Development 128, 45-56 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV5424

MAPK signaling by the D quadrant embryonic organizer of the mollusc *Ilyanassa obsoleta*

J. David Lambert and Lisa M. Nagy*

Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721, USA
*Author for correspondence (e-mail: lnagy@u.arizona.edu)

Accepted 25 October; published on WWW 27 November 2000

SUMMARY

Classical experiments performed on the embryo of the mollusc *Ilyanassa obsoleta* demonstrate that the 3D macromere acts as an embryonic organizer, by signaling to other cells and inducing them to assume the correct pattern of cell fates. We have discovered that MAP kinase signaling is activated in the cells that require the signal from 3D for normal differentiation. Preventing specification of the D quadrant lineage by removing the polar lobe disrupts the pattern of MAPK activation, as does ablation of the 3D macromere itself. Blocking MAPK activation with the MAP Kinase inhibitor U0126 produces larvae that differentiate the same limited complement of tissues as D quadrant deletions. Our results suggest that the MAP Kinase signaling cascade transduces the inductive signal from 3D and specifies cell fate among the cells that receive the signal.

Key words: *Ilyanassa obsoleta*, Mollusca, Embryology, Organizer, MAPK

INTRODUCTION

Molluscan embryogenesis is a classic example of mosaic development – most cell deletions result in partial larvae with predictable defects. This is because the cells of the embryo are specified at an early stage and rarely regulate to restore the missing structures. Mosaic development is often equated with cell-autonomous modes of cell fate specification such as cytoplasmic localization. However, in the embryo of the gastropod *Ilyanassa obsoleta*, the cells that give rise to most ectodermal structures are specified conditionally, by cell-cell interactions. Embryological manipulations have demonstrated that a particular cell in the D quadrant lineage of the embryo functions as an organizer of axial patterning by inducing various other cells to assume their proper spatial pattern of cell fates (Cather, 1971; Clement, 1976). Despite the importance of this induction in the axial patterning of the embryo, interest in the evolution of axial patterning in animal embryos and a long history of experimentation aimed at uncovering patterning molecules in molluscan embryos, nothing is known about the molecular mechanisms that underlie this event. Nonetheless, a century of experiments on molluscan embryos make specific predictions about the expected behavior of the molecules used by the molluscan organizer.

The D quadrant organizer functions at a early stage, when there are relatively few cells in the embryo, and the regularity of the preceding cell divisions allows unambiguous identification of all of these blastomeres. The first cell division is accompanied by the formation of a polar lobe – an anucleate extrusion of cytoplasm from the vegetal pole (see Fig. 1 for diagram of early *Ilyanassa* development). The contents of the polar lobe are shunted into one of the blastomeres at the four-cell stage. This produces one cell that is larger than the other three. The larger blastomere that inherits the polar lobe becomes the founder cell of the dorsal lineage known as the D quadrant. This event specifies the dorsal-ventral axis of the embryo. The three smaller blastomeres present at the four cell stage become the founder cells of different lineages, the A, B and C quadrants, respectively. During the next three cleavage cycles, each macromere divides towards the animal pole to produce a micromere and the four micromeres produced during a given cleavage cycle are referred to as a quartet. After the fifth cleavage cycle, there are three quartets of micromeres and their derivatives in the animal hemisphere and four vegetal macromeres. These micromeres give rise to all of the ectodermal structures of the larva, including velum, eyes, statocysts, shell, foot and operculum (Render, 1997). While the micromeres have predictable fates, they are not specified until later in development, when they are induced by the D quadrant macromere.

Ablation experiments have shown that the D quadrant macromere is required to establish the proper cell fates among the micromeres. Removal of the polar lobe prevents the specification of D quadrant macromere. The larvae that result from polar lobe ablation lack a dorsal-ventral axis (van den Biggelaar and Guerrier, 1983). They have disorganized ciliated velar bands, everted stomodea and esophageal tissue. They lack eyes, shell, heart, intestines, statocysts, opercula, larval kidneys and certain muscles (Atkinson, 1971; Clement, 1952). Killing the D macromere at the four, eight, or 16-cell stages (e.g. ablation of D, 1D or 2D) has the same effect as removal of the polar lobe, demonstrating that the requirement for the D

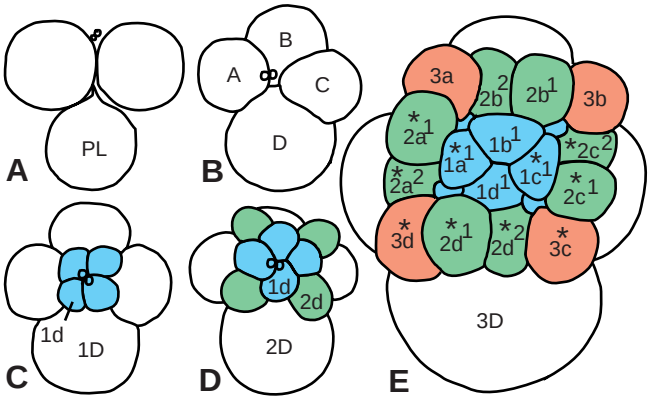

Fig. 1. Early cleavage of *Ilyanassa*. (A) The polar lobe is produced during the first cleavage. (B) The D macromere of the four cell stage is specified by inheritance of the polar lobe. (C-E) Successive cleavage cycles produce the first quartet (C, blue, '1q'), and second quartet (D, green, '2q'). The fifth cleavage cycle produces the third quartet (E, orange, '3q'). Up to this point, each cleavage cycle takes about 1 hour. After the production of the third quartet, the macromeres do not divide synchronously. 3D divides first (after about 1.5 hours) to produce the micromere 4d, and the macromeres in the other quadrants produce their fourth quartet micromeres about 3.5 hours later. After the production of the third quartet (E), when 3D signals to the micromeres, there are 24 cells in the embryo. In the absence of the signal from 3D, larvae do not differentiate eyes, shell or foot structures. The micromeres which are required (based on cell deletion studies) for the differentiation of these structures can thus be inferred to require induction by 3D. These cells (indicated by asterisks) are 1a and 1c, which are required for eye development, 2a, 2c, 2d, which are required for shell development, and 3c and 3d, which are required for foot structures (Clement, 1976). Additional cells contribute to these structures (Render, 1997) or partially disrupt development of these structures (Clement, 1976). This likely reflects interactions after the induction by 3D. The veliger larva that develops after about 7 days is shown in Fig. 6A,B.

macromere has yet to be fulfilled at these stages. The D quadrant lineage does not directly contribute to many of the structures that are missing after removal of the polar lobe, or ablation of the D macromere. This implies that the D quadrant macromere signals to other cells to induce these fates.

Additional ablation experiments have demonstrated that the D quadrant macromere exerts its effect on the micromeres after the production of the third quartet of micromeres. A critical transition in the phenotypes of D macromere ablations occurs at this time. Ablations of the 3D macromere soon after the production of the third quartet produce larvae similar to earlier D macromere ablations. However, ablations of 3D performed progressively later produce larvae with increasingly more complete sets of larval organs. This indicates that the induction of micromere fates begins during this period. When 3D is ablated shortly before it would divide to produce the fourth quartet micromere 4d, many larvae have a normal organization and full complement of ectodermal organs (Clement, 1962; Labordus and van der Wal, 1986; H. C. Sweet, PhD thesis, University of Texas at Austin, 1996). These results predict that the organizing effect of the D quadrant is mediated by the 3D macromere before the cell divides to produce 4d and that it acts via cell-cell signaling from 3D to the overlying micromeres. The polar lobe can thus be understood to function in part by partitioning substances into the D quadrant which allow the 3D macromere to signal to the micromeres.

The identity of the cells that are induced by 3D can be inferred from existing data. In the absence of the 3D signal, a defined set of structures are missing and micromere ablation studies have demonstrated which cells are required to give rise to these structures (Clement, 1976; see Fig. 1 legend). The micromeres that are required for the differentiation of these structures can thus be inferred to require induction by 3D. Notably, these cells are all in the A, C and D quadrant lineages. Micromeres in the B quadrant, which are furthest from 3D, do not seem to require induction for proper differentiation. While these results indicate which cells are induced by 3D, they do not indicate whether there are several parallel signals to different micromeres from 3D, or a single signal from 3D that is required in several different micromeres.

