**Drosophila** gastrulation: analysis of cell shape changes in living embryos by three-dimensional fluorescence microscopy

ZVI KAM², JONATHAN S. MINDEN¹*, DAVID A. AGARD¹, JOHN W. SEDAT¹ and MARIA LEPTIN³†

¹Department of Biochemistry and Biophysics and The Howard Hughes Medical Institute, University of California San Francisco, San Francisco, California 94143, USA
²Department of Immunology, Weizmann Institute of Science, Rehovot 76100, Israel.
³Max Planck Institut für Entwicklungsbiologie, Spemannstrasse 35, 7400 Tübingen, FRG

*Present address: Department of Biological Sciences, Carnegie Mellon University, Box 194, Pittsburgh, PA 15213, USA
†Author for correspondence

Summary

The first event of Drosophila gastrulation is the formation of the ventral furrow. This process, which leads to the invagination of the mesoderm, is a classical example of epithelial folding. To understand better the cellular changes and dynamics of furrow formation, we examined living Drosophila embryos by three-dimensional time-lapse microscopy. By injecting fluorescent markers that visualize cell outlines and nuclei, we monitored changes in cell shapes and nuclear positions. We find that the ventral furrow invaginates in two phases. During the first ‘preparatory’ phase, many prospective furrow cells in apparently random positions gradually begin to change shape, but the curvature of the epithelium hardly changes. In the second phase, when a critical number of cells have begun to change shape, the furrow suddenly invaginates. Our results suggest that furrow formation does not result from an ordered wave of cell shape changes, contrary to a model for epithelial invagination in which a wave of apical contractions causes invagination. Instead, it appears that cells change their shape independently, in a stochastic manner, and the sum of these individual changes alters the curvature of the whole epithelium.

Key words: Drosophila, gastrulation, epithelial folding, furrow formation, time-lapse microscopy, fluorescent markers.

Introduction

During the development of an organism, many organs and other complex structures are created by the folding of epithelial sheets. The formation of the germ layers in gastrulation is the earliest example during embryogenesis. The first morphogenetic process during Drosophila gastrulation is the formation of the ventral furrow, which leads to the invagination of the mesoderm (Poulsom, 1950; Sonnenblick, 1950). Drosophila ventral furrow formation is an example of epithelial folding that is especially well suited for histological, cell biological and genetical analysis. Compared with other epithelial foldings, it is simple and quick. The process, which takes less than 20 min, turns an epithelium of morphologically identical cells into an invaginated tube of cells. No cell division occurs during this period. Like other epithelial invaginations (reviewed in Ettenson, 1985; Schoenwolf and Smith, 1990), ventral furrow formation involves cell shape changes (Turner and Mahowald, 1977, Leptin and Grunewald, 1990). Studies on fixed embryos show that a population of prospective mesoderm cells constrict at their apical ends and their nuclei migrate basally (Leptin and Grunewald, 1990). Apical constriction of cells has been proposed to be a driving force for epithelial invaginations (Burnside, 1973), and computer models suggest that a wave of contractions transmitted from cell to cell might be sufficient to produce a furrow (Odell et al. 1981). However, it is not possible to determine the temporal and possible causal relationships of the events during epithelial invaginations from studies on fixed embryos. We therefore examined living embryos using three-dimensional time-lapse fluorescence microscopy (Hiraoka et al. 1989; Minden et al. 1989).

At the cellular blastoderm stage, the Drosophila embryo consists of an epithelial layer of ~6000 columnar cells (and ~40 spherical pole cells. Zalokar and Erk, 1976; Foe and Alberts, 1983). When cellularization is complete, a band of cells (~20 cells wide and ~60 cells long) invaginates to form the ventral furrow. This band is subdivided into two populations: a
central population (8–10 cells wide), whose nuclei move basally and whose apical sides constrict, and a peripheral population (4–5 cells wide on either side of the central population) which follows the central population into the furrow without these shape changes (Leptin and Grunewald, 1990). We have concentrated our analysis here mainly on the behaviour of the central cells, whose shape changes we believe to have an active role in ventral furrow invagination.

