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F-actin localization during trochoblast differentiation in *Patella vulgata* embryos

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Summary

We have studied the development of the ciliated, locomotory organ of *Patella vulgata* trochophore larvae. This organ, the prototroch, arises from different clones of trochoblasts. In each of these trochoblasts, a band of filamentous (F-) actin is formed at the time that ciliogenesis starts. This band, which we visualized with TRITC–phalloidin, is positioned at the base of the row of cilia that crosses each trochoblast. Isolated trochoblasts, as well as isolated quartets of animal micromeres (from which the trochoblasts are derived), similarly form rows of cilia and F-actin bands at the proper time in development. In whole embryos, the trochoblasts shift their position following ciliogenesis, and finally form a ring of differentiated prototroch cells with a continuous band of F-actin encircling the entire larva. At the dorsal side, a double row of prototroch cells and thus a double band of F-actin is present. In contrast, multiple regions with a double F-actin band are found in trochophores in which the establishment of a dorsoventral axis is inhibited experimentally. Confocal laser scanning microscopy shows that the F-actin band extends from the apical surface deep into the cytoplasm of the prototroch cells. At the ultrastructural level, a single striated rootlet connected to the basal body of each cilium can be seen to extend deep into the cytoplasm toward the nucleus, and a band of actin-like filaments is found to interconnect neighboring basal apparatus. Treatment of trochophores with cytochalasin B disrupts the organization of the F-actin band as visualized with TRITC–phalloidin, affects the angle of the effective stroke of ciliary beat and reduces their swimming capacity. This suggests that the F-actin band is essential for the normal locomotory behavior of the *Patella* trochophore larva.

Key words: F-actin; ciliogenesis; microfilament-microtubule interaction, molluscan development.

Introduction

Embryos of the gastropod mollusc *Patella vulgata* develop a ciliated organ, called the prototroch, which is responsible for the locomotion of the trochophore larva. The prototroch has a polyclonal origin: it arises from a well-ordered ring of two types of intercalated clones of trochoblasts, i.e. the primary and the accessory trochoblasts. The primary trochoblasts, formed at the 16-cell stage, and the accessory trochoblasts, formed at the 32-cell stage, divide only two more times, after which they start to differentiate: they become cell-cycle arrested, reduce their communication *via* gap junctions and develop cilia (Wilson, 1904; Van den Biggelaar, 1977; Janssen-Dommerholt *et al.* 1983; Serras *et al.* 1990). Later in development, these trochoblasts shift their position becoming organized into a ring of ciliated prototroch cells that encircles the entire embryo.

In this paper, we have studied the organization of F-actin during development of the prototroch in *Patella vulgata* embryos. We found that differentiation of the trochoblasts is accompanied by the formation of a distinct F-actin band, which underlies the row of cilia that develops on the surface of each trochoblast. The fate of this band during subsequent formation of the prototroch as well as its possible significance for locomotion of the trochophore larva were studied. In addition, the identity of the F-actin-containing structure was investigated at the ultrastructural level.

Materials and methods

Embryos

Patella vulgata was collected at Roscoff (Bretagne, France) and kept in aquaria at 15°C. Oocytes and sperm were obtained by dissection. Maturation of the oocytes was induced by treatment with Millipore-filtered sea water pH 8.9, for 7 min. The mature eggs were inseminated by adding a diluted solution of sperm collected from two or three males. Synchronously cleaving embryos were selected and kept in small Petri dishes. All experiments were carried out at 19°C.

Embryos used for cell isolation experiments, monensin-treatment and scanning electron microscopy were dejellied at the required stage by a 1–2 min treatment with filtered sea water pH 3.8.

The developmental stage of the embryos is indicated by the number of cells up to the 88-cell stage. At later stages, when the cell pattern becomes very complex and the number of cells cannot be determined accurately, time after first cleavage is indicated instead. In all experiments, groups of 20–30 synchronously cleaving embryos were fixed at the desired stage and further processed for fluorescence labeling or electron microscopy.

Labeling with TRITC–phalloidin and Hoechst 33258

Phalloidin is a toxin that stains F-actin in fixed material (Wulf *et al.* 1979; Barak *et al.* 1980). The pattern of filamentous (F-) actin was studied by fixing embryos in 3.7 % formaldehyde in buffer (10 mM Pipes pH 6.8, 100 mM KCl, 5 mM magnesium acetate, 5 mM EGTA pH 6.8) for 10 min, after which they were rinsed in buffer, transferred to 0.5 % Nonidet P-40 in buffer for 5 min, rinsed in buffer, incubated in 0.1 $\mu g\,ml^{-1}$ TRITC–phalloidin (Sigma, St Louis, MO) in buffer for 1 h and rinsed in buffer. Control groups were incubated in 0.1 $\mu g\,ml^{-1}$ TRITC–phalloidin in buffer to which 10 $\mu g\,ml^{-1}$ unlabeled phalloidin was added. Only slight background fluorescence was observed in such controls.

The TRITC–phalloidin-labeled embryos were subsequently stained with 10 $\mu g\,ml^{-1}$ of the fluorescent nucleic acid-specific dye Hoechst 33258 (Riedel de Haen AG, Hannover, FRG) in buffer. After 10 min, the embryos were rinsed extensively and mounted on a microscope slide in buffer. They were examined with a Zeiss Axiovert 35 M microscope using Differential Interference Contrast (DIC) optics as well as epifluorescence illumination (TRITC: exc. 510–560 nm, em. 590 nm; Hoechst: exc. 365 nm, em. 420 nm). Photographs were taken on Kodak Ektachrome 400 ASA and Tmax 100 ASA film.

Confocal laser scanning microscopy (CLSM)

Specimens were also examined with a Bio-Rad Lasersharp MRC-500 confocal laser scanning microscope (Bio-Rad Lasersharp Ltd, Oxfordshire, UK) to determine more precisely the position of the F-actin band relative to the apical surface of the prototroch cells. Series of optical sections at steps of 1.1 μm each were made using a Zeiss Axioplan microscope equipped with a 63×/NA 1.4 oil immersion objective.

Isolation of animal caps and trochoblasts

Pipettes with a tip diameter of about 10–20 μm were mounted on a micromanipulator attached to a stereo microscope. The back of the pipet was connected to a Teflon tube, through which suction was applied. By means of the micromanipulator, the tip of the pipet was brought to the surface of the embryo, and single cells were removed from the embryo. A first series of operations was performed at the 8-cell stage. By removing the four vegetal macromeres, the remaining cluster of four animal micromeres was isolated. During a second series of operations, single stem cells of the primary trochoblasts were isolated from 16-cell embryos. The isolates were transferred to small Petri dishes with filtered sea water and cultured for 16–18 h, after which they were fixed for TRITC–phalloidin and Hoechst staining.

Inhibition of dorsal induction

Monensin is an Na^+-ionophore that inhibits the establishment of a dorsoventral axis in *Patella* embryos (Kühtreiber *et al.* 1988; Serras and Speksnijder, 1990). Embryos were incubated for 90 min in 10 μM monensin in Millipore-filtered sea water, starting at 5 min after fifth cleavage. After incubation, the embryos were rinsed extensively in filtered sea water and cultured until they had reached the trochophore stage. They were subsequently fixed for TRITC–phalloidin and Hoechst labeling.

Cytochalasin treatment

The effects of cytochalasin on the organization of the band of F-actin in trochophore larvae was investigated by incubating trochophores in 1, 5 or 10 $\mu g\,ml^{-1}$ cytochalasin B in sea water. The latter concentration causes an altered swimming behavior within 10 min, and was therefore used for all further experiments. Treatment with cytochalasin D gave similar results, indicating that the effects are actin-specific. After cytochalasin treatment, the trochophores were fixed for TRITC–phalloidin labeling or transmission electron microscopy. Cytochalasin was used from a stock of 1 $mg\,ml^{-1}$ in DMSO; control embryos incubated in 1 % DMSO develop normally.

The swimming characteristics of both control and cytochalasin-treated larvae were registered with a DAGE-MTI Newvicon camera mounted on a Zeiss Axiovert 35 M microscope and recorded with a Panasonic AG-6720-E VHS time-lapse videorecorder.