Taken together, these experiments make several predictions about the molecules that are employed by the D quadrant organizer in *Ilyanassa*. The D macromere inherits molecules from the polar lobe that are required for normal development of cells in several different lineages. These molecules are not active before the production of the third quartet; after this point they enable the D macromere (3D) to signal to several different micromeres. The micromeres that can be inferred to require this signal are in the dorsal and lateral quadrants of the embryo. In the course of examining conserved cell signaling systems in *Ilyanassa*, we discovered that activation of the mitogen-activated protein kinase (MAPK) signal transduction cascade fulfills many of the expectations predicted for a molecular component of the organizer. The MAPK cascade functions in inductive signaling pathways during embryogenesis of many animals (reviewed in Ferrell, 1996; Garrington and Johnson, 1999). It transduces one of several extracellular signals, via sequential phosphorylations, to various targets in a given cell. These targets regulate various cellular behaviors, including the cell cycle and cell fate specification.

We report that MAPK is initially activated in the 3D macromere and subsequently in the dorsal-most micromeres. Activation eventually reaches all micromeres that are known to require the D quadrant induction for normal fate specification. By ablating the 3D macromere, we show that this cell is required for the normal activation of MAPK in the micromeres. Conversely, inhibition of MAPK activation creates larvae phenotypically similar to D quadrant ablations. By inhibiting MAPK activation at successive stages, we demonstrate that the progressive activation of MAPK correlates with a progressive specification of cell fates. Our results enable us to interpret classical cell ablation and lineage tracing studies from a molecular perspective. We propose a model wherein MAPK is activated within specific micromeres upon reception of the signal from 3D and instructs those micromeres to pursue different fates depending on their quartet.

MATERIALS AND METHODS

Snail husbandry

Adult snails were obtained from the Marine Resources Center (Marine Biological Laboratories, Woods Hole, MA) and were fed every other day on frozen clams. Embryo collection was performed as described (Collier, 1981). All experiments were performed with embryos from

at least three capsules. (Each capsule contains a brood of 50-200 synchronously developing zygotes laid by a single female.) For most experiments, embryos from each capsule were staged from the appearance of the third quartet of micromeres. Embryos were reared in small groups at 23°C (±1°C) in 0.2 μm-filtered artificial sea water (FASW, Instant Ocean). For periods longer than about 14 hours, penicillin (100 units/ml) and streptomycin (200 μg/ml) were added, and embryos were moved to fresh FASW every 2 days.

Immunohistochemistry

Embryos were fixed in 3.7% formaldehyde in 90% FASW for 30-35 minutes. Some embryos were dehydrated and stored in methanol at –20°C. Embryos used for phalloidin staining were washed after fixation in PBS+0.1% Tween 20 (PBTw) and stored at 4°C for short periods. For staining with anti-active (double phosphorylated) MAPK monoclonal antibody (Sigma), embryos were blocked with PBTW+2% bovine serum albumin (PBTw+BSA) for 1-4 hours and incubated in antibody diluted in PBTw+BSA for 9-20 hours at 4°C. Embryos stained for dp-MAPK were washed six times over 1-2 hours in PBTw, and the secondary antibody (anti- mouse Cy5, or anti-mouse HRP) was added in PBTw+BSA. After 2-15 hours incubation with secondary antibody, embryos were washed as above, developed (for HRP detection), counterstained and mounted in PBS+80% glycerol+4% n-propyl galate. For phalloidin staining, fixed embryos in PBTw were incubated in phalloidin Alexaflor 568 (Molecular Probes) dissolved in PBTw at 33 nM, for 1 hour, and washed 3 times for 30 minutes in PBTw. For staining with the DNA stain YOYO-1 (Molecular Probes), fixed embryos were treated with DNAse-free RNAse in PBTw at 10 μg/ml for 1.5 hours, washed twice in PBTw, incubated with YOYO-1 in PBTw at 2.5 nM for 30 minutes, washed in PBTw and mounted. DAPI (Molecular Probes) was used at 1 μg/ml. Samples were mounted in PBS+80% glycerol. For embryos which were not stored in methanol, orientation of embryos on the microscope slide was aided by dipping the coverslips in 0.2% poly-L-lysine and drying.

Imaging and analysis

Most assays for MAPK activation were visualized with HRP. In these preparations, cells can be identified by observing nuclei stained with DAPI and cell boundaries that can be seen with DIC optics. To

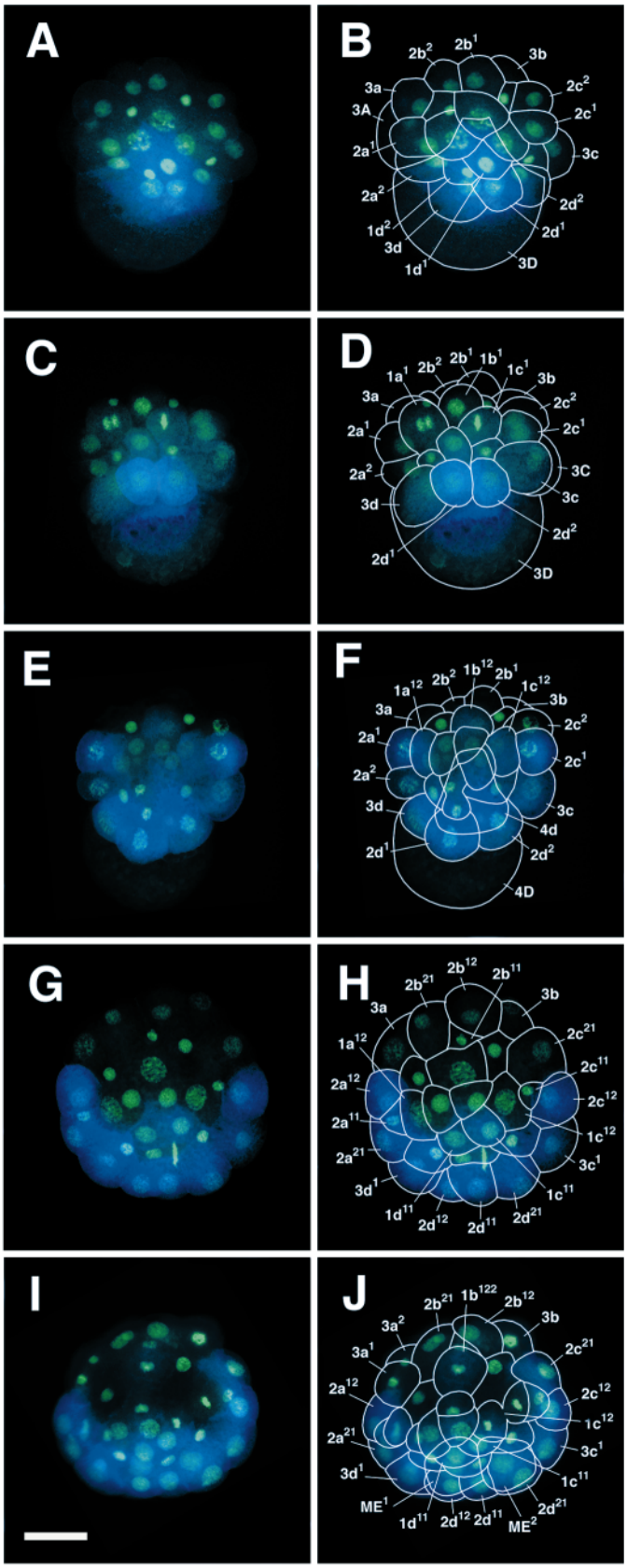

Fig. 2. Pattern of MAPK activation during early *Ilyanassa* development. (Left column) YOYO-1 stained nuclei in green and activated MAPK detected with anti-mouse-Cy5 in blue. (Right column) The corresponding image with cell boundaries and cell identities. (A-F) Dorsal-animal views. (G-J) Animal views. Dorsal (D quadrant) is down in all panels. (A,B) 30 minutes after the production of the third quartet of micromeres (3q+30). The 3D nucleus underlies $2d^1$. Di-phosphorylated, activated MAPK is detected in throughout 3D, with strong signal around the nucleus. Signal in 3D is observed through 1a-d^1, $1d^2$, $2d^1$, $2d^2$ and 3d. Confocal sectioning demonstrated that these cells were not stained. (C,D) 60 minutes after the production of the third quartet (3q+60). Strong MAPK activation is detected in 3D, $2d^1$ and $2d^2$. (E,F) 100 minutes after the production of the third quartet (3q+100), shortly after the birth of the 4d micromere. MAPK activation is observed in $2a^1$, $2a^2$, $2c^1$, $2c^2$, $2d^1$, $2d^2$, 3c and 3d. There is also weak MAPK activation in 1a-c^{12}. In this view, 4d is underneath the micromeres, and the outline of the cell is shown. MAPK activation is observed in 4d but not 4D. (G,H) 150 minutes after the production of the third quartet (3q+150), at the 39 cell stage. Activation is detected in $1c^{11}$, $1d^{11}$, $2a^{12}$, $2a^{21}$, $2c^{12}$, $2d^{11}$, $2d^{12}$, $2d^{21}$, $3c^1$ and $3d^1$. 4d is in metaphase. (I,J) 210 minutes after the production of the third quartet (3q+210), about 2 hours after the birth of 4d. 3a has divided and the chromosomes of 3b are condensed. Activation is detected in $1d^{11}$, $2a^{12}$, $2a^{21}$, $2c^{12}$, $2c^{21}$, $2d^{11}$, $2d^{12}$, $2d^{21}$, $3c^1$ and $3d^1$. Weak activation is detected in the daughter cells of 4d, ME^1 and ME^2 which are underneath the micromeres. $1c^{12}$ is in cytokinesis, and weak activation is detected in $1a^{122}$ and $1b^{122}$. Times are approximate, owing to variation between capsules. Scale bar: 50 μm.

