Protein Tyrosine Kinase Activity Following Fertilization Is Required to Complete Gastrulation, but Not for Initial Differentiation of Endoderm and Mesoderm in the Sea Urchin Embryo

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The egg activation process functions to implement developmental programs that act much later in embryogenesis. One example of this is the fact that application of protein tyrosine kinase inhibitors to the fertilized sea urchin egg for a 15-min period results in a defect in the gastrulation process occurring over 24 h later (Kinsey, W. H., Dev. Biol. 172, 704–707, 1995). In the present study, we show that the window of sensitivity is not due to differential uptake of inhibitor, and establish that the inhibitor inhibits tyrosine kinase activity at the time of application. We also demonstrate that inhibition of protein tyrosine kinase activity in the zygote causes a specific defect in the morphogenetic movements associated with gastrulation without interfering with the initial specification and differentiation of endoderm and mesoderm. Differentiation events occurring concurrent with or subsequent to gastrulation were also suppressed in embryos derived from treated zygotes. These findings indicate that fertilization initiates a signaling cascade involving protein tyrosine kinase activity that is required specifically for events at gastrulation. This signaling event is required to complete the developmental program of both endoderm and mesoderm, but is different from those events necessary for initial specification of endodermal and mesodermal cell fate.

INTRODUCTION

Egg activation entails a series of sequential biochemical steps that trigger the block to polyspermy and activate egg metabolism and cause the egg to reenter the cell cycle. In addition, egg activation initiates the "developmental program" (Epel, 1989) which directs the zygote to give rise to the different cell types found in later embryos and to undergo the morphogenetic movements that result in the final body plan of the animal. A model for how this occurs in sea urchins (Davidson, 1989) proposes that a series of cell-cell signaling events initiated by the micromeres at the vegetal pole of the 16-cell embryo modifies the activity of maternal transcription factors, leading to the activation of tissue-specific sets of genes in the five embryonic territories. However, there is also evidence that developmental information is present as early as the 1-cell stage. The oral/aboral axis is specified by the first cleavage (Cameron et al., 1989), and there are asymmetrically localized maternal components in the egg (DiCarlo et al., 1994, Vlahou et al., 1996). The signal transduction events that activate the egg's developmental program are not known, but in a recent study, evidence was presented that one or more fertilization-activated protein tyrosine kinases (PTK) were important in activating development (Kinsey, 1995). In that study, treatment of fertilized zygotes with various PTK inhibitors during a limited 30 min period (between 30 and 60 min postfertilization) caused effects on embryonic development that did not become apparent until gastrulation, over 24 h later. Identical treatments before or after that "window" of sensitivity had no effect and we have termed this sensitive period the "gastrulation-sensitive window" (GSW). Although the effect was specific for inhibitors of tyrosine kinases, it was not directly established that the

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2 Abbreviations used: PTK, protein tyrosine kinase; GSW, gastrulation-sensitive window; GSW embryos, embryos derived from zygotes treated with tyrosine kinase inhibitors; DMSO, dimethyl sulfoxide; PMCs, primary mesenchyme cells.
inhibitors had an effect on embryonic PTKs. Neither was it clear whether the GSW was due to inhibition of kinase activity at a specific time, or due to differences in the ability of the zygote to take up inhibitors at different times. We report here that the inhibitor genistein is taken up equally well by zygotes at all times administered and that it effectively inhibits endogenous PTKs at the time of application.

The morphology of embryos derived from the inhibitor-treated zygotes (GSW embryos) resembled a mesenchyme blastula in that a group of cells had migrated into the blastocoel and remained at one pole of the embryo. This rather ambiguous morphology left open several important questions regarding the specific nature of the defect. For example, it was not clear whether a true vegetal pole was established, whether the cells in the blastocoel were primary mesenchyme cells (PMCs) or endoderm and whether any mesoderm or endoderm had formed at all. In short, it was not clear whether a defect had occurred in cell differentiation or only in morphogenesis.

In the present study, we have examined the hypothesis that PTK inhibition during the GSW allowed the primary mesenchyme to differentiate, but prevented normal differentiation of the endoderm and formation of signals necessary for spicule formation. We have used cDNAs homologous to tissue-specific mRNAs and antibodies to tissue-specific proteins or glycoproteins as probes to ascertain whether endoderm and mesoderm are formed in embryos derived from treated eggs. The results clearly demonstrate that the GSW defect was not at the level of initial specification and differentiation of mesoderm or endoderm. The GSW defect did result in the inhibition of the cell movements associated with gastrulation and with subsequent differentiation of mesoderm and endoderm.