Scanning electron microscopy (SEM)

Groups of embryos were dejellied at 6 h 10 min, 6 h 40 min, 7 h, 7 h 30 min and 8 h after first cleavage and fixed for 20 min in 2.5 % glutaraldehyde in 0.1 M sodium-cacodylate buffer pH 7.4, postfixed in 1 % OsO_4 plus 2.5 % glutaraldehyde in 0.1 M sodium-cacodylate buffer for 1 h at 4 °C, rinsed in buffer and dehydrated in a graded series of ethanol. After critical-point drying with CO_2, the embryos were mounted on specimen holders with double-sided adhesive tape and sputter-coated with gold. Preparations were examined in a Cambridge Camscan scanning electron microscope. Photographs were taken on Ilford FP4.

Transmission electron microscopy

For TEM, different fixation protocols were tested, two of which proved to give satisfactory results. For fixation I (see Lacalli, 1985), 22 h old trochophore larvae were transferred to 1 ml of seawater, to which 1 ml of 3 % glutaraldehyde, 0.2 M sodium-cacodylate buffer (pH 7.4), 6 % sucrose was subsequently added. After about 3 min, 2 ml of ice-cold 2 % OsO_4 was added and the trochophores were fixed in this mixture for 30 min at 4 °C, after which they were fixed in a fresh solution of 2 % OsO_4, 0.1 M sodium-cacodylate at 4 °C for 30 min. The embryos were rinsed in distilled water and stained overnight in 2 % uranyl acetate at 55–60 °C.

For fixation II (see Tamm and Tamm, 1987), the larvae were fixed for 1 h in 2.5 % glutaraldehyde, 1 % paraformaldehyde, 1 % OsO_4, 0.075 M NaCl, 0.01 M $CaCl_2$, 0.2 M sodium-cacodylate buffer (pH 7.5). They were then washed in 0.3 M NaCl, 0.2 M sodium-cacodylate (pH 7.5) for 1–2 h, postfixed in 1 % OsO_4 in 0.37 M NaCl, 0.1 M sodium-cacodylate (pH 7.5) for 15–20 min, washed in distilled water, and stained overnight in 1 % uranyl acetate. All steps were carried out on ice.

For both procedures, the embryos were subsequently rinsed in distilled water and dehydrated in a graded series of ethanol, after which they were embedded in Spurr's resin. Semi-thin sections stained with methylene blue were used for light microscopical observations. Ultrathin sections were cut

on a Reichert ultramicrotome using glass knives, and examined in a Zeiss EM 10 electron microscope.

Results

Development of the prototroch

The 16-cell *Patella* embryo possesses four primary trochoblasts which, together with the four accessory trochoblasts that are formed at the 32-cell stage, constitute the stem cells of the locomotory organ of the trochophore larva, i.e. the prototroch. The two types of stem cells lie alternately around the animal pole (Fig. 1A) and are derived from the animal micromeres of the 8-cell stage. These stem cells divide twice only, so that a total of 8 clones of 4 trochoblasts each are present at the 88-cell stage. After their final division, the DNA of the trochoblasts becomes highly decondensed. From the 64-cell stage onwards, the large nuclei of the primary trochoblasts are clearly visible (Fig. 1B). The accessory trochoblasts, which are one cell cycle behind in their development, show the same phenomenon from the 88-cell stage onwards.

The development of cilia starts in the primary trochoblasts at the 72-cell stage. The accessory trochoblasts similarly develop cilia after their second division at the 88-cell stage (Fig. 1D–F). During further development, the ciliated trochoblasts become repositioned, and finally a continuous ciliated ring of prototroch cells is formed around the trochophore larva (Serras *et al.* 1990). The prototroch cells remain easily distinguishable due to their large size and large interphase nuclei (Fig. 1C). In contrast, most of the other domains within the larva continue to show cells in mitosis, suggesting an ongoing proliferative activity.

Localization of F-actin in the trochoblasts

The organization of F-actin was studied during development of the prototroch in *Patella* embryos by labeling different embryonic stages with TRITC–phalloidin. Between the 16- and 32-cell stage, a homogeneous cortical labeling of all the cells in the animal hemisphere of the embryo was observed, without any differences in fluorescence intensity. However, in embryos fixed 5 h 30 min (i.e. late 72-cell stage) and 6 h (i.e. 88-cell stage) after first cleavage, a small patch with higher fluorescence intensity was observed at the apical surface of each primary trochoblast (Fig. 2A–C). The site of this

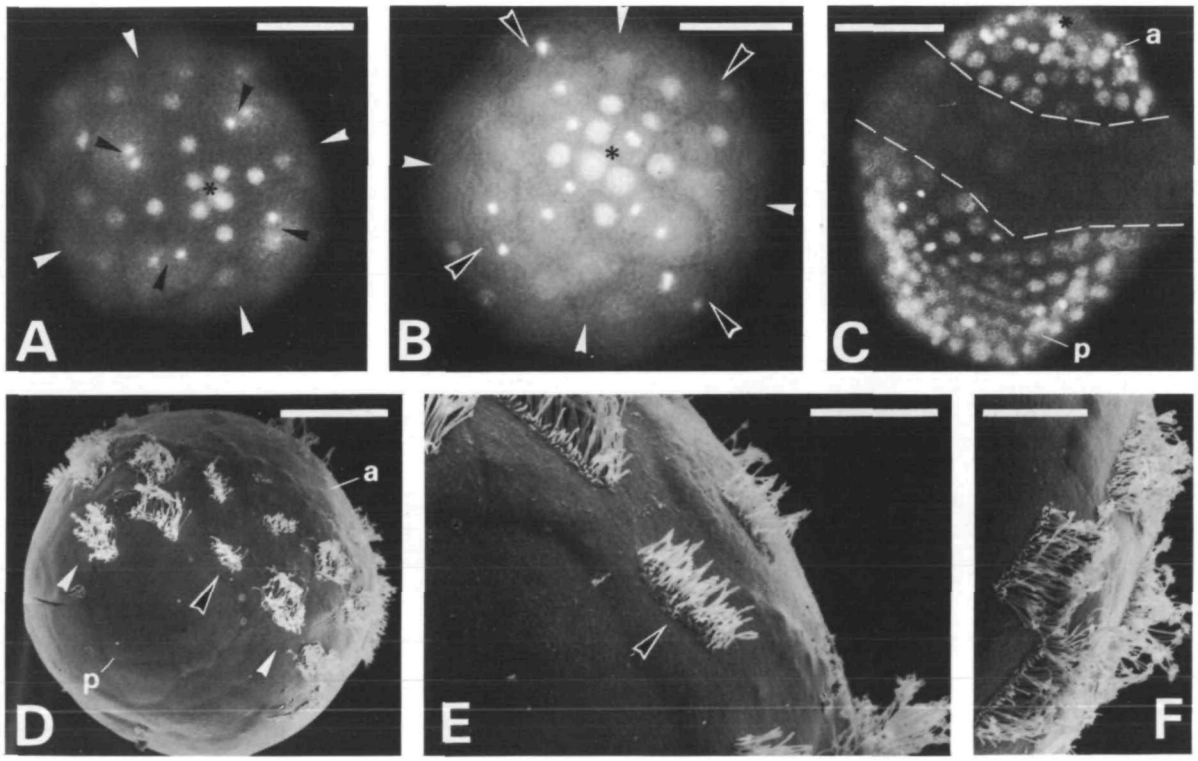
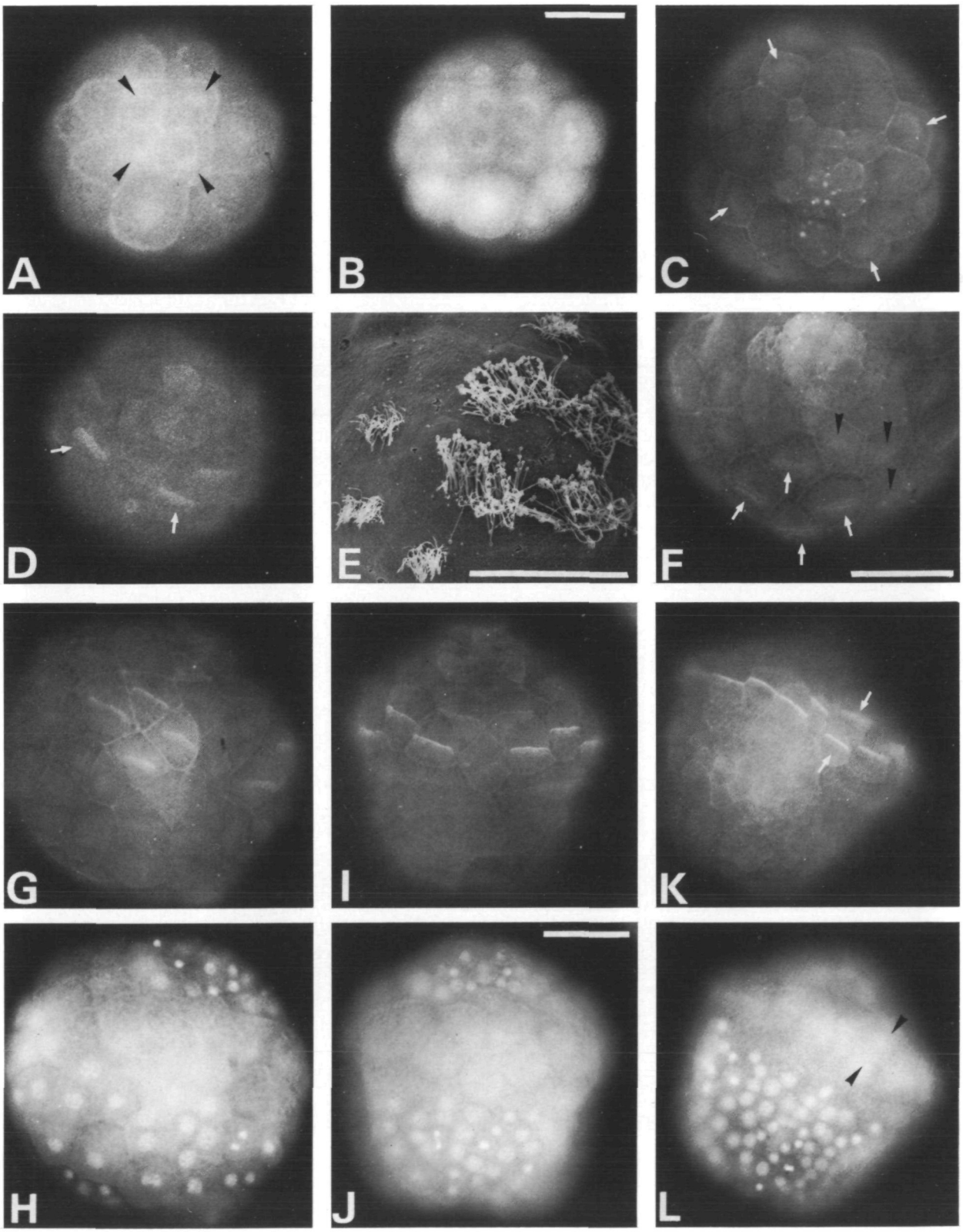