confirm identities of stained cells, to reduce distortion of the embryos because of compression required for conventional photomicrography and to present cell boundaries for publication, the following protocol was followed. Embryos were stained with Cy5 secondary antibody, YOYO-1 nuclear dye and Alexaflor 488-phalloidin as described above. Embryos were observed on a BioRad 1024 confocal microscope. A Z-series was captured for each embryo, and for nuclei and MAPK staining respectively, relevant sections were merged into one image, and then these were pseudocolored and merged together. Cell outlines were drawn on these images by loading all phalloidin sections, in order, into Photoshop 5.0 as individual layers, and each cell was drawn by comparing these sections and tracing cell outlines, revealed by phalloidin labeling, onto the image.

Ablations and MAPK inhibition

Polar lobes were removed by gentle pipetting in artificial sea water containing 1/10 the normal concentration of Ca^{2+} and Mg^{2+} (Collier, 1981). 3D deletions were performed freehand with a glass needle, as described (Clement, 1952). The killed cell was removed from the embryo by gentle pipetting within 10 minutes of deletion. The MAPKK inhibitor U0126 (Promega) was dissolved in DMSO at 10 mM, and diluted in FASw to 10 μM. Controls received the same dilution of DMSO. The drug was used within 7 days of resuspension. For the experiments presented here, embryos were removed from treatments and washed after 12-15 hours. Shorter exposure times produced the same phenotype, and treatments at 3q+180 (180 minutes after the production of the third quartet; Table 1) produced normal larvae, demonstrating that the phenotypes that we describe here are not the result of blocking later MAPK activation. Larvae were all reared for 8 days (unless otherwise noted), anaesthetized as described (Clement and Cather, 1957), and fixed using the same conditions as embryos. Larvae were scored with DIC optics to visualize birefringent materials (shell, statocysts and operculum).

RESULTS

Pattern of activated MAPK antibody expression

Using an antibody that specifically recognizes diphosphorylated, activated MAPK in a variety of eukaryotes (Gabay et al., 1997; Gould and Stephano, 1999; Yung et al., 1997), we have characterized the temporal expression of activated MAPK in individual cells during cleavage stages of the *Ilyanassa* embryo (Fig. 2). No activated MAPK was detected at the 2-16-cell stages, but 10-20 minutes after the production of the third quartet of micromeres, weak activation was observed throughout the 3D cell, with a strong signal in the cytoplasm around the nucleus (Fig. 2A,B). This is the stage when 3D is thought to be signaling to the micromeres.

MAPK activation was next detected in the dorsal-vegetal micromeres which directly overlie the 3D nucleus ($2d^1$ and $2d^2$; Fig. 2C,D). Activation then spread further away from 3D, into more animal and ventral micromeres. After the division of the first quartet cells 1a-c^1, activation was detected in the vegetal daughter cells of $1a^1$ and $1c^1$ (first $1c^{12}$ and then $1a^{12}$) and subsequently in $1b^{12}$ (Fig. 2E,F). Activation was often noticeably stronger in $1c^{12}$ than in $1a^{12}$ or $1b^{12}$. About 90 minutes after the production of the third quartet, 3D divides to produce the 4d micromere. After this division, activation was detected in 4d but activation was no longer detected in the D macromere (4D; Fig. 2G,H). After the birth of 4d, the ventral spread of MAPK activation continued in the micromeres. By 150 minutes after the production of the third quartet, the micromeres with activated MAPK were arranged in an arc that was centered on the presumptive dorsal midline (Fig. 2I,J). The activated cells were derivatives of second and third quartet cells in the A, C and D quadrants. At this stage, no activation was observed in derivatives of 2b, or in the third quartet cells, 3a and 3b.

Subsequent experiments (below) demonstrated that the MAPK activation observed later than 180 minutes after the third quartet was not required for normal patterning. At about 3q+270 (270 minutes after the production of the third quartet), MAPK activation was detected for the first time in micromeres of the B quadrant: $2b^{12}$ and $3b^1$ (data not shown). By the time 3A, B, C are dividing (about 3q+300), activation was lost from $3b^1$ and $2b^{12}$. After this point, activation does not spread to new cells and is undetectable 6 hours after 4d formation (3q+7.5h). Activation was never detected in derivatives of $2b^2$ or 3a, or in the macromeres 3A, 3B or 3C.

Experiments on *Ilyanassa* are performed on snails from wild populations that are not isogenic. In lots of embryos derived from a single mother (i.e. from the same capsule), the temporal and spatial patterns of MAPK activation were very consistent. We did, however observe some variation between lots of embryos laid by different mothers. The main variation in the pattern was in the timing of onset of activation in the micromeres. Activation in 2d derivatives was observed as early as 40 minutes after the production of the third quartet (3q+40), and as late as 3q+90. There was also some variation in the order of activation among the micromeres. Activation was always observed in 2d derivatives first, and activation was usually observed next in $2a^1$ and $2c^1$, but sometimes activation was observed in 3c and 3d earlier than $2a^1$ and $2c^1$. Activation was usually observed in 3c and 3d at the same time, but sometimes 3d was activated first. The variation in the timing of the pattern of MAPK activation is consistent with earlier observations of variation between capsules in the signaling behavior of 3D (Clement, 1962; H. C. Sweet, PhD thesis, University of Texas at Austin, 1996).

The role of the D quadrant in MAPK activation

In order to test whether the observed pattern of MAPK activation is dependent on the D quadrant, we ablated the polar lobe and assayed for MAPK activation. Around 3q+120, when the ventral progression of MAPK activation was underway in controls, no activation was detected in embryos lacking the polar lobe (Fig. 3A,B). Later, around 3q+240, MAPK was weakly activated in a subset of micromeres in each quadrant (Fig. 3C,D). The strongest activation was observed in four equivalent second quartet cells ($2q^{12}$). The pattern is radialized in the sense that the same cells in all four quadrants show activation, but it is not more extensive than the normal pattern, since we did observe activation in the B quadrant in those cells which are activated in polar lobe deletions, i.e. $2b^{12}$. We did not detect activated MAPK in the third order macromeres (3Q). Thus, without D quadrant identity, the normal dorsal-vegetal MAPK activation pattern is weakened, delayed and radialized.

Ablation of the 3D macromere soon after the production of the third quartet prevents the proper differentiation of micromeres in which MAPK is activated. To see if ablation of 3D prevents MAPK activation in these cells, we killed 3D during the 15 minutes following the production of the third quartet.

These embryos were fixed about 3q+120, and scored for MAPK activation (Fig. 3E-F). Among 11 embryos from two capsules, nine had no detectable MAPK activation and two had very weak activation in $1a^{12}$. Thus, the 3D macromere is required for proper temporal and spatial activation of the MAPK pathway in the micromeres, consistent with MAPK activation in the micromeres being the result of a signal emitted by 3D.

Consequences of inhibiting MAPK activation

Since ablation of the polar lobe or the D quadrant macromere prevents the differentiation of micromeres that normally activate MAPK, we wondered if MAPK activation was required for normal differentiation of these cells. To test this, we blocked MAPK signal transduction using the MAPK kinase (MAPKK) inhibitor U0126 (Favata et al., 1998; Gould and Stephano, 1999). U0126 inhibits MAPKK, the kinase that activates MAPK in the conserved MAPK pathway (Ferrell, 1996). In vertebrate systems, UO126 has been found to be specific to this class of kinase – it does not inhibit other, related kinases (Favata et al., 1998). We found that exposure of embryos to U0126 effectively blocked MAPK activation. Blocking MAPK activation resulted in disorganized and incomplete larvae that strongly resemble polar lobe deletion larvae, both in general appearance and in the specific complement of tissues that each class of larvae is able to differentiate.