Materials and methods

Preparation of embryos

Drosophila melanogaster (strain Oregon R) embryos were dechorionated and mounted ventral side up with double-sided tape (Scotch no. 665) on oxygen permeable teflon membrane (see below). The embryos were dehydrated slightly, covered with 700 weight halocarbon oil (HC Co. Hackensack NJ) and injected as described (Mindell et al., 1989). calf thymus histones H2A and H2B were prepared as previously described (Mindell et al. 1989) and used. Most of the embryos were injected with 0.4 mg mL⁻¹ Fl-dextran (70000 M₉, Molecular Probes, Eugene OR) at 1 mg mL⁻¹ was injected into the perivitelline space shortly after the Rh–histone injection. Recordings were done at approximately 22°C.

Microscopy and image and data processing

Low light level images were recorded with a cooled, scientific grade charge coupled device (CCD) camera operated by a self-contained computer workstation. Electronic shutters, focus position and fluorescence filter selection were also controlled by the computer workstation. In order to focus more than 30 μm into the embryo at high magnification, it was necessary to image directly through the halocarbon oil in which the embryo was immersed rather than through a coverslip. The embryos could not be mounted on a glass coverslip because they became anoxic when placed under the microscope objective. Instead, they were mounted ventral side up on a thin oxygen permeable teflon membrane (YSI high sensitivity film, Yellow Springs Instruments, Yellow Springs, OH) stretched over a specially designed platform. Under these conditions, the embryos developed normally at the same rate as control embryos. A Zeiss PlanNeofluar ×63/1.2 water coverslip correction objective (Zeiss 461832) was used. The correction ring was turned to 0.22 mm to match the index of refraction of 1.414 for the halocarbon oil, as described by Hiraoka et al. 1990. Ten optical sections, 3 μm apart, each taken in both fluorescein (0.2 s exposure) and rhodamine (0.1 s exposure) channels (20 images altogether), were recorded for each time point. Because of the large distances between focal planes, fluorescent background was subtracted by image enhancement filters rather than by deconvolution. The large dynamic range of the data (12 bits) allows such enhancement even for the high background caused by the large amount of out-of-focus fluorophores.

The nuclei in Figs 2 and 4 are modelled as ellipsoids with their centres, axial ratios and tilt angles calculated from the raw data as first and second moments of the corresponding volume defined by histone fluorescence. The nuclear volume parameters were automatically computed by an adaptation of the 2D ridge algorithm of Soferman and Shamir (Soferman, 1989). The cell outlines were drawn using the PRISM 3D modelling software (Chen et al. 1990) by connecting the Fl–dextran marked vertices between cells.

Results and discussion

To observe nuclear movement and cell shape changes simultaneously, two fluorescent probes were injected into the same embryo. Nuclear behaviour was observed by injecting rhodamine-labelled histones (Rh–histones) into embryos during the nuclear division cycles 9 and 10. The Rh–histones are incorporated into chromatin during the subsequent cycles of DNA replication (Mindell et al. 1989). Cell outlines were visualized by injecting fluorescein-dextran (Fl–dextran) into the space between the plasma membrane and the vitelline membrane. The Fl–dextran diffuses throughout the perivitelline space and reveals a bas-relief of the surface of the embryo (Warn and Magrath, 1982). Depressions and spaces between cells appear brightly fluorescent, while the areas where the dextran is excluded from the cells appear as dark regions. The Fl–dextran is carried into intercellular spaces as the plasma membranes extend down between nuclei during cellularization.