Fig. 1. Division pattern and ciliogenesis of primary and accessory trochoblasts. a, anterior or pre-trochal domain of the ectoderm; p, posterior or post-trochal domain of the ectoderm. Asterisks indicate the position of the animal pole. Bar, A–D=50 μm; E and F=20 μm. (A) Embryo during the transition from the 32- to the 64-cell stage viewed from the animal pole after labeling with Hoechst. The 4 primary trochoblasts have each divided twice, thus giving rise to 4 clones of 4 cells each (white arrowheads). The four accessory trochoblasts are in telophase of their first division (black arrowheads). (B) 88-cell stage embryo labeled with Hoechst and viewed from the animal hemisphere; the accessory trochoblasts (black arrowheads) have completed their second division. (C) Lateral view of a trochophore larva labeled with Hoechst. The prototroch cells with large nuclei are located in between the broken lines. (D–F) Scanning electron micrographs of 6 h 40 min old embryos: (D) lateral view, showing the row of cilia on each of the primary (white arrowheads) and accessory (black arrowheads) trochoblasts. At this stage the cilia of the accessory trochoblasts (E; black arrowheads) are shorter than those of the primary trochoblasts (F).



patch corresponds to the site where cilia are just beginning to develop.

This patch of F-actin becomes more distinct in the trochoblasts of embryos of 7 h and older: in embryos fixed between 6 h 40 min and 8 h 30 min, the fluorescent patch is clearly present in all four clones of primary trochoblasts and has become more intense (Fig. 2D,E). The intensity of fluorescence in this patch continues to increase as development proceeds.

At 8 h after first cleavage, a distinct fluorescent band

Fig. 2. Localization of F-actin during trochoblast differentiation and prototroch formation. Bar=50 μm. (A,B) TRITC-phalloidin and Hoechst labeling of a 5 h 30 min old embryo. Arrowheads indicate one clone of 4 primary trochoblasts. Note that each of these cells contains a small patch with a higher fluorescence intensity close to its large, decondensed nucleus. (C) 6 h old embryo viewed from the animal pole after labeling with TRITC-phalloidin. Arrows indicate the most animal primary trochoblast of each of the four clones, in which a more intensely labeled patch is present. (D) 7 h old embryo labeled with TRITC-phalloidin. Arrows indicate the patch of F-actin in 2 primary trochoblasts of different clones. (E) SEM image of a clone of 4 primary trochoblasts of an embryo of the same age as in D, showing the row of cilia present on their surface. The cells with shorter cilia are the accessory trochoblasts. (F) TRITC-phalloidin labeling of a 8 h old embryo, animal view. White arrows: primary trochoblasts; black arrowheads: accessory trochoblasts. (G-L) TRITC-phalloidin and Hoechst labeling of 8 h (G,H), 10 h (I,J) and 12 h (K,L) old embryos, showing the repositioning of the trochoblast cells during prototroch development. Arrow(head)s indicate a double row of prototroch cells.

running through the middle of each primary trochoblast becomes visible, which suggests that F-actin becomes organized as a band in these cells (Fig. 2F–H). At the same time, a patch of F-actin appears in the four intercalated clones of accessory trochoblasts (Fig. 2F). Thus in both primary and accessory trochoblasts, the formation of a patch of F-actin occurs at the time ciliogenesis is initiated after their final division.

In 10 h old embryos, both primary and accessory trochoblasts possess a distinct band of F-actin. However, we found that in each embryo, four clones of trochoblasts display a band with somewhat higher intensity than the remaining four, intercalated clones. We assume that the clones with the strongly fluorescent band are the primary trochoblasts, since they are somewhat ahead in their development (Fig. 2I,J).

Since control experiments with unlabeled phalloidin (see Materials and methods) show only weak background fluorescence, we conclude that F-actin becomes localized in these trochoblasts at the time when and the place where ciliation is initiated.

Localization of F-actin in the trochophore larva

At 12 h after first cleavage, the embryo has developed into a young trochophore larva. From that time onwards, a ring of ciliated prototroch cells is present and the F-actin band in adjacent cells is aligned such that it is visible as a continuous fluorescent ring around the entire larva after TRITC-phalloidin labeling (Fig. 2K,L; Fig. 3A–C). In addition, a double fluorescent band is always found at one side of the trochophore larva (Fig. 2K,L; Fig. 3D–H).

At about 14 h after first cleavage, the dorsal side of the embryo can be recognized due to the differentiation of the mantle edge and the mantle epithelium. In all embryos studied (several hundred) except two, this double band of F-actin is located at the dorsal side of the prototroch (Fig. 3D,E). Analysis of the nuclear pattern *via* Hoechst labeling and of the position of cells *via* bright-field images reveals that such a double actin band corresponds to a double row of ciliated prototroch cells (Fig. 3F–H).

From 14 to 22 h after first cleavage, the organization of the F-actin band does not change visibly (Fig. 3A–H). At the later stages, however, TRITC-phalloidin labeling was also found to be present in the cilia of the prototroch cells (Fig. 3I). This is in agreement with the observations of Hard and Rieder (1983) that axonemes contain actin.