Activation of MAPK in 3D occurs 10-30 minutes after the production of the third quartet. We incubated the embryos in sea water supplemented with U0126, starting within 15 minutes after the production of the third quartet. We assayed for MAPK activation in these embryos at about 3q+90. When the spread of MAPK activation was underway in controls, no activation was detectable in embryos treated with U0126 (Fig. 4A-D). Other experiments demonstrated that inhibition of MAPK activation with U0126 was very rapid; 5 minutes after treatment, MAPK activity was substantially reduced and 15 minutes after treatment activation was undetectable (data not shown).

To determine the phenotypic consequences of inhibiting MAPK activation, we treated embryos with U0126 within 15 minutes after production of the third quartet, and reared them until the controls were well-developed veliger larvae (7 or 8 days). We examined 54 U0126-treated larvae aged either 7 days (14 larvae, three capsules) or 8 days (40 larvae, eight capsules; a representative larva is shown in Fig. 6C,D). Like polar lobe ablations, U0126-treated embryos developed everted stomodea, multiple patches of esophageal tissue, some endodermal structures, larval muscle and disorganized ciliated bands. With few exceptions, U0126-treated animals lacked the same structures as polar lobe deletion larvae: external shell, eyes, foot structures, intestine, heart and muscle. External birefringent material (shell or operculum) was observed in one, an eye was observed in one, and internal shell material or statocyst was observed in five embryos in three capsules. We did not observe any intestinal structures, and in ten living larvae examined, we did not observe any beating heart. While isolated muscle cells were observed, organized bands of muscle characteristic of the larval retractor muscles were not (Clement, 1952). These results demonstrate that inhibiting MAPK signaling prevents the differentiation of the same set of tissues as polar lobe ablation or early 3D deletion, implying that MAPK activation is required for the embryonic patterning attributed to the D quadrant.

Table 1. Larval structures differentiated after inhibiting MAPK signaling at successive timepoints*

Larval structure	3q+60	3q+100	3q+120	3q+150	3q+180	Control
Esophagus	12/12	14/14	15/15	14/14	15/15	15/15
Stomodeum	12/12	14/14	15/15	14/14	15/15	15/15
Bi-lobed velum	1/12 (C, 1/4)	13/14 (C, 5/6)	15/15	14/14	15/15	15/15
At least one eye‡	1/12 (C, 1/4)§	9/14	15/15	14/14	15/15	15/15
Two eyes	0/12	3/14 (B, 2/4; C, 1/6)	11/15 (A, 4/5; B, 3/5; C, 4/5)	10/14 (A, 2/4; B, 3/5)	14/15 (A, 4/5)	15/15
Internal shell (no external shell)	0/12	3/14 (A, 1/4; B, 2/4)	4/15 (A, 1/5; B, 1/5; C, 2/5)	0/14	0/15	0/15
External shell	0/12	0/14	11/15 (A, 4/5; B, 4/5; C, 3/5)¶	14/14	15/15	15/15
Intestine	0/12	1/14 (B, 1/4)	14/15 (A, 4/5)**	14/14	15/15	15/15
Operculum	0/12	0/14	3/15 (A, 1/5; C, 2/5)	14/14	15/15	15/15
At least one statocyst with statolith	0/12	0/14	2/15 (A, 1/5; C, 1/5)‡‡	14/14	15/15	15/15
Two statocysts with statoliths	0/12	0/14	0/15	9/14 (A, 3/4; B, 3/5; C, 3/5)	15/15	15/15

*For each timepoint, the proportion of larvae with each larval structure is shown. The three capsules used are referred to as A, B and C, and the subtotals for each capsule are shown when applicable.

‡We observed a total of 16 embryos with only one eye. In all cases where it could be determined whether a single eye was the right or the left (13/16 single eyes), it was found to be the right eye.

§The same larva possessed a bi-lobed velum and single eye.

¶Treatment at 3q+120 produced a range of shell shapes and sizes, including small disks, small uncoiled cup-shaped shells, and shells with normal coiling which were also smaller than controls.

**When an intestine was present, it was generally accompanied by a fully developed digestive tract, but we did not score the other organs individually.

‡‡Of the seven larvae with one statocyst/statolith, six were on the left side, one was on the right. We did not observe any statocysts in the absence of statoliths, and vice versa.

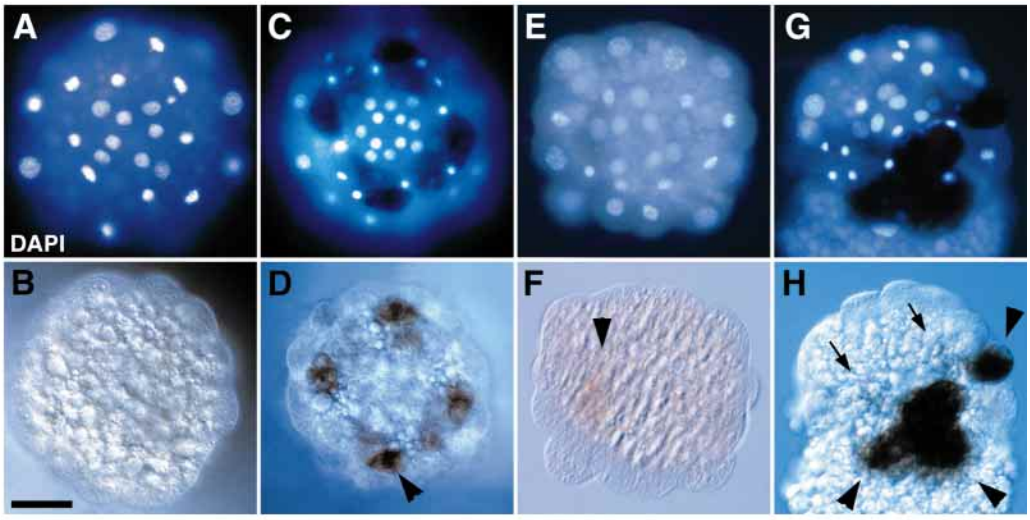

Fig. 3. Perturbation of MAPK activation by ablation of the polar lobe, or ablation of the 3D macromere. (A,C,E,G) Nuclei stained with DAPI. (B,D,F,H) The same mounts in brightfield DIC images showing activated MAPK detected with HRP. All panels are animal views. In E-H, the dorsal quadrant is oriented downwards, and in A-D, polar lobe deletion embryos are oriented arbitrarily, since they do not have a D quadrant. (A,B) An embryo lacking the polar lobe (PL–), at about 3q+120 minutes. Controls were similar to G,H. (C,D) A PL– embryo around 3q+240 minutes. Arrowhead indicates activation in one of the $2q^{12}$ cells. (E,F) For this embryo, 3D was removed less than 15 minutes after the production of the third quartet, and the embryo was fixed at 3q+120. Arrowhead indicates weak activation in $1a^{12}$. (G,H) Control embryo for E,F, fixed at 3q+120 and processed together with experimentals. Activation is detected in 3d (lower left arrowhead), $2d^1$ and $2d^2$, 4d (lower right arrowhead), and $2c^{12}$ (upper right arrowhead). Weak activation is detected in $1a^{12}$ and $1c^{12}$ (arrows). Scale bar: 50 μm.

Development following inhibition of MAPK activation at successive timepoints

The pattern of MAPK activation in the micromeres suggested that there might be a progressive determination of micromere fates. However, the activation observed in the micromeres might not be required for normal fate specification and the phenotype observed above might instead result solely from blocking activation in 3D, thereby preventing signaling from 3D. To examine this, we treated embryos with U0126 at a series of timepoints after the production of the third quartet. Four to six embryos from each of three capsules were treated at 30 minute intervals during the spread of MAPK activation in the micromeres. From each capsule, four to six untreated embryos were fixed at each timepoint and assayed for the state of MAPK activation. Representative embryos from this time course were presented in Fig. 2, to allow comparison with this experiment.