The mechanism by which PTK activity in the fertilized zygote can affect subsequent morphogenesis much later in development is not clear, but it is obvious that defects in the egg activation process can result in highly specific developmental defects in the gastrula stage embryo.

MATERIALS AND METHODS

Culture and Treatment of Embryos

Gametes of Strongylocentrotus purpuratus were obtained and fertilized by standard methods (Hinegardner, 1967, Hall, 1978, Lutz and Inoue, 1986). One-celled embryos were treated for 25 min with 25 μM of the PTK inhibitor genistein (Calbiochem) in DM20 20 min after fertilization as described by Kinsey (1995). Control embryos were treated with DM20 alone. The inhibitor was then washed out with artificial seawater. The development of the embryos was monitored by light microscopy.

Uptake of Genistein by Zygotes

Iodination of genistein was carried out in 50 mM Tris containing 100 μCi of 125I (Amersham) and catalyzed with Iodobeads (Pharmacia). The reaction was started by addition of genistein to a concentration of 1 mM and was carried out at 25°C for 30 min. The reaction products were fractionated on a C-18 Sep Pak cartridge (Waters) to remove the unincorporated 125I. The 125I was eluted with 80% acetonitrile and used immediately for uptake studies. Purification of genistein on Sep Pak cartridges was monitored by HPLC. For this experiment, 70,000 cpm of labeled genistein was added to 500 μl of a 2% culture of eggs in artificial seawater before fertilization and at 15-min intervals following fertilization. Each time point was incubated for 15 min following addition of genistein and then centrifuged 30 s at 3000g. The supernatant was removed, the embryos were resuspended in 200 μl artificial seawater and recentrifuged, and the supernatant was removed. The pelleted embryos were counted directly in a Beckmann Gamma 5500.

PTK Assay

Samples of eggs or embryos (50 μg protein) were pelleted in a microfuge, suspended in NP-40 lysis buffer (50 mM Tris, pH 7.4, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 1 mM Na3VO4, 5 mM NaF and 10 μg/ml aprotonin (U.S. Biochemical)). After centrifugation at 70,000g for 15 min, the detergent-soluble material was assayed for PTK activity as described by Moore and Kinsey (1994) using a synthetic peptide substrate.

Northern Blot Analysis

RNA was isolated from prism gastrula using methods described by Childs et al. (1979). RNA blotting was carried out as described by Benson et al. (1987) using 15 μg of total RNA/sample well. 32P-labeled random-primed DNA probes were prepared using a Pharmacia Ready-to-Go kit (Pharmacia). Hybridization was as described by Pharmacia. The PMC-specific cDNA clone, PH572, was isolated by Benson et al. (1987). The PMC-specific clone, SM30 was isolated by George et al. (1991). The endoderm-specific cDNA, Endo16, was isolated by Nocente-McGrath et al. (1989). A probe to the 16S mitochondrial ribosomal RNA was used to normalize loading of RNA in the sample wells. After hybridization, bands were visualized using autoradiography. Three different batches of embryos were used in these experiments.

Immunoblotting

Electrophoresis was carried out in 10% acrylamide gels as described by Laemmli (1970) and modified by Dreyfus et al. (1984). Immunoblotting was carried out as described by Towbin et al. (1979), with modifications by Johnson et al. (1984). Detection of antibody bound to proteins on the nitrocellulose was carried out using a chemiluminescent detection system as described by the manufacturer (Amersham). The rabbit anti-SM 50 polyclonal antibody was provided by F. Wilt (University of California, Berkeley). The goat polyclonal anti-Endo16 antibody was provided by S. Ernst (Tufts University). Both antibodies were used as a 1/1000 dilution of whole serum in PBS. Three different batches of embryos were used in these experiments.