Apart from the prototroch cells, a few other structures were also found to contain a higher density of F-actin. For example, the ciliated prototroch cells are flanked anteriorly, and in some places also posteriorly, by poorly ciliated large cells which are called the supporting cells (Fig. 3B,C). At higher magnification, a small yet clear band of F-actin is visible in such cells (Fig. 3F). In addition, an increased density of F-actin is present at the dorsal side of the posterior (post-trochal) ectoderm, which will develop the mantle and shell of the adult. A ring of F-actin was found to be present in the mantle cells (Fig. 3J,K), which can be clearly distinguished because of their large nuclei (not shown). Finally, the most apical cells of the trochophore larva, which possess a tuft of very long cilia, also show a distinct localization of F-actin at their apical surface. Fig. 3L,M shows an example of a TRITC-phalloidin-labeled larva in which localization of F-actin in the apical cells in addition to the prototroch cells is clearly visible.

3-D organization of the F-actin band as studied with CLSM

While studying the F-actin band in the prototroch cells at higher magnification with a high NA objective, we noticed that the band is not entirely cortical, but also seemed to extend deeper into the cytoplasm. We investigated this possibility further by making use of a confocal laser scanning microscope (CLSM). This instrument allows one to make thin optical sections of an object without interference of out-of-focus fluorescence (White *et al.* 1987). A series of optical sections of the band area starting at the apical surface and descending into the cytoplasm of the prototroch cells clearly reveals that the fluorescent band extends about 7 μm deep into the cytoplasm. In addition, the band is somewhat granular and dispersed at the surface, but becomes more concentrated deeper in the cytoplasm and seems to converge into a narrower band (Fig. 4).

Ultrastructural organization of the prototroch

We attempted to identify the structure responsible for the phalloidin labeling pattern by fixing and embedding 22 h old trochophore larvae for transmission electron microscopy. In semi-thin sections for light microscopy, the large ciliated prototroch cells as well as the cells that carry the apical tuft can be easily recognized (Fig. 5A). The prototroch cells display a distinct polarity common to all epithelial cells: at the apical side of the cell, dense

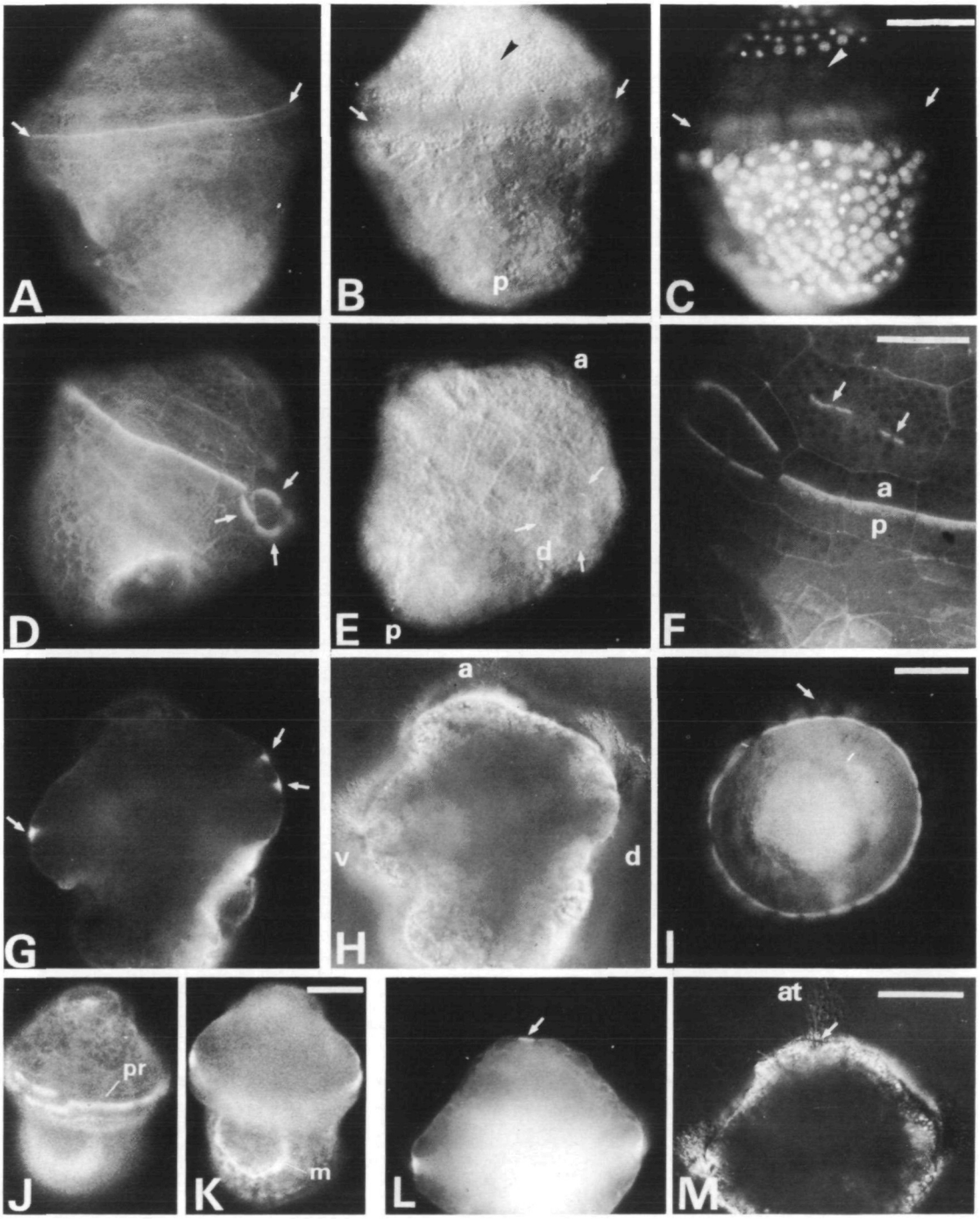


Fig. 3. Localization of F-actin in the trochophore larva. a, anterior; p, posterior; d, dorsal; and v, ventral side of the embryo. Bar=50 μm in all except F: 25 μm; A–E and G–H same magnification. (A–C) 15 h old trochophore larva labeled with TRITC–phalloidin (A) and Hoechst (C) viewed in fluorescence and bright-field (B) optics. The white arrows indicate the row of ciliated prototroch cells; the arrowheads show the row of supporting cells at the anterior side of the prototroch. (D,E) Fluorescence and transmission image of a 17 h old trochophore labeled with TRITC–phalloidin. Arrows indicate the two rows of prototroch cells – each with a band of F-actin – at the dorsal side of the larva. (F) Higher magnification of the F-actin band in a 14 h old trochophore labeled with TRITC–phalloidin. At left the dorsal side of the larva with two rows of prototroch cells. Arrows indicate the localization of F-actin in an anterior supporting cell. (G,H) Fluorescence and bright-field image of a TRITC–phalloidin labeled, 18 h old trochophore focussed on its contour. Arrows indicate the ciliated prototroch cells that possess the F-actin band; at the dorsal side of the larva a double row of prototroch cells is present. (I) Anterior view of a 22 h old trochophore labeled with TRITC–phalloidin showing the continuous ring of F-actin that encircles the entire larva. Arrow indicates labeling of the cilia. (J,K) Dorsal views of a TRITC–phalloidin-labeled trochophore focussed on the prototroch (pr) and on the mantle (m) respectively. (L,M) Fluorescence and bright-field image of a TRITC–phalloidin-labeled trochophore showing the presence of F-actin in the ciliated apical cells (arrow) in addition to the prototroch cells.

