skeletogenic mesenchyme ingestion (the initial gastrulation-associated cell movement to occur in sea urchin embryos). Dil labeling results show that activated RhoA did not induce precocious convergent extension events in the sea urchin. The RhoA inhibitor C3 failed to inhibit convergent extension events, and antibody staining indicated that RhoA is not localized at the
apical membrane at the time of convergent extension movements. Thus, LvRhoA does not appear to regulate convergent extension events during sea urchin gastrulation. The data demonstrate that invagination and convergent extension are separable events, and that invagination does not automatically trigger subsequent convergent extension, which therefore must rely on independent signals. Currently, there are no known molecular triggers for convergent extension in the sea urchin, although likely candidates, such as the GTPase Rac which participates with RhoA in vertebrate convergent extension (Bakkers et al., 2004; Habas et al., 2003; Tahinci and Symes, 2003), have not yet been studied.

This finding is in contrast to vertebrate embryos, in which non-canonical Wnt signaling has been implicated specifically in controlling convergent extension events during gastrulation through RhoA-mediated control of actomyosin contractility (Heisenberg et al., 2000; Tada and Smith, 2000; Tada et al., 2002; Wallingford et al., 2000, 2002). However, the results herein are consistent with observations in Drosophila, where RhoA has also been identified as a regulator of invagination. Both the expression of dominant negative DRhoA and the inhibition of the Rho-activating DRhoGEF2 result in a failure to invaginate the ventral furrow and posterior midgut, although germ band elongation was initiated (Hacker and Perrimon, 1998), indicating a similar separation of invagination and convergent extension movements. This suggests that control of invagination might be an ancient function for Rho that has been adapted more recently to the control of convergent extension.

Vertebrate convergent extension movements rely on the Wnt-mediated planar cell polarity (PCP) pathway which signals through RhoA (Mlodzik, 2002; Wallingford, 2005; Zhu et al., 2006). The non-canonical Wnt pathway is activated by Wnt signals that bind to Frizzled receptors, which in turn activate RhoA through Frizzled-associated Disheveled proteins. The PCP pathway also appears to be responsible for the RhoA activity described herein. First, the Rho effector ROCK, a vital component of the PCP pathway, is required for archenteron invagination in sea urchin embryos, as demonstrated in this investigation. Further, a Frizzled receptor, Fz5/8, which signals through the PCP pathway, is necessary for initiation of sea urchin invagination and appears to mediate this function through activation of RhoA (Croce et al., in press). Thus, the PCP pathway appears to function generally in the morphogenetic changes associated with gastrulation, whether in vertebrate or sea urchin embryos. Interestingly, vegetal Fz5/8 is first expressed at mesenchyme blastula stage, possibly explaining the normal delay in RhoA activation, despite the earlier competence of the vegetal plate to respond to RhoA and undergo invagination movements.

Since the first cells to invaginate are secondary mesenchyme cells (SMCs) (McClay et al., 2004), it seems reasonable to hypothesize that RhoA is activated in these cells to initiate invagination. Mosaic analyses are consistent with this proposition, since RhoA activity is required only in the vegetal hemisphere to regulate invagination. And although LvRhoA expression at the blastopore is maintained throughout gastrulation, treatment with C3 demonstrates that once invagination is initiated, RhoA activity is no longer required for gastrulation to proceed. Further, expression of vegetal Fz5/8 (which apparently signals through LvRhoA to regulate invagination) is limited to SMCs (Croce et al., in press). Since LvRhoA is apically localized throughout the embryo prior to invagination, this also strongly argues for the necessity of local activation of LvRhoA in a restricted cell lineage predetermined to undergo blastopore formation.

Wnt8, a Frizzled ligand, is also a positive regulator of sea urchin invagination (Wikramanayake et al., 2004). Wnt8 is normally expressed in vegetal cells and, when overexpressed, produces embryos with multiple, ectopic invaginations, even in isolated animal halves (Wikramanayake et al., 2004). Wnt8 is probably not the PCP pathway ligand, since it normally acts early in endomesoderm specification. The Wnt8-induced extra archenterons are apparently a consequence of ectopic endomesoderm specification. Further, since none of the Wnt8-induced ectopic invaginations occur precociously, it is very unlikely that RhoA regulation of invagination is a direct response to this signal. Thus, an important remaining question concerns the mechanism of RhoA activation at the blastopore. Understanding this mechanism will provide a molecular link connecting endomesodermal specification to the onset of morphogenesis.

Acknowledgments

We would like to thank Drs. Cynthia Bradham and Jennifer Croce for many invaluable discussions and suggestions, both about the studies performed herein and the manuscript itself. We thank Shu-yu (Simon) Wu for technical assistance with phylogenetics and Dr. Gregory Wray for DiI labeling methods. Thanks for support also goes to JLB, as well as the members of the McClay laboratory. This work was supported by NIH grants HD 14483 and GM 61464 to D.R.M.

References

Berg, L.K., Chen, S.W., Wessel, G.M., 1996. An extracellular matrix molecule that is selectively expressed during development is important for gastrulation in the sea urchin embryo. Development 122, 703–713.


Nakajima, Y., Burke, R.D., 1996. The initial phase of gastrulation in sea urchins is accompanied by the formation of bottle cells. Dev. Biol. 179, 436–446.