The eight day-old larvae were fixed and scored for the presence of various organs. The results are shown in Table 1 and Fig. 5. Representative larvae from each timepoint are shown in Fig. 6. Larval phenotypes were consistent within sets of embryos treated at given time and these larval phenotypes were reminiscent of larvae that develop after ablation of 3D (Clement, 1962). Treatment at 3q+30 produced the same phenotype as treatment at 3q+0-15 (described above; Fig. 6C,D). Treatment at 3q+60 (not shown) was essentially the same, except that one of 12 larvae had an eye and a bi-lobed velum, rather than the disorganized ciliated bands observed with earlier treatments. Inhibition of MAPK at 3q+100 (Fig. 6E,F) resulted in the formation of two normal velar lobes in almost all larvae, and at least one eye was present in most larvae. These larvae lacked external shell, but internal shell masses were present in some. Treatment at 3q+120 (Fig. 6G,H) produced larvae which uniformly developed at least one eye. Most had external shell, and the remainder had internal shell masses. Almost all had differentiated intestine, but few had foot structures such as operculum or statocysts. In all of these cases, the dorsal mantle edge was behind the right velar lobe. The anus was in the correct (right-dorsal) position with respect to the shell, but was ventral in relation to the head. This unusual configuration suggests a defect in torsion, the dramatic morphogenetic event in gastropods whereby the anterior of the body is twisted counter-clockwise relative to the posterior. Similar defects have been observed after 3c deletion (Clement, 1986b; cf. Figs 3, 4, 7). Treatment at 3q+150 (Fig. 6I,J) resulted in mostly normal larvae which usually had a complete foot. Several were lacking half of the foot and some lacked one

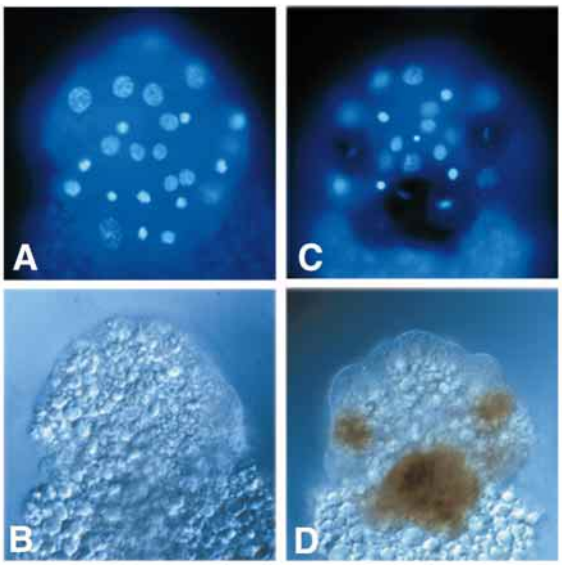

Fig. 4. MAPK expression in U1026-treated embryos. (A,C) Nuclei stained with DAPI. (B,D) Brightfield DIC images of the same mounts showing activated MAPK detected with HRP. Embryos were fixed at about 3q+120. All panels are animal views. (A,B) Embryo treated with U0126 shortly after the production of the third quartet. No MAPK activation is detectable. (C,D) Control embryo with MAPK activation in $2a^1$, 3d, $2d^1$, $2d^2$, 3c, $2c^1$ and 4d.

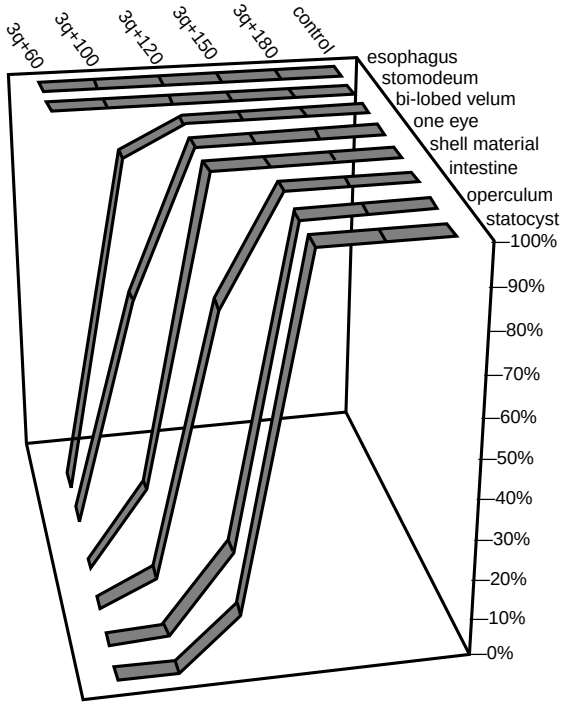

Fig. 5. Progressive acquisition of micromere cell fate following application of U1026. Plot of the frequencies of differentiation of larval organs after inhibition of MAPK activation at successive timepoints. Values from Table 1 were converted to percentages and plotted.

eye. Treatment at 3q+180 (and 3q+210, not shown) produced larvae that were essentially the same as controls. In sum, the differentiation of micromeres improved in a progressive fashion, consistent with a requirement for the observed spread of MAPK activation for micromere differentiation.

When U1026 is applied after activated MAPK is no longer detected in 3D (around 3q+90), micromere fates are nonetheless altered. Thus, the U0126 phenotypes are not simply the result of blocking MAPK activation in 3D and consequently preventing this cell from signaling. MAPK activation is required in the micromeres themselves. This is shown directly for the second and third quartets, since structures descended from these cells – external shell and foot structures – were not differentiated when U0126 was added after 3q+90 (Fig. 6E,F). However, the earliest treatment that allowed eyes to differentiate (3q+100; Fig. 6G,H) coincided with the loss of MAPK activation in 3D. Thus, we cannot distinguish whether the lack of eyes after U0126 treatment reflects a role for MAPK exclusively in 3D or exclusively in

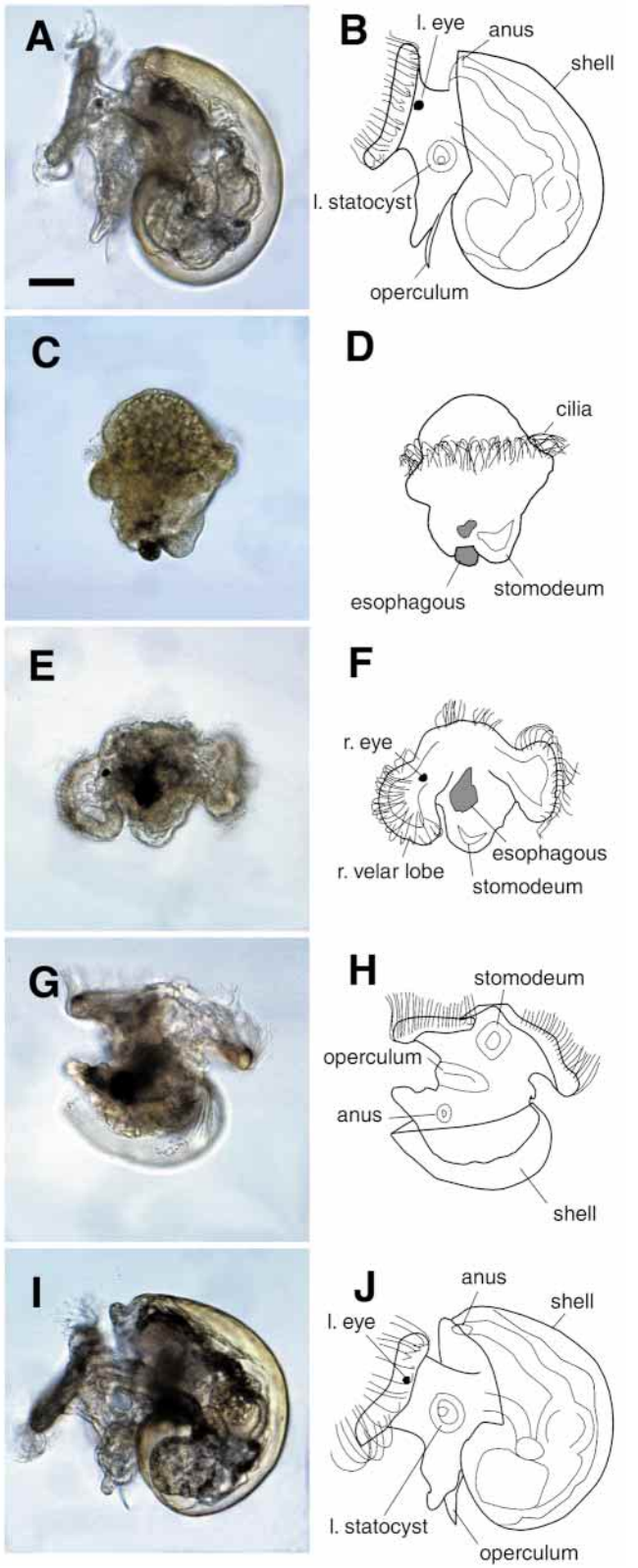

Fig. 6. Eight-day-old larvae treated with the MAPK inhibitor, U1026, at successive timepoints after the production of the third quartet. (A,C,E,G,I) Brightfield images of larvae. (B,D,F,H,J) Labeled drawings of the same larvae. (A,B) Left lateral view of a control eight-day-old larva. Statocysts are paired larval balance organs that contain biomineral granules called statoliths. The operculum is a biomineral plate on the posterior face of the foot. The larval eyes are on the anterior surface of the head. The velum is the ciliated swimming organ that surrounds the head. (C,D) Lateral view of larva treated with U0126 at 3q+15 minutes. (E,F) Anterior view of a larva treated with U0126 at 3q+100 minutes. (G,H) Ventral view of larva treated with U0126 at 3q+120. This larva has two eyes (not shown) but no statocysts or statoliths. (I,J) Left lateral view of a larva treated with U0126 at 3q+150. This animal has a full complement of larval organs. In A, G,I, the mantle has separated from the shell during processing. Scale bar: 50 μm.