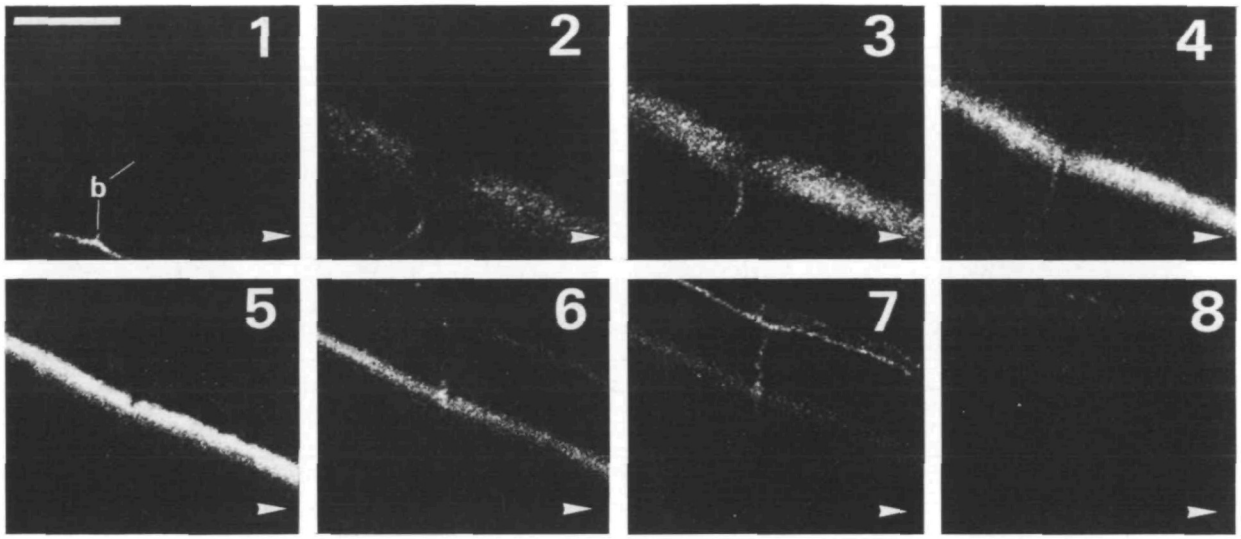


Fig. 4. Optical sections of the F-actin band in two prototroch cells obtained with a confocal laser scanning microscope. The sections were taken starting at the surface (1) at steps of 1.1 μm each. Note that the F-actin band extends about 6–7 μm into the cytoplasm and converges from a wide band just underneath the surface into a narrower band deeper in the cytoplasm (2–7). b=border between the two prototroch cells; Bar=10 μm.

structures are present underneath the row of cilia. The large nucleus is positioned slightly toward the basal side, and sits on top of large yolk granules. In ultrathin sections, many mitochondria can be seen between the nucleus and the apical surface (Fig. 5B, 6A).

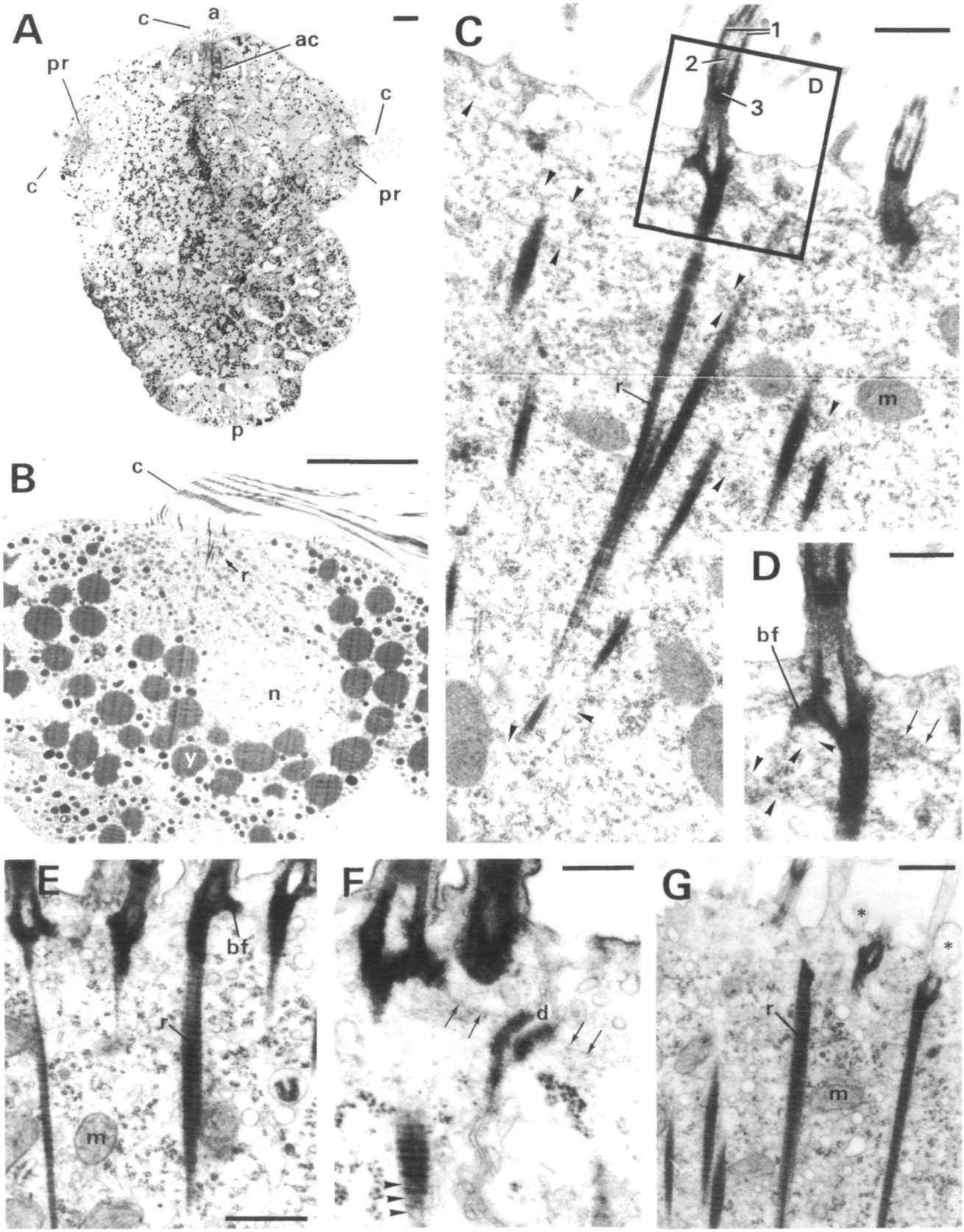
The ciliary apparatus of the prototroch cells displays characteristics typical of ciliated epithelial cells (see Dentler, 1987; Sandoz *et al.* 1988). The very long cilia (about 50 μm) are each composed of a '9+2' arrangement of microtubules surrounded by the ciliary membrane. The outer doublet microtubules are continuous with the microtubules of the basal body, whereas the two central microtubules end in an electron-dense structure distal of the basal body (Fig. 5C,D). The basal foot, an electron-dense structure on the basal body, points in the direction of the effective stroke (Fig. 5A–D), similarly to what has been described for gill cilia of other mollusks (Gibbons, 1961). From each basal body, a single striated rootlet about 7–8 μm long projects toward the nucleus (Fig. 5B, 6B). The rootlets display a typical 58 to 65 nm periodicity and intraband fine structure (Fig. 5E,F) – similar to the banding pattern found in other metazoan rootlets (see Dentler, 1987). In oblique sections, their fibrillar nature is clearly visible (Fig. 5F). These rootlets extend at an angle of about 150–160° from the basal bodies central axes in the direction of the basal foot (Fig. 5C). Two to three adjacent rootlets are often seen to converge and to continue as a single striated fiber extending toward the nucleus (Fig. 5C). The apical cells, which carry extremely long cilia on their surface, similarly possess striated rootlets associated with the basal bodies, which reach lengths up to 16 μm (not shown).

In the prototroch cells, the basal apparatus is linked to various cytoskeletal microtubules and filaments. From the basal foot cap (see Reed *et al.* 1984), microtubules can be seen to radiate into the cortical region of the cell (Fig. 5D). Microtubules are also closely associated with the striated rootlets, contacting them along their entire length at many different angles (Fig. 5D). In the more central part of the cell, microtubules can be seen to extend from the rootlet toward and past the nucleus (Fig. 6B–E). These cytoplasmic microtubules are labile structures, since they are lost upon fixation at 4°C (Fig. 5E,F) (see also Sandoz *et al.* 1988).

Microfilament-like thin filaments, which are retained best after a simultaneous fixation with aldehyde and osmium at 4°C (fixation II; see Materials and methods), are found in a layer parallel to the apical surface at a depth of about 0.4–0.6 μm (Fig. 6B,H). These filaments interconnect the basal part of the basal bodies and the apical regions of the striated rootlets, and can sometimes be seen to be closely associated with the basal foot cap (Fig. 5F). In addition, these thin filaments are inserted into the belt desmosome at the apical part of the lateral surface (Fig. 5F). Finally, septate as well as gap junctions are found along the plasma membranes of neighboring prototroch cells (Fig. 6F,G), This fits our previous demonstration of intercellular communication between prototroch cells *via* gap junctions (Serras *et al.* 1990), and preliminary evidence of the presence of gap junctions between prototroch cells in freeze-fracture replicas (F. Serras and M. J. Good, unpublished observations).

