Changes in the distribution of cortical myosin during the cellularization of the *Drosophila* embryo

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SUMMARY

Changes in the distribution of myosin during the formation of the cellular blastoderm of *Drosophila melanogaster* were followed by staining sections of embryos with antibodies to myosin. These were visualized with indirect immunofluorescence. Prior to the start of cell membrane extension myosin is distributed between the nuclear caps as a thin sub-plasmalemma layer. There is also myosin present beneath the surface of the caps. When plasmalemma growth occurs, myosin is associated with the furrow canals, the tips of the advancing membranes. The fluorescence is distributed in an approximately hexagonal pattern around the growth points of each cell. The hexagons are joined up forming a network. It is suggested that this myosin is associated with bundles of microfilaments, orientated parallel to the surface, to form many interlocking contractile rings. The simultaneous contraction of these rings causes the cleavage of the blastoderm. During the first phase of membrane growth, myosin is also associated with the apical surfaces of the forming cells. At this stage these surfaces are rich in microvilli. However, by the time the furrow canals have reached the bases of the cells much of this myosin has disappeared. At about this time the apical surface becomes taut with a loss of the microvilli.

INTRODUCTION

For a wide range of organisms and particularly for many types of vertebrate and invertebrate egg, contractile rings have been described during cell cleavage. (For a review see Schroeder, 1975). These contractile rings are belts of ordered microfilaments, parallel to the plane of cleavage, and present immediately under the plasmalemma where cytokinesis is occurring. There is now good evidence that these provide the contractile force required for dividing cells in two after nuclear division. Several studies have shown the existence of contractile proteins in the cleavage furrows of dividing cells. Heavy-meromyosin-binding studies have demonstrated that the microfilaments of the contractile rings contain actin (Perry, John & Thomas, 1971; Schroeder, 1973; Forer & Behnke, 1972). Using fluorescent antibodies it has been possible to demonstrate the

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existence of both myosin and α-actinin in the cleavage furrows of dividing HeLa cells (Fujiwara & Pollard, 1976; Fujiwara, Porter & Pollard, 1978). Myosin is recruited into the contractile ring as it forms and remains there until the mid-body forms. α-Actinin follows a similar pattern except that it remains in the mid-body region, suggesting that it is directly attached to the membrane. Tropomyosin has also been reported in the furrows of cleaving sea urchin eggs (Ishimoda-Takagi, 1979). Direct evidence that cleavage depends on an actin-myosin contraction has been given by Mabuchi & Okuno (1977), who demonstrated that anti-myosin antibodies inhibited cleavage when injected into sea urchin eggs.

However, up to now the evidence for a contractile ring organization in Drosophila embryos has been tenuous. Because cell membranes form simultaneously around approximately 6000 nuclei in the cortex of the embryo a highly organized system for cleavage must exist. Mahowald (1963) and Fullilove & Jacobson (1971) have reported fibrillar material around the advancing furrow canal, the tip of the extending plasmalemma. This probably consists of microfilaments. Fullilove & Jacobson (1971) hypothesized that a network of microfilaments around the furrow canals of each cell all linking together over the whole surface of the embryo could act as many contractile rings. These would cleave the embryo into the required number of cells as a result of gradual contraction. In this paper we demonstrate that fluorescent antibodies against the heavy chain of Sarcophaga muscle myosin selectively stain the area around the furrow canals of forming blastoderm cells forming a hexagonal pattern. The hexagons may link up over the whole surface of the embryo. It is suggested that this network represents the contractile ring apparatus.

MATERIALS AND METHODS

The method of production of the antibodies and the demonstration of their specificity have been described elsewhere (Warn, Bullard & Maleki 1979). Briefly, antibodies were prepared against the heavy chain of electrophoretically purified Sarcophaga muscle myosin and shown to react both with Sarcophaga muscle myosin and with locust oocyte myosin.