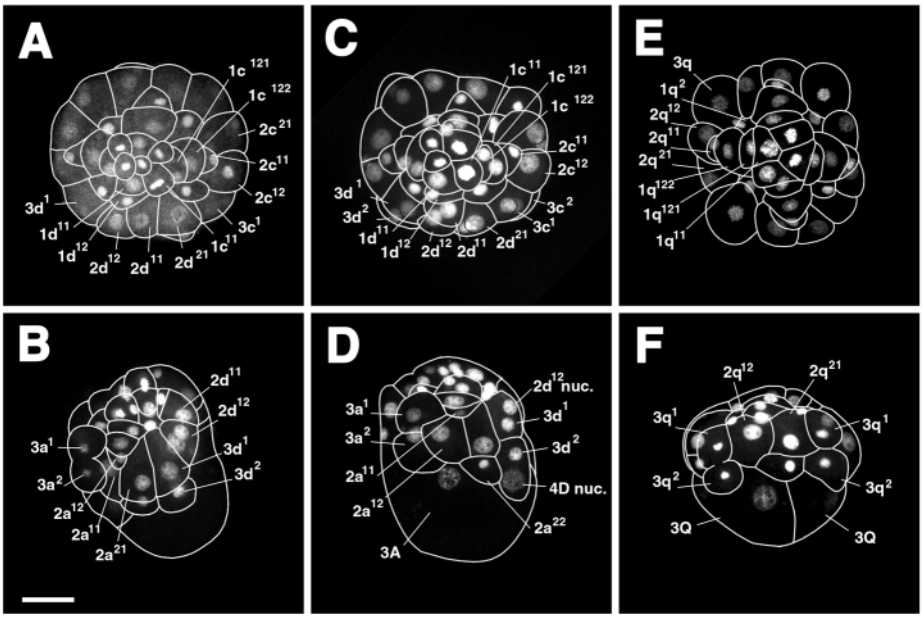

Fig. 7. Diagrams of cell cleavage patterns in U0126-treated embryos, polar lobe deletion embryos and controls. All panels show nuclei stained with YOYO-1, and cell boundaries drawn from optical sections of phalloidin staining. All embryos were fixed at approximately 3q+210 minutes (2 hours after 4d formation in controls). (A) Animal view of control embryo. The micromeres 1a-c^{12}, which were larger than 1d^{12} have divided to produce 1a-c^{121} and 1a-c^{122}. The divisions of 1d and its progeny are delayed compared with 1a-c (Clement, 1952, and data not shown),. 2d^{11} is approximately the same size as 2d^{12} but 2a-c^{11} are much smaller than 2a-c^{12}. (B) Left lateral-animal view of control embryo. 3a is in cytokinesis, and cleaving equally. 3d^{1} is much larger than 3d^{2}. (C) Animal view of U0126-treated embryo. As in controls, the divisions of 1d and its progeny are delayed compared to 1a-c, 1a-c^{12} are larger than 1d^{12}, and 2d^{1} has divided equally. (D) Left-lateral view of U0126 embryo. The 4D nucleus is behind 3d^{2} and the 2d^{12} nucleus is behind 3d^{1}. Unlike controls, 3d^{1} is approximately the same size as 3d^{2}. (E) Animal view of polar lobe deletion embryo. All first quartet cells divide at about the same time, and daughter cells are similarly proportioned in all quadrants. 2q^{1} divides unequally in all four quadrants. (F) Lateral view of polar lobe deletion embryo. The division of 3q cells occurs at same time and produces equally sized daughter cells. Scale bar: 50 μm.

first quartet micromeres, or whether MAPK is required in both macromere and micromeres for eye development. Finally, these results show that U0126 is not preventing normal differentiation by inhibiting MAPK signaling in downstream events, since treatment at 3q+180 resulted in normal patterning (Fig. 6I,J).

Effect of inhibiting MAPK activation on cleavage patterns

Since MAPK plays a role in the regulation of cell division in some systems, we wondered if U0126 treatment was inhibiting differentiation by a general disruption of cell division among the micromeres. About 20 cell divisions occur between the production of the third quartet and the end of the U0126-sensitive period (3q+180). In order to determine whether these cell divisions proceeded normally, we examined cell division patterns in U0126-treated embryos, polar lobe deletion embryos and controls. For each of these classes of embryo, we fixed two or three embryos from each of three capsules, at two arbitrary time points (3q+150 and 3q+210; 44 embryos total). For most micromeres, U0126-treated embryos were identical to controls in the timing of micromere divisions, as well as in the proportions of daughter cells of unequal divisions. The two exceptions are described below. The general congruence between micromere cleavage patterns in the U0126-treated embryos and controls shows that the phenotype observed after inhibition of MAPK signaling is not a simple consequence of a global disruption of the cell cycle in micromeres.

The most significant difference between U0126-treated embryos and controls was in the third quartet micromeres. In normal embryos, the division of the 3rd quartet micromeres 3c and 3d is different from 3a and 3b. About 1 hour after the production of 4d, 3c and 3d divide unequally, producing a much smaller daughter cell towards the vegetal pole (Fig. 7A,B). 3a and 3b divide equally about an hour later. After either polar lobe deletion or U0126 treatment, all four third quartet cells (3a, 3b, 3c, 3d) divide equally, at the time when 3a and 3b are dividing in controls (Fig. 7C,D). The difference in cleavage time and proportion between dorsal (3c and 3d) and ventral (3a and 3b) third quartet cells may be an early diagnostic of differential cell fate specification by MAPK activation.

In normal embryos, the 3D macromere divides to produce 4d 3.5 to 4 hours before 3ABC divide to produce 4abc. About 1 hour after its birth, 4d divides to produce two equal daughter cells. In embryos lacking the polar lobe, the fourth quartet is produced synchronously, at about the same time as 4a-c are produced in normal embryos (Fig. 7E,F; Clement, 1952). In U0126 embryos, the birth of 4d was delayed compared with controls, but 4d was still born before 4abc. In embryos fixed at 3q+210, there was also a delay in the division of 4d, such that in U0126-treated embryos, 4d was in metaphase when cytokinesis was complete in controls (4d normally divides at about 3q+160). Thus, the behavior of 4d in U0126-treated embryos is intermediate between normal embryos and embryos lacking the polar lobe: without activated MAPK, 4d divides later than in controls, but before the fourth quartet is produced in embryos lacking the polar lobe. This suggests that activated MAPK is partly responsible for the early division of 3D to produce 4d, but other signals or lineage-dependent mechanisms are also involved. The difference in cleavage of 4d observed after treatment with U0126 may reflect disruption of fate specification of this cell, since the tissues normally produced by 4d, such as intestine and heart, are not produced after U0126 treatment.

DISCUSSION

In *Ilyanassa*, the dorsal-ventral axis of the embryo is specified at the four-cell stage, when one blastomere inherits material from the polar lobe. This event itself, however, is not sufficient for normal axial patterning of the embryo. The polar lobe mainly functions later, by enabling the D quadrant macromere to signal to the micromeres after the production of the third quartet. The source of this signal – the 3D macromere – has been characterized as an embryonic organizer, because it induces multiple cell fates and is required for the normal axial organization of the larva. We have identified the first known molecular component of this signaling event. Using an antibody that recognizes phosphorylated, activated MAPK, we have found that MAPK is differentially activated in the *Ilyanassa* embryo at the stage when 3D is signaling to the micromeres. MAPK activation is initially detected in 3D; shortly afterwards, activation is detected among the dorsal, vegetal micromeres. Among the micromeres, activation then spreads from dorsal-vegetal cells that directly overlie 3D, to more ventrally located cells. The sequence of activation intuitively suggests the spread of a diffusible signal from 3D.