Effects of cytochalasin

Normal trochophore larvae swim actively right underneath the water surface and are rarely found on the bottom of the dish. We found that treatment with 10 $\mu g\,ml^{-1}$ cytochalasin B greatly affects their swimming behavior and results in a high percentage of trochophores on or near the bottom of the dish, even though their cilia can be seen to beat seemingly normal



under the stereo microscope. In DIC optics, a normal wave pattern of ciliary beat is visible in cytochalasin-treated embryos. Video microscopy revealed that this wave progresses along the prototroch surface in a clockwise direction – when viewed from the anterior pole – at a speed of about 170 $\mu m\ s^{-1}$ in both normal and

Fig. 5. Ultrastructural organization of the prototroch as seen in sections through the anterior–posterior axis of the trochophore larva. A–D: fixation I; E–G;: fixation II. bf, basal foot; d, desmosome; m, mitochondrion; n, nucleus; r, striated rootlet; y, yolk granule. (A) Semi-thin section showing the ciliated prototroch cells (pr), as well as the apical cells (ac) at the anterior pole (a) of the larva. c, cilia; p, posterior pole; Bar=10 μm. (B) Thin section of a prototroch cell; the image is rotated 90° clockwise with respect to A, and the long cilia are pointing in the direction of the anterior pole. Bar=10 μm. (C,D) Higher magnifications of the apical part of the same cell shown in B. Arrowheads indicate cytoplasmic microtubules associated with the striated rootlets (r) at different levels in the cytoplasm. 1: outer doublet microtubules of the cilium; 2: central microtubule; 3: electron dense structure at the base of the central microtubules; Bar=0.5 μm. (D) Detail of the basal apparatus showing cytoplasmic microtubules (arrowheads) radiating from the basal foot (bf). Thin filaments (arrows) are associated with the basal part of the basal body. Bar=0.25 μm. (E) Apical part of a prototroch cells fixed according to procedure II: the rootlets as well as the basal foot are better preserved, but cytoplasmic microtubules are absent. Bar=0.5 μm. (F) Detail of the basal apparatus displaying thin filaments (arrows) associated with the basal foot cap and a desmosome on the lateral surface of the cell. Arrowheads show the filamentous nature of the striated rootlet. Bar=0.25 μm. (G) Prototroch cell of a larva treated with cytochalasin B for 110 min. Seemingly normal, long rootlets are present; the apical surface displays large blebs (asterisks). Bar=0.5 μm.

cytochalasin B-treated trochophores. However, we found one striking difference between treated and untreated trochophores, i.e. the angle between the direction of the cilia after the effective stroke and the anterior–posterior axis of the embryo. In all cytochalasin B-treated trochophores studied, the cilia were found to be tilted toward the pretrochal (anterior) region in such a way that the effective stroke never extended to the most posterior side of the larva (Fig. 7A–C). This observation may explain why cytochalasin-treated embryos are not capable of swimming up toward the surface and remain on the bottom of the dish instead. This prompted us to investigate the effects of cytochalasin B on the organization of the F-actin band.

Phalloidin labeling of trochophores treated with cytochalasin B for 10–20 min revealed a seemingly normal band of F-actin, even though the cortical labeling had become very punctate (not shown). However, in embryos treated for 80 min, the actin band has become less pronounced, suggesting that some disassembly of F-actin had taken place (Fig. 7D). In embryos incubated with cytochalasin B for 110 min, no band of F-actin could be found (Fig. 7E).

The effects of cytochalasin B on the ultrastructure of the prototroch cells was studied in thin sections for electron microscopy. The surface of such treated cells displays many blebs, and the area of contact between neighboring cells has become limited to the belt desmosome. In addition, the layer of thin filaments is no longer observed in the apical part of the prototroch cells. However, striated rootlets were still found to be present, and their organization does not seem to be affected by treatment with cytochalasin (Fig. 5G).

Organization of F-actin in isolated trochoblasts and animal caps

From 7 different embryos a primary trochoblast was isolated at the 16-cell stage and cultured in filtered sea water. Such isolated cells divide twice and all four daughter cells develop cilia, as would occur in the whole embryo. Phalloidin labeling of these isolates show the presence of a localized band of F-actin at the base of the cilia (Fig. 8A–C). The F-actin band usually crosses the entire cell, although in some cells the band was not quite continuous. In other cells, a double band was sometimes observed.

From 15 embryos, the four animal micromeres of the 8-cell stage were isolated. These micromeres divide further and develop into two types of cells: small proliferating cells with nuclei often in mitosis, and ciliated cells with large interphase nuclei and less concentrated Hoechst labeling, which are the prototroch and apical cells. After TRITC–phalloidin labeling of these isolates, we found an F-actin band localized in each of the ciliated prototroch cells. However, these bands were never organized in a continuous band running from cell to cell; rather they seemed to be random in their direction relative to each other (Fig. 8D,E).

Organization of the prototroch and the actin band in the absence of a dorsoventral axis

The inductive process that causes the establishment of the dorsoventral axis occurs at the 32-cell stage (Van den Biggelaar, 1977) and is inhibited by treatment with monensin (Kühtreiber *et al.* 1988; Serras and Speksnijder, 1990). This treatment does not affect the anterior–posterior axis. Such monensin-treated embryos develop into trochophore larvae that possess a ring of ciliated prototroch cells. After labeling of such larvae with TRITC–phalloidin, we observed that in contrast to normal larvae, which display a double row of prototroch cells and a double actin band at one (the dorsal) side only, a double band of F-actin was found either at two opposite sides, or on all four sides of the embryo (Fig. 8F,G). *Via* bright field-optics and Hoechst labeling, we determined that these multiple regions with a double band represent multiple regions with a double row of prototroch cells. In addition, each of these regions is always found associated with an intensely fluorescent cluster of ectodermal cells in the post-trochal domain (Fig. 8F–I). It has been described that in monensin-treated embryos multiple (often four) mantle primordia are present (Kühtreiber *et al.* 1988). This suggests that the TRITC–phalloidin labeling found in the post-trochal domain (Fig. 8H,I) may correspond to multiple mantle primordia, which in normal embryos would develop the mantle and shell.