Antibody Staining of Embryos

Fixation of embryos was carried out as described by Ettensohn and McClay (1988). Antibody staining was performed as described by Ettensohn and McClay (1988) and modified by Livingston and Wilt (1990). Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were used to visualize the location of the primary
antibody. A Zeiss LSM4 confocal microscope was used to record the staining pattern. The 1- to 2-μm sections were taken using either fluorescence alone or fluorescence and Nomarski optics simultaneously. Five different batches of embryos were used in these experiments; 100–200 embryos/batch were stained with each antibody. SM 50 and SM 30 were used at a 1/500 dilution of whole serum in PBS. 4C12 was used at a 1/100 dilution of ascites fluid in PBS. Endo1 was used at a 1:1 dilution of ascites fluid in PBS. The mouse monoclonal 4C12 antibody was provided by M. C. Lane (University of California, Santa Barbara). The mouse monoclonal antibody Endo1 was provided by D. McClay (Duke University).

RESULTS

Genistein Treatment

Embryos were treated for 25 min with 25 μM genistein between 20 and 45 min after fertilization. As reported previously, this treatment had no effect until 22–24 h later at the start of gastrulation (Kinsey, 1995). While control embryos underwent gastrulation, the GSW embryos had some cells ingress into the blastocoel, but in the majority of treated embryos the vegetal plate did not undergo invagination and guts were not formed (Fig. 1). The ingressed cells in GSW embryos did not form spicules. While these effects were observed consistently in all batches of embryos treated with genistein, occasionally treatment produced batches of embryos of which 10–20% exhibited some level of gut formation. Often what was seen was small aggregates of columnar cells with a lumen at their center. These small aggregates were not connected to a blastopore and were formed by cell migration and association rather than invagination. Spicules were seen at even lower frequencies. The low level of guts formed in GSW embryos was observed with all PTK inhibitors tested (Kinsey, 1995).

Uptake of Genistein by Embryos and Inhibition of PTK Activity

The uptake of 125I-labeled genistein was significantly higher following fertilization (P < 0.001) than that in unfertilized eggs (Fig. 2). However, following fertilization there was no difference in the ability of zygotes to take up genistein from seawater at any time during the first 75 min of development. The genistein treatment resulted in some inhibition of tyrosine kinase activity at each period tested (Fig. 2). Measurement of tyrosine kinase activity in zygote extracts did not reveal significant differences in the level of inhibition of endogenous kinase activity when genistein was added at different times following fertilization. It is clear that 25 μM genistein does not completely inhibit all tyrosine kinase activity in the zygote. However, when higher concentrations were used, subsequent development was irregular and a large proportion of embryos did not survive; therefore 25 μM genistein was chosen for use.

Expression of Tissue-Specific RNAs

In order to assess whether genistein treatment abolished cell type-specification, we assayed the accumulation of tissue-specific mRNAs. RNA was isolated from control and GSW embryos when control embryos reached the prism gastrula stage and the level of various RNAs was determined by Northern blot analysis (Fig. 3). The levels of a primary mesenchyme-specific mRNA, SM 50 (Benson et al., 1987), and an endoderm-specific marker, Endo16 (Nocente-McGrath et al., 1989), were unchanged in GSW embryos relative to controls. Both of these transcripts are known to appear prior to gastrulation already localized to their respective tissues. Another primary mesenchyme-specific mRNA, SM 30 (George et al., 1991), was not detected in GSW embryos. This mRNA appears later in development than the others, and may require specific cell–cell or cell–extracellular matrix interactions during gastrulation to be expressed (F. Wilt, personal communication).

Appearance of Endoderm- and Mesenchyme-Specific Proteins

Control and GSW embryos were homogenized at the gastrula stage in SDS–PAGE sample buffer and analyzed by Western blots. Antibodies specific to SM 50 and Endo16 were used on separate blots to detect the presence of these proteins. Figure 4 shows that there is no difference in the level of these tissue-specific proteins accumulating in the GSW embryos relative to the control embryos. We can conclude that normal transcription and translation of early markers of mesoderm and endoderm differentiation occur in GSW embryos.