Two colour, three-dimensional data sets were obtained by alternately recording fluorescein and rhodamine images over 10 optical sections at 3 μm increments once every minute until the ventral furrow had formed. Fig. 1 shows a sample of these data with alternating rows of Fl–dextran and Rh–histone images, each pair of images taken at the same focal plane. The columns represent four time points at 5, 20, 24 and 28 min after the beginning of the recording. Furrow formation began at approximately 10 min in this experiment. The Fl–dextran is found above the apical surfaces (Fig. 1, 1A) and at the interstices of the polygonal cells (Fig. 1, 3A and 5A). The leading edge of the cellularization furrow appears as a crown-like ring of fluorescence around the base of each cell (Fig. 1, 7A). The intestinal Fl–dextran signal persists throughout gastrulation, while the crown-like rings disappear at the completion of cellularization (Fig. 1, compare 7A and 7B). All analysis shown in this paper was derived from a single recording, but other experiments gave the same results.

The first visible cell shape change is the flattening of the hemispherical apical cell surfaces, seen as the accumulation of bright patches of Fl–dextran above individual cells (Fig. 2A; see arrows). The corresponding Rh–histone image 6 μm below the surface shows that these patches lie above cells whose nuclei have begun to migrate basally. The brightly labelled chromocentres at the top of the nuclei are visible in this plane of section, while the neighbouring nuclei are seen at mid-section (Fig. 2B, see arrows). Computer models of three cells tracing cell outlines and nuclear positions are shown in Fig. 2C–E (these three cells were among the first to flatten their apical surfaces). For clarity, the nuclei are shown as ellipsoids, whose centres, dimensions and tilt angles are calculated from the histone images (see figure legend). Initially, the nucleus is at the apical side of the cell, and the cell is narrower at its basal end. The average ratio of apical to basal cross-sectional area is 1.6±0.2 (n=20 cells). The nuclei descend at an average rate of approximately
Cell shape changes in Drosophila gastrulation

Fig. 1. A montage of optical sections of a developing ventral furrow. Column A shows a time point late in cellularization, 5 min after the beginning of the recording. The first signs of ventral furrow formation were visible between 8 and 10 min. Columns B–D represent the 20, 24 and 28 min time points, ending at the closure of the furrow. The diagram at the top of each column (based on sections of fixed embryos; Leptin and Grunewald, 1990) shows the shape of the ventral furrow in the developing embryo (ventral side up), at similar stages as the fluorescent images below. Four focal planes separated by 6 μm are shown, each pair of rows representing Fl-dextran (labelled D at the right margin; rows 1,3,5,7,) and Rh–histone (labelled H; rows 2,4,6,8) images at the same focal plane. The Rh–histones label nuclei. The chromocentre appears as a particularly bright region at the apical tip of each nucleus. Dextran is confined to the extracellular space. On the surface of the embryo it marks cell outlines, until the apical surfaces begin to flatten (row 1). It accumulates over areas where the cell surfaces flatten and over the invaginating ventral furrow (columns B and D). Deeper in the epithelium, the dextran is found mainly at the vertices of the hexagonal cells. No nuclei are initially visible at the lowest level (row 8), but as they disappear from the top level (row 2), they begin to appear at the lowest level.
2.5 \mu m \text{min}^{-1} \text{ (but not always continuously). As the nucleus moves away from the top of the cell, the apical end contracts, and when the nucleus reaches the basal end of the cell, the cell is wider at its base than at the apex (average ratio of apical to basal cross-sectional area is 0.7\pm0.2; n=10 cells). The nuclei do not change their orientation within the cell during basal movement (as illustrated by the position of the chromocentres). Occasionally, the apical surface expands in cells that are adjacent to those whose apices have contracted. It is not possible to discern whether nuclear movement and cell shape changes are caused by active contraction of the apical surface, or by a release of nuclei from the apical surface and passive compression of the cell. In some cases (Fig. 2C is an example), we see the nucleus beginning to move basally in the absence of visible apical constriction, suggesting that nuclear migration might not depend entirely on apical constriction.