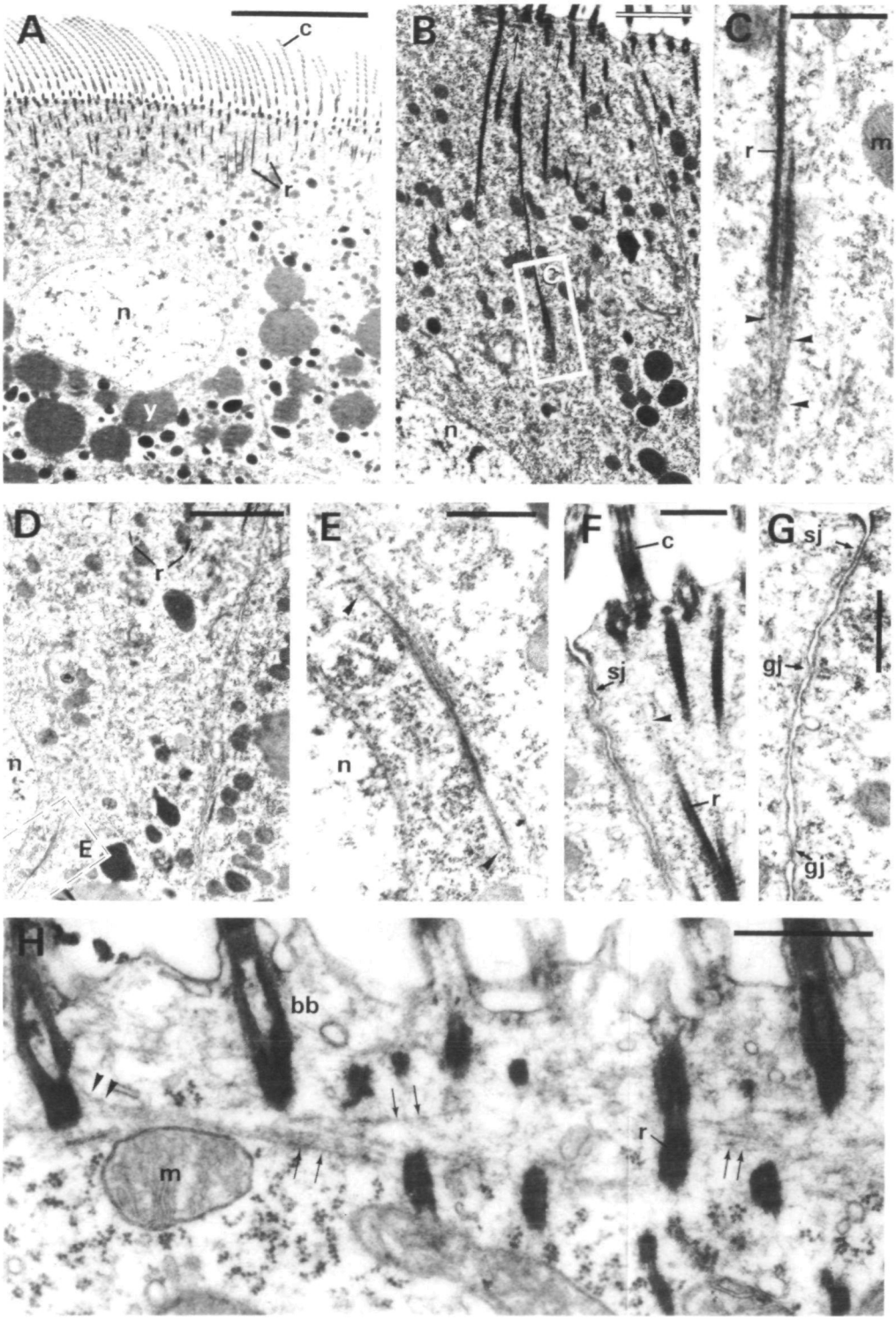


Fig. 6. Ultrastructural organization of the prototroch as seen in equatorial sections through the larva. A–G: fixation I; H: fixation II. bb, basal body; c, cilium; gj, gap junction; m, mitochondrion; n, nucleus; r, striated rootlet; sj, septate junction; y, yolk granule. (A) Overview of a prototroch cell showing the rootlets at the base of the cilia; Bar=10 μm. (B) Higher magnification showing an electron-dense layer (arrows) interconnecting the basal bodies and apical part of the rootlets, and a very long rootlet (about 8 μm) that extends toward the nucleus. Bar=2 μm. (C) Detail of the rootlet shown in B. Note the microtubules (arrowheads) that extend from it. Bar=0.5 μm. (D) Central part of a prototroch cell with rootlets at the top and nucleus at bottom left; Bar=2 μm. (E) Detail of D showing parallel cytoplasmic microtubules (arrowheads) that run past the nucleus toward the basal part of the cell. Bar=0.5 μm. (F) and (G) Junctional complexes between the lateral membranes of ciliated prototroch cells. Bar=0.5 μm. (H) Layer of thin filaments (arrows) in the apical part of the prototroch cell that interconnects the apical part of the rootlets and the basal part of the basal bodies (see arrowheads). Bar=0.5 μm.

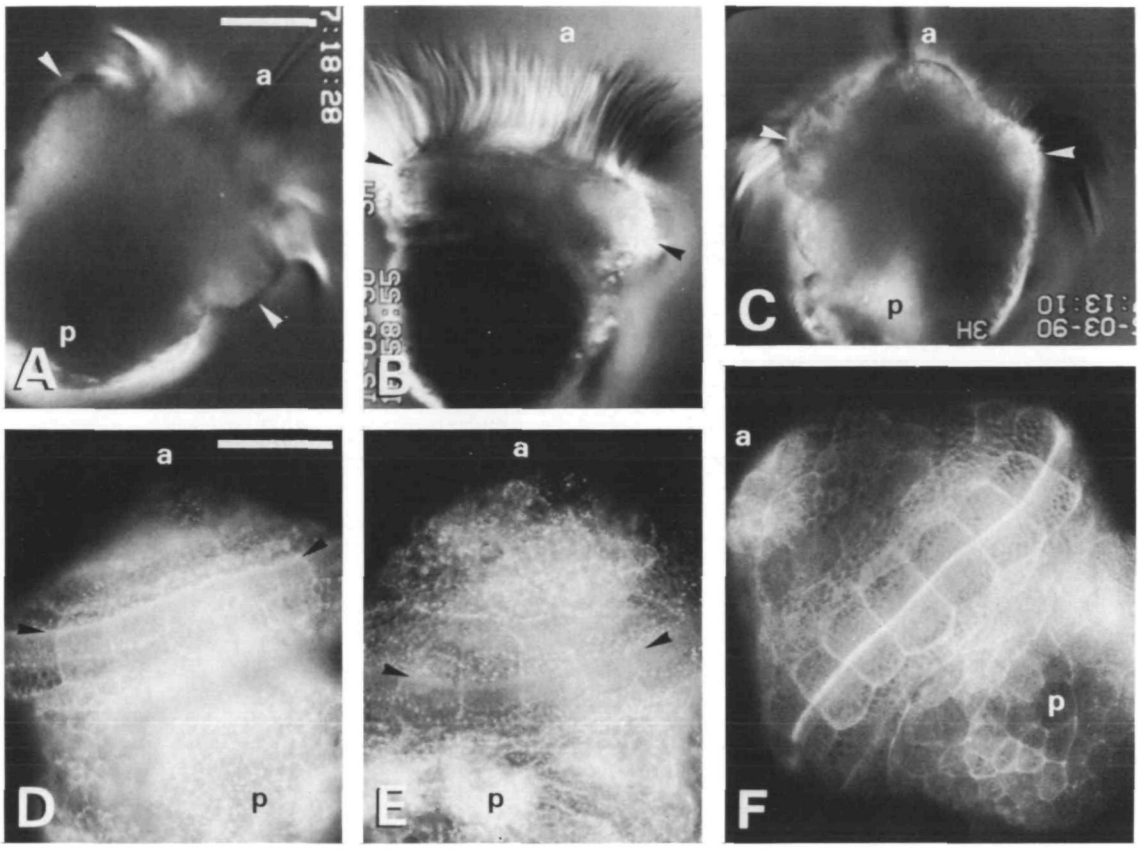


Fig. 7. Effects of cytochalasin on ciliary beat and organization of the F-actin band. a, anterior; p, posterior. Arrowheads indicate the position of the ciliated prototroch cells. Bar=50 μm. (A–C) Living trochophores photographed from a video monitor. (A,B) Cytochalasin B-treated trochophores. (C) Untreated control. Note that the cilia of the cytochalasin-treated trochophores are pointing more upwards toward the anterior pole than in the control. (D–F) Fluorescence images of TRITC–phalloidin labeled trochophores after treatment with cytochalasin B (D: 80 min; E: 110 min treatment; F: untreated control). Note the reduced intensity of the band at 80 min, and the absence of a band at 110 min.

Discussion

Our results show that filamentous actin becomes localized as a patch in both primary and accessory trochoblasts of the *Patella* embryo after the occurrence of cell cycle arrest in these cells (i.e. after the 64-and 88-cell stage, respectively). This patch of F-actin coincides in time and place with the onset of ciliation in these cells. As development proceeds, the cilia become organized as a row, and F-actin forms a distinct band, which crosses the entire trochoblast and is positioned at the base of this row of cilia. A repositioning of the trochoblast cells finally leads to the formation of a continuous ring of ciliated prototroch cells with an F-actin band that encircles the entire trochophore larva.

Three different aspects of the differentiation process of these trochoblasts are of particular developmental interest. First, the formation of an F-actin band in individual trochoblasts is an autonomous process, as is their ciliation (Wilson, 1904). This is clearly demonstrated by the capacity of isolated quartets of animal micromeres as well as of isolated primary trochoblasts to form a more or less straight actin band underlying a row of cilia. The animal micromeres of the 8-cell *Patella* embryo are thought to contain developmental determinants for ciliation (Janssen-Dommerholt *et al.* 1983), which are segregated to the trochoblasts at the following cleavages.