Localization of PMC-Specific Proteins

GSW embryos arrest at a stage equivalent to control mesenchyme blastula and resemble control embryos at this stage. A major question, however, is whether the cells in the interior of the GSW embryos had in fact differentiated into primary mesenchyme cells. To address this question, we have used antibodies to three different proteins expressed specifically in PMs to detect expression by immunofluorescence. Each of these proteins begins being expressed in PMC cells at different developmental stages. In Fig. 5, a monoclonal antibody to the carbohydrate portion of a PMC-specific cell surface glycoprotein, 4C12 (C. Lane, personal communication; Livingston and Wilt, 1992), was used. The 4C12 protein first appears on the PMC cell surface following ingestion. In control mesenchyme blastula, ingressed PMCs were seen to bind the 4C12 antibody (Figs. 5A and 5B). In GSW embryos fixed when controls were at mesenchyme blastula, no cells within the blastocoel bound the 4C12 antibody (Figs. 5C and 5D). In control gastrulae, the PMCs have fused to form a syncitium surrounding the skeletal spicule. The entire syncitium binds the 4C12 antibody (Figs. 5E and 5F). In GSW embryos fixed when controls were at gastrula, again no cells in the blastocoel bind the 4C12 antibody (Figs. 5G and 5H). Occasionally, one or two cells in the blastula of GSW embryos bound the 4C12 antibody, but these cells were never fused into syncitia. The 4C12 antibody also binds a component of the hyaline layer.
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FIG. 1. Genistein treatment. (A) The protocol used for treatment with 25 μM genistein. Embryos were examined using light microscopy after 48 h of development (B). a, control embryo that has completed normal gastrulation. b, a GSW embryo that has arrested prior to gastrulation. The arrow indicates the embryonic gut. Arrowheads indicates cells which have ingressed into the blastocoel. Scale bar, 10 μm.

surrounding the embryos, and this remains unchanged in GSW embryos.

Another marker for PMC differentiation is SM 50. The SM 50 RNA and protein are first synthesized at the blastula stage, with an increase in expression following ingress of PMCs. Since the SM 50 gene was shown to be transcribed and translated in GSW embryos, we examined the spatial arrangement of cells expressing SM 50. A polyclonal antibody to the SM 50 protein (Richardson et al., 1989), which is not glycosylated (Livingston et al., 1991), was used to detect the SM 50 protein. In control gastrula the SM 50 antibody bound along the entire syncitium formed by PMCs (Figs. 6A and 6B). In GSW embryos fixed when controls were at gastrula, individual cells in the blastocoel bound the SM 50 antibody (Figs. 6C and 6D). These cells were usually arranged in a ring at one end of the embryos, but were occasionally scattered throughout the blastocoel. The SM 50 protein did not appear to be secreted, but was found concentrated in localized areas of the individual cells (Fig. 6E). Cells expressing SM 50

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FIG. 2. (A) Uptake of \(^{125}\)I-labeled genistein from seawater. Unfertilized eggs and zygotes were incubated with 70,000 cpm of \(^{125}\)I-labeled genistein for 15 min at 15-min intervals following fertilization. Eggs and zygotes were then washed twice and counted. The values shown represent the average of triplicate assays ± SE. UF = unfertilized eggs. (B) Genistein treatment of zygotes inhibits PTK activity. Fertilized eggs were incubated with genistein at 25 \(\mu\)M or with DMSO as a control for a 15-min period beginning at different times following fertilization. At the end of each incubation period, an aliquot of fertilized eggs (50 \(\mu\)g protein) was assayed for PTK activity using a synthetic peptide substrate. The inhibitory effect of genistein was calculated as (control activity - genistein activity)/control activity and is expressed as percentage inhibition. Values represent the average of duplicate assays ± SE.

FIG. 3. Expression of tissue specific RNAs. RNA blots of RNA from 48-h embryos were probed with cDNAs homologous to different tissue-specific RNAs as indicated in the figure. 1, control embryos; 2, GSW embryos. In all cases the amount of RNA loaded in each lane was determined using a probe to 16S mitochondrial ribosomal RNA. An example of this using the blot first probed with SM30 is shown.

FIG. 4. Expression of endoderm- and mesoderm-specific proteins. Protein extracted from equivalent amounts of gastrula-stage embryos were fractionated and blotted to nitrocellulose. Duplicate blots were probed separately with antibody to either SM50 protein or Endo16 protein, as labeled in the figure. 1, control embryos; 2, GSW embryos. Equivalent amounts of samples were run on the same gels and stained with Coomassie blue in order to normalize the amount of protein in control and treated samples (not shown). Three different batches of embryos were used in these experiments and none showed any significant difference between control and GSW embryos.