The above cellular changes do not happen simultaneously in all cells of the central population. A graphical representation of the behaviour of 40 nuclei is shown in Fig. 3. The position of each nucleus along the left–right axis of the embryo is projected onto the horizontal axis for each time frame recorded, and the vertical axis represents time. Images of five time points are shown alongside the graph, with four representative traces to demonstrate how the traces were constructed. Each trace terminates with a circle at the time that the nucleus moves below the plane of focus. For the cells of the central population, this represents the basal movement of the nucleus within the cell (the approximate edges of the central region are marked by triangles in Fig. 3). For the peripheral cells, the disappearance of nuclei corresponds to the movement of the whole cell into the furrow. Two stages of ventral furrow formation can be distinguished. During the first stage (min 10 to min 22 in Fig. 3), more than half of the nuclei in the central population gradually move basally. There is little movement of the lateral nuclei towards the centre of the furrow, and only a moderate change in the curvature of the cell sheet. During the second stage, the remaining nuclei of the central population move basally almost simultaneously and the furrow deepens quickly (the Fl–dextran can be seen at a depth of 18 \mu m; Fig. 1 7D). The peripheral cells now move laterally towards the furrow at a rate of about 10 \mu m \text{min}^{-1} and fold over the invaginated central population. The transition from the first to the second stage could either be due to the activation of new cellular processes, or it could simply be a mechanical collapse of the cell sheet as a consequence of the continuing cellular processes of the first stage.

To give an integrated view of the data, Fig. 4 shows a computer-generated three-dimensional reconstruction of the position and orientation of nuclei. Before the beginning of ventral furrow formation, the nuclei are regularly aligned at the surface of the embryo (Fig. 4A). In Fig. 4B, many nuclei of the central population in apparently random positions have moved away from the apical surface (highlighted nuclei have migrated more than 6 \mu m from the surface). Four
Fig. 4. A model of the positions of nuclei at four points of ventral furrow formation, derived from the same set of data as those shown in Fig. 1. The nuclei are presented as ellipsoids with their centres and tilts computed from the histone images as in Fig. 2. Nuclei that have migrated away from the apical surface are shown in red. The time points are 1, 19, 23 and 26 min.
minutes later, the majority of the nuclei in the central population have moved basally (Fig. 4C), while at the same time the epithelium begins to invaginate, as seen by the accumulation of FI-dextran in an intense band over the furrow (Fig. 1. 1C). Subsequently, the peripheral population of cells folds over the invaginated central population. The chromocentres of the peripheral population remain at the apical surface and point towards the furrow (Fig. 1, panels 2D, 4D, 6D, and 8D).

The order in which cells begin to change their shape during the first phase of ventral furrow formation is inconsistent with models that postulate a wave of contractions propagating from cell to cell as the triggering event for invagination (Odell et al. 1981). Others have also noted an irregular appearance of cells in invaginating epithelia, consistent with a stochastic initiation of shape changes (Hardin and Keller, 1988; Schoenwolf and Franks, 1984). Since, in the ventral furrow, neighbours of early constricting cells do not necessarily constrict sooner than other cells in the epithelium, it appears that there are also no small local waves of apical contractions (however, an extensive statistical analysis would be required to exclude them completely). Instead, our results suggest that the initial cellular events in furrow formation are part of an independent program of activity of each cell in the central population. Thus, the question of how the ventral epithelium begins to invaginate is reduced to the analysis of how individual cells change their shape. The techniques described here will be useful in complementing genetic and biochemical approaches to answering this question.

We thank Bruce Alberts, Daniel St Johnston and Eric Wieschaus for critical reading of the manuscript. J.S.M. is a Lucille P. Markey scholar and his work was supported by a grant from the Lucille P. Markey Charitable Trust.

References


POULSON, SCHOENWOLF, MINDEN, LEPTIN, HIRAOKA (1989). ODELL

Development and invagination. pl68-274 (Meigen). Development 110, 73-84.


Note added in proof

Observations on fixed embryos reported by Sweeton et al. (Development 112, 1991 in press) also suggest an initial slow stochastic and a later fast phase of ventral furrow formation.