If MAPK activation in the micromeres is a result of the inductive signal from 3D, then the normal pattern of activation should not be observed when the signal does not reach the target cells. We showed this in two ways. Ablation of the source of the signal, the 3D macromere, prevents the normal activation of MAPK in the micromeres. Moreover, ablation of the polar lobe, which prevents the specification of the entire D quadrant, results in a delayed and radial pattern of MAPK activation. The simplest explanation of these data is that MAPK activation in the micromeres is downstream of the inductive signal from 3D.

At the outset of these experiments, there was no indication as to whether 3D was sending several parallel signals to different micromeres, or sending one signal to several different target cells. If a cell gives rise to a structure that does not differentiate without a signal from 3D, and if that cell is required for normal development of that structure, then we infer that the cell requires an induction from 3D for normal fate specification. We found that MAPK is ultimately activated in all the cells for which an inductive influence of 3D can be thus inferred. Thus, this signaling cascade could be the critical inductive signal in all of the target cells of 3D induction.

Preventing the induction of the micromeres by ablation of the polar lobe or a D quadrant macromere has a well-defined larval phenotype. These animals have disorganized velum, esophagus and stomodeum, but lack eyes, foot structures and external shell. The phenotype obtained when MAPK signaling is inhibited at its onset precisely mimics D macromere ablations, in the general appearance of the larvae, as well as in the complement of tissues that are differentiated. This demonstrates that MAPK activation is required for establishment of all of the fates that require D quadrant induction.

MAPK activation is observed in all cells that require the induction from 3D, and all of the tissues that are lacking in D macromere deletions are missing when MAPK activation is inhibited. These results suggest that MAPK activation is required in the micromeres for normal fate specification. However, an alternative explanation remained, that the phenotype obtained when MAPK was inhibited was a result of inactivating MAPK in 3D and thereby blocking signaling. To distinguish between these possibilities, we blocked MAPK activation at successive timepoints. Blocking MAPK activation at timepoints when there was no MAPK activation in the D macromere (3q+100 to 3q+150) still blocked differentiation of micromeres, demonstrating that the effects of inhibiting MAPK are not solely the result of perturbing 3D. Rather, the micromeres are specified progressively during the spread of activated MAPK, indicating that the MAPK activation observed in the micromeres is required for the proper fate specification of these cells. In this experiment, we recovered the same series of larval phenotypes that is produced by ablation of 3D at successive stages (Clement, 1962; H. C. Sweet, PhD thesis, University of Texas at Austin, 1996). This suggests that later 3D deletions result in better patterning because a longer period of contact between 3D and the micromeres allows sufficient signal to be secreted for the mature pattern of MAPK activation to develop in the micromeres.

Model for the specification of multiple fates by MAPK

Our experiments suggest that the same response – MAPK activation – elicits different cell fates in different micromeres. Since equivalent levels of MAPK activation are observed in cells with different fates, we rule out a morphogen model. Instead, we propose that MAPK activation serves as a trigger to allow cells to follow different fates in different quartets based on competencies established prior to induction by 3D. Within a quartet the presence or absence of MAPK activation can, with few exceptions, predict micromere fate. The exceptions are those few cells which express MAPK, but do require the 3D signal. These exceptions implicate additional signals or alternative mechanisms, described below, in the patterning of all micromere fates.

Several lines of evidence suggest that the micromere quartets have different developmental potencies before the induction by 3D. First, the cell lineage of *Ilyanassa* shows that the micromeres within a quartet share cell cleavage programs that are more similar to each other than they are to micromeres in other quartets, and this is even true without induction from 3D (Clement, 1952; Crampton, 1896). Also, the set of fates adopted by cells in one quartet is different from the set of fates adopted by cells in other quartets. For instance, cells of the second quartet generally contribute to shell, velum or stomodeum, while cells in the third quartet generally contribute to foot structures, velum or esophagus (Render, 1997). Quartet-specific cleavage patterns and fates are both consistent with the existence of quartet-specific identities. However, the most convincing evidence for a quartet-specific pre-pattern comes from an elegant series of cell transplantation experiments (Sweet, 1998). Sweet's primary discovery was that all cells in the first quartet are equally competent to form eyes after induction by 3D, but that 1b was prevented from doing so by its position in the embryo, and that 1d was insensitive to induction because of substances in the polar lobe. However, she also found that the ability to form eyes after induction was specific to the first quartet; when transplanted into the position of a first quartet cell, second quartet cells formed shell material after induction, rather than eye. Sweet thus showed the signal from 3D induces different fates in different quartets, regardless

of position in the embryo. Rigorous understanding of the patterns of competency among the micromeres will only come after Sweet's experiments are repeated with the second and third quartets. However, her transplantation data, combined with the similarities of cleavage patterns and fates within each quartet, strongly argue that micromeres acquire quartet-specific identity at, or soon after their birth, and that the compentency of D-quadrant cells can be further modified. If there are pre-existing differences among the micromeres in the response to induction, then a single signal, transduced by MAPK activation, could specify several different fates among different quartets.

We propose that cells of each quartet share the potential to follow two different developmental pathways: a default developmental pathway, which includes fates that do not depend on the signal from 3D for differentiation, and an inducible pathway, which includes fates that depend on the 3D induction. Since the quartets are arranged in tiers along the animal-vegetal axis, this quartet specific pre-patterning represents differentiation along the primary axis of the embryo. The signal from the dorsal macromere 3D reaches the dorsal and lateral micromeres and activates MAPK in those cells. This selects for the inducible quartet-specific pathway in those cells. Since they lack the appropriate MAPK activation, the ventral cells carry out the default quartet-specific program. In this way, the signal from 3D integrates anterior-posterior patterning with positional information along the dorsal-ventral axis, and thus spatially organizes the micromere fates.

In our model, the cells in a quartet initially have equivalent fates and MAPK activation selects for an inducible pathway among dorsal and lateral cells. We have direct evidence that MAPK activation is required in second and third quartet micromeres, since inhibition of MAPK activation at time points after the end of MAPK activation in 3D disrupts development of tissues descended from these cells. We propose that MAPK activation in the second quartet derivatives specifies shell production. All second quartet cells contribute, in varying degrees, to mantle, which secretes shell. Despite this, deletion of 2b has no effect on shell development, while deletions of 2a, 2c or 2d result in shell defects (Clement, 1986a). The different requirements of second quartet cells for shell development suggests that 2a, 2c and 2d are specified differently from 2b. We propose that MAPK activation in the 2a, 2c and 2d directs these cells to play a primary role in shell development. The development of external shell is complex, and probably requires further interactions between these shell-producing cells after induction by 3D (McCain, 1992), as well as D quadrant-specific properties of 2d.

In the case of the third quartet, the two dorsal micromeres, 3c and 3d, give rise to foot structures, while the ventral cells, 3a and 3b, produce esophagus (Render, 1997). In our model, the esophageal contribution made by the ventral cells is the default pathway for third quartet cells and the MAPK activation observed in 3c and 3d induces these cells to produce foot structures. This model is supported by the observation that U0126-treated embryos often have several patches of esophageal tissue, consistent with an over-abundance of esophageal precursor cells.

In the first quartet, we hypothesize that the MAPK activation observed in the lateral cells $1a^{12}$ and $1c^{12}$ directs them to produce eyes. However, since the MAPK activation in 3D overlaps with the MAPK activation in these cells, our experiments do not directly show that MAPK activation in these cells is required for differentiation. It is possible that there is another signal from 3D that specifies differential fates among the first quartet micromeres and that this signal is not sent when MAPK activation is blocked in 3D, resulting in a lack of eye specification. Two additional observations, however, suggest that MAPK activation in $1a^{12}$ and $1c^{12}$ is required for eye differentiation. Activation is observed in $1c^{12}$ before $1a^{12}$, and activation is usually noticeably stronger in $1c^{12}$. Accordingly, when U0126 treatment produces larvae with one eye, it is always the right eye that is produced, which develops from 1c. Also, when one eye develops after 3D deletion, it is usually the left, which develops from 1a (Clement, 1962). When we deleted 3D, $1a^{12}$ was the only cell in which MAPK was activated. These experimental correlations between eye differentiation and MAPK activation in $1a^{12}$ and $1c^{12}$ argue that MAPK activation may be required for eye fate.