SM30 is a PMCs-specific gene whose expression begins in the gastrula, 8–10 h later than SM50 is first seen. The protein can clearly be seen accumulating in the spicule-forming syncitia of control prism gastrula (Figs. 7A and 7B). The SM30 protein is not visible in any cells of the GSW embryos, although the same batches of embryos were used as those that stained positive for SM50 (Figs. 7C and 7D).

It is clear from these experiments that inhibition of tyrosine kinases 20–45 min postfertilization does not inhibit the initial specification and differentiation of PMCs. PMCs in GSW embryos ingress into the blastocoel and can form the characteristic ring at the vegetal pole; they also express SM50, a molecular marker of differentiation. However, PMCs in GSW embryos do not complete their develop-
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Appearance at Gastrulation of an Endoderm-Specific Protein

The normal appearance of Endo16 mRNA and protein in GSW embryos indicates that normal differentiation of endoderm is occurring. Endo16, however, appears early in development, prior to gastrulation. Since gastrulation does not occur in GSW embryos, we examined the appearance of an endoderm-specific cell surface epitope, Endo1, that is not expressed until the gastrulation is underway. Endo1 is a monoclonal antibody to a 350-kDa glycoprotein that appears on the cell surface of invaginating endoderm (Wessel and McClay, 1985). In control gastrula the antibody binds to cells in the mid- and hind-gut of gastrulae (Figs. 8A and 8B). In GSW embryos, the antibody did not bind to any cells (Figs. 8C and 8D). These experiments clearly show that initial specification and differentiation of endoderm are not affected by genistein treatment of one-celled embryos, as assayed by expression of Endo16 mRNA and protein. The morphogenetic movements of endoderm during gastrulation are blocked, as well as the appearance of Endo1, a cell surface marker specific to endoderm that appears during invagination.

DISCUSSION

Fertilization initiates a complex sequence of events that leads to both cell differentiation and morphogenetic movements, culminating in a distinct three-dimensional body plan. While a number of studies have examined the role of cell-cell interactions and signal transduction during cleavage stages in differentiation of cells in sea urchin embryos (Horstadius, 1973; Livingston and Wilt, 1990, 1992, 1995; Wikramanayake, 1995, 1997; Ransick and Davidson, 1993, 1995), little is known about the very early events that initiate these processes. We show here that the PTK activity shortly after fertilization is essential for normal morphogenetic movements at gastrulation, but not for initial specification and differentiation of endoderm and mesoderm.

The identification of a narrow window of time following fertilization where application of PTK inhibitors had an effect much later in development was an unusual and intriguing discovery (Kinsey, 1995). However, it was possible that the sensitive window was due to a difference in the ability of zygotes to take up inhibitor at different times. We show here that zygotes takes up the inhibitor genistein very well at all times it is present in the seawater. We also show that genistein inhibits endogenous tyrosine kinase activity in the zygote at the time it is taken up from the seawater. The specificity of the GSW for inhibitors of tyrosine kinases, and not other kinases, was shown previously (Kinsey, 1995). Taken together, this is strong evidence that the activity of one or more tyrosine kinases at this one particular time following fertilization is necessary to complete normal development. It is not clear how many PTKs are affected, or if some are inhibited more than others. As specific kinases...
FIG. 6. Localization of the PMC-specific protein SM 50. The 48-h embryos were fixed and stained with a polyclonal antibody that binds a protein, SM 50, that is an integral part of the organic matrix of the spicule formed by PMCs. Binding of the primary antibody was detected using an FITC labeled secondary antibody. Images are 2-μm confocal sections showing simultaneous FITC emission and Nomarski images (A,B,C,D) or FITC emission alone (E). A and B show a control embryo. C and D show a GSW embryo. E shows an 2× enlargement of two GSW embryos. Scale bars, 10 μm.

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FIG. 7. Expression of the PMC-specific protein SM30. The 48-h embryos were fixed and stained with a polyclonal antibody that binds a protein, SM30, that is an integral part of the organic matrix of the spicule formed by PMCs. Binding of the primary antibody was detected using an FITC-labeled secondary antibody. Images are 2-μm confocal sections showing simultaneous FITC emission and Nomarski images. A and B show a control embryo. C and D show a GSW embryo. Scale bar, 10 μm.

active in the zygote are identified, it may be possible to study their role in the GSW.