Second, the F-actin band is orientated perpendicular to the anterior–posterior axis of the embryo. During normal development of the prototroch, the trochoblasts shift their position and form an organized ring of ciliated cells (Serras *et al.* 1990). During this process each individual trochoblast retains the anteroposterior orientation of its F-actin band. However, isolates consisting of four animal micromeres from the 8-cell stage fail to organize the F-actin band in a circular ring perpendicular to the anterior–posterior axis. At present, it is unclear whether these micromeres are already polarized and require the posterior cells as a mechanical support to keep them in their proper orientation or whether the posterior cells are necessary to provide information concerning the anterior–posterior polarity of the embryo.

Third, the fully developed prototroch displays a distinct dorsal–ventral polarity. All embryos at 12 h of development or older possess a region where two rows rather than one row of prototroch cells are present, and

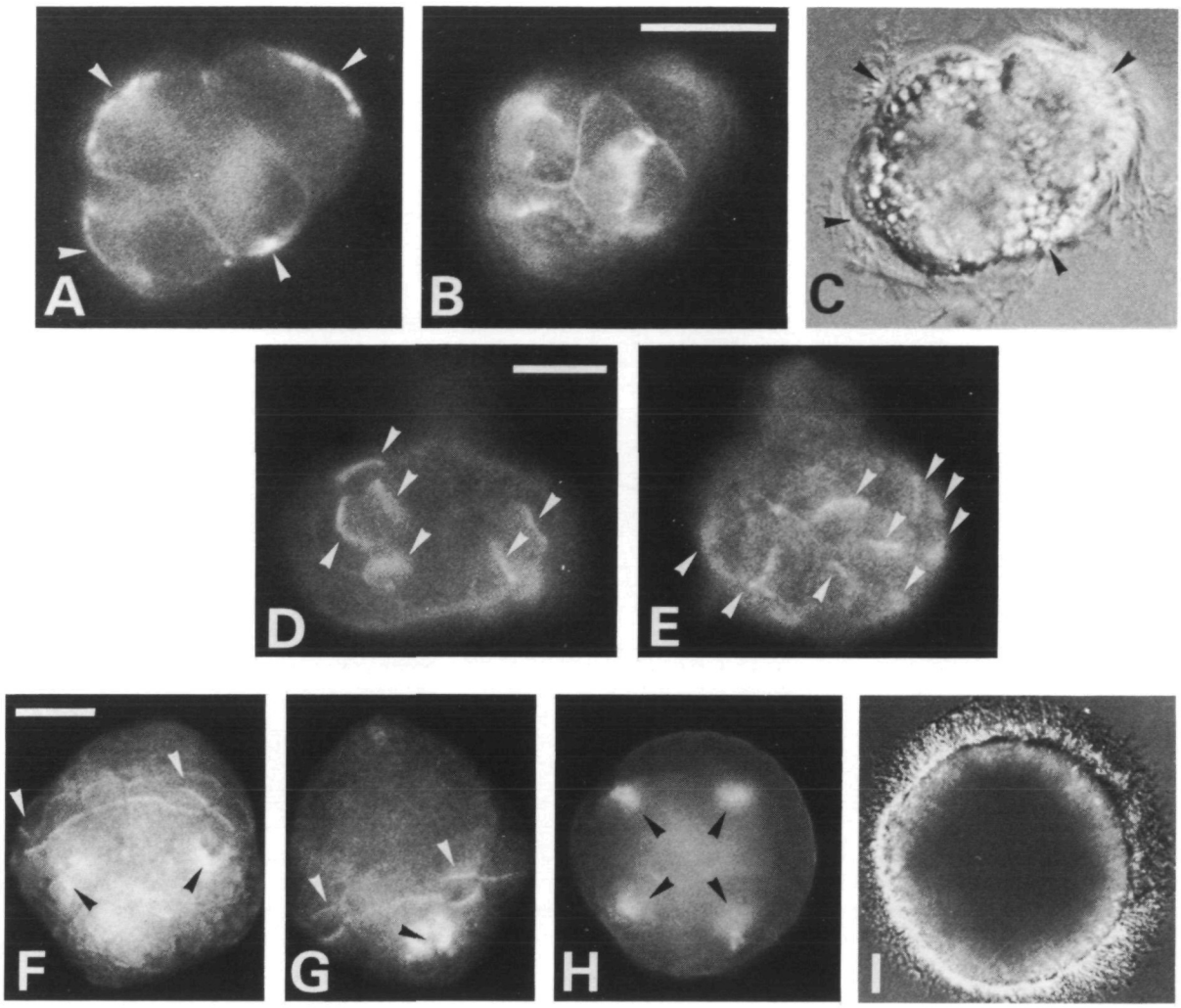


Fig. 8. Organization of the F-actin band in isolated trochoblast cells, isolated animal caps, and monensin-treated embryos. (A–C) Four ciliated cells (arrowheads) that developed from a primary trochoblast isolated at the 16-cell stage and fixed and stained with TRITC–phalloidin 16 h after isolation. Bar=25 μm. (A,B) Fluorescence images showing the F-actin bands in all 4 cells at 2 different focus levels; (C) shows the same cells in bright-field optics. (D,E) Two different partial embryos derived from the animal micromere cap isolated at the 8-cell stage viewed in fluorescence optics after labeling with TRITC–phalloidin. Arrowheads indicate the cells with an F-actin band. Bar=25 μm. (F–I) Localization of F-actin in monensin-treated embryos in which the development of the dorsal–ventral axis is inhibited. White arrowheads: double row of prototroch cells; black arrowheads: localization of F-actin in the post-trochal domain. Bar=50 μm. (F,G) Same trochophore viewed laterally from both sides. Note the 4 sites with a double F-actin band; two on each side of the trochophore. (H,I) Trochophore viewed from the post-trochal domain in fluorescence and bright-field optics; note the 4 areas with increased fluorescence intensity.

as a result two bands of F-actin are found. In normal embryos, this 'double region' is located at the dorsal side of the embryo as identified by the presence of mantle structures. However, in monensin-treated embryos, in which the establishment of the dorsoventral axis is inhibited (Kühtreiber *et al.* 1988), this dorsal–ventral pattern of the prototroch is disturbed, and one finds not one but two or four 'double regions', each one in a different quadrant of the embryo. In addition, two or four intensely fluorescent post-trochal domains are usually found underneath these double bands. This suggests that the inductive interactions that take place at the 32-cell stage and establish the dorsal–ventral axis (Van den Biggelaar, 1977; Van den Biggelaar and Guerrier, 1979), inhibit the formation of multiple 'double regions' in the prototroch and post-trochal ectodermal domains. This possibility is supported by the observation that multiple shell masses are formed in *Ilyanassa* embryos after deletion of the polar lobe which is known to contain dorsal information (Atkinson, 1971).

As to the nature of the structure that corresponds to the concentration of F-actin seen after TRITC–phalloidin labeling, our studies do not provide a conclusive answer. One candidate is the layer of thin filaments that interconnects the basal bodies and the apical parts of the striated rootlets and insert into the belt desmosome on the lateral surface. Such a layer of

phalloidin-staining actin filaments has also been found – although in a much more extensive form – in the macrociliary cells of the ctenophore *Beroë* (Tamm and Tamm, 1987). This possibility is supported by the observation that after treatment with cytochalasin, which disrupts the band labeled with TRITC–phalloidin, such thin filaments are no longer found in thin sections for electron microscopy. On the other hand, this layer of thin filaments extends only about 0.6 μm into the cytoplasm, whereas the fluorescent band found after TRITC-labeling extends about 7 μm deep. An alternative explanation would be that F-actin associated with the striated rootlets (which extend about 8 μm into the cytoplasm) is responsible for the fluorescent labeling pattern observed. Efforts to isolate the structural proteins of the striated rootlets from scallop gill epithelial cells have revealed that actin is probably closely associated with these structures (Stephens, 1975), although no evidence exist that actin forms an integral part of the rootlet (see Dentler, 1987). Since cytochalasin B does not affect the organization of the rootlets, but does destroy the F-actin band found after TRITC–phalloidin labeling, we would therefore suggest that TRITC–phalloidin binds predominantly to actin filaments closely associated with the striated rootlets.

In any case, our results clearly demonstrate an important role for actin in the organization of the microtubule-based motile apparatus of the *Patella vulgata* trochophore larva, and thus provide a striking example of microtubule–microfilament interactions and their significance for cellular functioning.

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