In general, there is good correspondence between the set of micromeres that require induction by 3D, and the set in which MAPK is activated. However, there are a few cells in which MAPK activation is observed, but which follow ventral fates that do not require induction. For instance, in the case of the third quartet, activation is detected in the ventral cell $3b^{1}$, well after activation is detected in 3c and 3d. Why is this cell not induced to form foot structures? The fact that MAPK activation is observed in this cell and additional ventral cells (e.g. $1b^{12}$ and $2b^{12}$) seems inconsistent with our model. However, activation is always observed in these ventral cells later than in the dorsal and lateral cells of a given quartet, so it is possible that this difference in timing could prevent MAPK activation from affecting ventral cells. This could happen in at least two ways. The effects of activated MAPK might be limited in ventral cells by additional signals from dorsal and lateral cells once they are specified. For instance, we suggest that MAPK activation in $1a^{12}$ and $1c^{12}$ specifies eye fates in these cells, but activation is subsequently observed in $1b^{12}$, which does not produce an eye, but is competent to do so if it receives the inductive signal from 3D (Sweet, 1998). The lateral cells may signal to $1b^{12}$ after they are specified and inhibit the ability of this cell to form an eye. The existence of such an inhibitory mechanism is supported by the results of experiments with the 'half-embryos' that only contain cells of the B and D quadrants (McCain and Cather, 1989; Sweet, 1998). In these cases, 1b and 1d are able produce eyes, and this may reflect the absence of a normal inhibitory signal from 1a and 1c derivatives.

Another way that the effects of late MAPK activation could be suppressed is by a restricted window of sensitivity. For example, the MAPK activation in $3b^{1}$ is after the division of 3a and 3b. We found that the division of 3a and 3b differs from the division of 3c and 3d, in timing and the relative sizes of daughter cells, and that the all third quartet cells follow the ventral cleavage pattern in polar lobe deletion embryos. This suggests that the third quartet cells are already specified by the time of their first division. Hence, the division of 3a and 3b may mark the end of a window of sensitivity, after which MAPK activation has no effect on their fate specification.

Activation of MAPK in the 3D macromere

The observation that MAPK is initially activated in 3D was surprising, since this cell is thought to be sending a signal, not receiving one. We do not know whether MAPK activation in

3D is required for this cell to signal to the micromeres. However, we have shown that MAPK activation is required for a different behavior of 3D, its division to produce 4d at the appropriate time. Since MAPK activation is observed in 3D very soon after the production of the third quartet, MAPK activation in this cell could be required for signaling to the micromeres. In some other molluscs, the D quadrant is specified after the production of the third quartet, by signaling from the micromeres to the presumptive 3D macromere (van den Biggelaar and Guerrier, 1979; Martindale et al, 1985). It will be interesting to learn if this signaling event is associated with the activation of MAPK in 3D in these other embryos.

In *Ilyanassa*, the activation of MAPK in 3D is dependent on the polar lobe, whose contents are inherited by the D macromere. Unlike the case of MAPK activation in the micromeres, which seems to be a direct consequence of signaling from 3D, MAPK may be activated in 3D in a cell-autonomous fashion by molecules inherited from the polar lobe. For many years, polar lobe determinants were sought as differentially expressed mRNAs or proteins (reviewed in Collier, 1983). Activated MAPK may not have been easily identified in these screens, since MAPK transcript and protein might be present in similar amounts in embryos lacking the polar lobe versus controls.

MAPK activation after removal of the polar lobe

We observed radial MAPK activation in polar lobe deletion embryos at 3q+210. If the normal pattern of MAPK activation is a consequence of a signal from 3D and the D quadrant is not specified after the polar lobe is removed, then why is MAPK activation observed after polar lobe ablation? We do not know whether these cells autonomously activate MAPK at this stage or if activation is a result of signaling by other cells. Cather (1967) provided evidence that the internal shell masses that are common after polar lobe deletion are a result of signaling from the macromeres to the micromeres, even though 3D is not specified after polar lobe ablation. The MAPK activation observed after polar lobe ablation may be a result of such signaling. Consistent with MAPK transducing this signal, U0126 treatment results in a much lower frequency of internal shell material (6/54 or 11%, conservatively assuming all of the internal birefringent masses were shell material) than polar lobe deletion (60%; Atkinson, 1971).

MAPK activation in 4d

The 4d micromere, or mesentoblast, gives rise to mesodermal and endodermal organs such as muscle, heart, kidney and intestine. It is born after the initiation of signaling by 3D, and deletions of the D macromere after its birth (4D) have no effect on development (Clement, 1962). These results suggest that it may be specified by a lineage-dependent mechanism. We observed that MAPK activation in 3D was concentrated in the animal part of the cell which contains the yolk-free cytoplasm that will be inherited by 4d. After the division of 3D to produce 4d and 4D, MAPK activation is only detected in 4d. This is consistent with activated MAPK being segregated into 4d at its birth. Our results also suggest that activated MAPK in 4d is required for normal fate specification; one tissue produced by this cell, intestine, is absent when U0126 is added right after the birth of 4d (3q+100), but does differentiate when MAPK activation is allowed to persist longer (until 3q+120).

Nature of the signal transduced from 3D to the micromeres

Our results are consistent with a single signal emanating from 3D to the micromeres to specify cell fate. Sweet analyzed the cell contacts in the *Ilyanassa* embryo during induction of the micromeres by 3D (H. C. Sweet, PhD thesis, University of Texas at Austin, 1996). Some cells that activate MAPK were found to contact 3D, e.g. first quartet derivatives, 2d derivatives, 3c, and 3d. Others, such as the 2a and 2c derivatives were not found in contact with 3D. This suggests that the signal is a secreted protein that can diffuse short distances in the extracellular matrix. The FGF and EGF class ligands are obvious candidates for this role, because they are known to signal through the MAPK cascade in other systems. Indeed, during early embryogenesis in *Drosophila* and *Xenopus*, all detectable MAPK activation is associated with EGF or FGF signaling (Christen and Slack, 1999; Gabay et al., 1997). EGF and FGF ligands bind receptor tyrosine kinases, which activate the MAPK pathway in a Ras-dependent fashion. Therefore, a proposed role for these ligands is contradicted by the observation that an antibody against a human Ras protein does not recognize an epitope during this stage in *Ilyanassa* (Yan and Collier, 1993). Resolution of this question awaits further analysis of the components that activate MAPK signaling in *Ilyanassa.*

The evolution of spiralian development

In the current view of metazoan phylogeny, there are three clades of bilaterian phyla, the deuterostomes, ecdysozoans and lophotrochozoans (Aguinaldo et al., 1997; Halanych et al., 1995). Since very little is known about the molecular events that pattern lophotrochozoan embryos, most comparisons of the molecular basis of embryonic patterning have focused on the deuterostomes and ecdysozoans. Embryos of at least six lophotrochozoan phyla display the patterns of cell division and cell fate that characterize spiralian development; these are the annelids, platyhelminths, echiurans, nemerteans, sipunculans and molluscs (Aguinaldo et al., 1997; Boyer et al., 1998; Halanych et al., 1995; Henry and Martindale, 1998; Newby, 1932; Rice, 1967; Wilson, 1892). The conserved patterns of cell division and fate maps in these spiralian embryos may reflect some conservation in the mechanisms of patterning in these taxa. Indeed limited experimental data suggest that the role of the D quadrant in embryonic organization is conserved among some embryos (Damen and Dictus, 1996; Dorresteijn et al., 1987; Henry and Martindale, 1987; Martindale, 1986; Render, 1983).

Nevertheless, despite the apparent conservation of the function of the D quadrant, modifications in the cellular mechanisms by which the D quadrant is specified have appeared in spiralian lineages (Boyer and Henry, 1998; Freeman and Lundelius, 1992). Because the antibody that recognizes activated MAPK in *Ilyanassa* is likely to recognize activated MAPK in other spiralian embryos (Gould and Stephano, 1999), we can now begin to examine the extent to which the similarity in cell cleavage patterns and fate maps in spiralian embryos reflect conserved patterning mechanisms.

We wish to thank the following colleagues for comments on the manuscript, D. Brower, S. Ward, M. Goulding, E. Jockusch, T. Williams, M. Martindale, E. Seaver, J. van den Biggelaar, J. Wandelt

and E. Wilk. We would especially like to thank M. Gibson and N. Tax for helpful discussions throughout the course of this work. We acknowledge J. Wandelt for the initial observation of the 3D cleavage delay. J. D. L. was supported by an NSF Graduate Research Fellowship, and an NSF Doctoral Dissertation Improvement Grant (IBN-9902278). L. M. N. was supported by the Alfred P. Sloan Foundation.

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