PTK activity during the GSW is not required for normal development to the mesenchyme blastula stage, including ingestion of PMCs. It is required for invagination of the gut as well as for fusion of PMCs and elaboration of the skeletal spicule. Initial differentiation of endoderm is not affected by inhibition of PTK activity during the GSW, as assayed by the normal transcription and translation of the gene encoding Enod16. The endoderm in GSW embryos is not completely normal, as a cell surface glycoprotein, Enod1, expressed at a later step in differentiation is not present.

Differentiation of mesoderm is initiated despite inhibition of PTKs during the GSW as assayed by transcription and translation of the gene encoding SM 50. Cells expressing SM 50 in GSW embryos can be found in rings near one pole. These cells do not fuse, and do not have on their surface a carbohydrate epitope recognized by the 4C12 antibody. They also do not express the product of the SM 30 gene. This indicates that although PMCs are specified and begin to differentiate in GSW embryos, they do not complete their morphological movements or the process of differentiation.

We can conclude that a chain of events is initiated shortly after fertilization that leads to proper morphogenetic movements at gastrulation. One or more tyrosine kinases are required as part of this cascade of events. This process is separate from the mechanisms utilized by the embryo for initial specification of endoderm and mesoderm cell fate. In GSW embryos, the inability of endoderm to invaginate or PMCs to fuse, as well as the missing cell surface molecules on both endoderm and mesoderm, suggests that cell surface interactions important in morphogenetic processes have been affected. In contrast to the effect on gastrulation, early development in GSW embryos is normal. This, along with the presence of an intact epithelia and the ingestion of PMCs in GSW embryos, demonstrates that the genistein effect is not a completely general effect on all cell interactions.

Developmental biologists have identified several types of perturbations that, when applied during cleavage stages, cause arrest of development at mesenchyme blastula and result in a morphology similar to GSW embryos. Disruption of the extracellular matrix also causes arrest of sea urchin development at the mesenchyme blastula stage (Solursh et al., 1986; Wessel and McClay, 1987; Benson et al., 1991). Similarly, disruption of signaling through growth factors and their receptors can also disrupt gastrulation (Ramachandran et al., 1993, 1995; Govindarajan, 1995). Cell–cell interactions between the blastula and mesenchyme blastula stage have been shown to be required for endoderm differentiation (Chen and Wessel, 1996), and skeletal formation requires the presence of intact collagen (Butler et al., 1987). All of these studies suggest that interactions between cells...
FIG. 8. Expression of an endoderm-specific marker, Endo1. The 48-h embryos were fixed and stained with a monoclonal antibody, Endo1, to an endoderm-specific glycoprotein. Binding of the primary antibody was detected using an FITC-labeled secondary antibody. Images are 2-μm confocal sections showing simultaneous FITC emission (A, C) and Nomarski images (B, D). A and B show control embryos. C and D show GSW embryos. Scale bar, 10 μm.

and between cells and the extracellular matrix are involved in morphogenetic movements during gastrulation.

The difference between previous studies using agents that inhibit gastrulation and the present one is that the GSW occurs in the zygote 24 h prior to gastrulation. This indicates that the required tyrosine kinase activity is a very early event in a cascade of information leading to downstream events necessary for the morphogenetic movements of gastrulation. The tyrosine kinase activity in the zygote could be part of a chain of signaling molecules. It is also possible that the tyrosine kinase activity is required for processing or redistribution of maternal factors that are sequestered for later use.

We suggest that there are two possible mechanisms for the downstream effects caused by inhibition of PTKs during the GSW: (1) Inhibition of PTK activity during the GSW leads to the specific inhibition of expression of molecules in the ECM or on the surface of cells that are necessary for cell recognition events leading to formation of the gut and skeleton, or (2) inhibition of PTK activity during the GSW results in the disruption of the processing of some types of cell surface molecules, which results in the loss of any developmental processes requiring that class of molecules. In either case, it is clear that a very early signaling event is required for proper morphogenetic movement and subsequent differentiation of the gut and skeleton, and that this signaling event does not affect initial specification of endoderm and mesoderm. These results are consistent with a report by Chen and Wessel (1996) that shows there are at least two steps in endoderm differentiation: an initial conditional fate restriction to “preendoderm,” followed by commitment to endoderm at the time PMCs ingress into the blastocoe!.

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