Eggs of Drosophila melanogaster (Oregon R strain) were collected from mass cultures on grape plates smeared with yeast. They were collected after 30 min laying, aged at 25 °C until the required stage of development was reached and manually dechorionated. The embryos were then desiccated by drying for 5 min at 25 °C. This improves subsequent embedding without damage to the structure. They were then transferred to a formaldehyde:alcohol:distilled water (6:16:22) mixture and punctured with a fine glass needle usually towards the centre of the embryo. In order to ensure that the fixative and the dehydrating and clearing reagents could freely enter the embryo, a double puncturing method was used. Firstly, a fine prick was made. This rapidly killed the embryo
and coagulated the cytoplasm. After 1–5 min a second puncture was made at the same position as the first, making a small hole through the embryo. If done carefully this operation did not damage the major part of the embryo. Unfortunately the method of Zalokar & Erk (1977) of permeabilizing Drosophila embryos with heptane or octane could not be used, because in our hands there was a significant increase in background fluorescence. Embryos were dehydrated and embedded by a modification of Sainte-Marie’s method (Sainte Marie, 1962) as previously described. Sections were decorated firstly with either anti-myosin antibodies or non-immune antibodies at a final concentration of 2 mg/ml for 1 h and then washed for 15 min with PBS, pH 9.5. The sections were incubated in fluorescein-isothiocyanate-conjugated sheep anti-rabbit IgG (Wellcome) at 0.8 mg/ml for 1 h at 37 °C, washed again, and mounted in a 9:1 mixture of glycerol:1 M Trizma base, pH 9. Fluorescence was visualized using an epi-fluorescence attachment (Zeiss) with the appropriate filters for fluorescein. Photographs were taken using Tri-X film with an exposure time usually of 40 sec for fluorescence. The film was developed in Acuspeed (Paterson) which gave an effective ASA of 1250.

Various problems were encountered during the course of this work with non-specific fluorescence. The vitelline membrane was always brightly fluorescent, due to autofluorescence. Yolk granules were also ringed with weak fluorescence in controls. On occasion nuclei too would autofluoresce weakly (e.g. Figs. 3A, 4A). Unlike Sarcophaga or Drosophila myofibrils which showed no fluorescence when PBS at pH 9.5 was used, there was always faint background fluorescence when sections of Drosophila embryos were treated with non-immune sera (Figs. 1A, B). During the course of this study non-immune IgG (obtained either
prior to immunization or from non-treated animals) was purified from the serum of five animals. Four of these gave only the kind of weak background fluorescence seen in Fig. 1A. One, however, sometimes gave somewhat increased fluorescence in the cortical regions of the embryo. Sometimes this was all over the cortex, sometimes it was stronger towards the periphery of the embryo. The fact that a pH 9.5 wash reduced this fluorescence but did not affect that obtained with the anti-myosin antibodies suggests a binding effect described by Bennett et al. (1978). This indicates that further work should be done with affinity-column-purified antibodies as recommended by Herman & Pollard (1979).

RESULTS

After the migration of the bulk of the nuclei into the cortex of the syncytial embryo at stage 9 (stages of Zalokar & Erk, 1976) small protruberances or caps are present over these nuclei. (Fullilove & Jacobson, 1971; Turner & Mahowald, 1976). These remain during the four successive blastema cleavages although at each cleavage the area of each cap becomes reduced and the spaces between them are smaller (Turner & Mahowald, 1976). Figs. 2A–D show a cross-section and an oblique section of nuclear caps at stage 10. Each pair of photographs show the section firstly with epifluorescent optics which reveal the distribution of myosin antibodies and then in phase contrast. A thin line of fluorescence is visible under the plasmalemma of each cap. This appears to thicken somewhat in the regions between caps (Fig. 2A). In the oblique section, which cuts through the caps, the fluorescence appears round the edges and seems to be in association with what appear to be microvilli projecting from the surface of the cap (Fig. 2C). However, it is difficult to judge what the exact relationship is, given the thickness of the section. Similar results were obtained for stages 11, 12 and 13 (not shown). Figs. 3A–D show cross and oblique sections of caps at stage 14 immediately before the cellularization of the blastema. At this stage the areas between the caps are most probably where the cleavage furrow commences. The relationship of the fluorescence to the nuclear caps is much the same as for earlier stages. Fig. 3A shows a thin line of fluorescence above the caps with a thickening between them. This is seen again in Fig. 3C, where the section cuts through different levels of the caps. At the bottom left the tops of the caps are in focus and a thin layer of fluorescence is visible. What are probably microvilli are visible round the sides of the caps. Further to the right and higher up, the level of the bases of the caps is reached. Here, there is an increase in fluorescence associated with the edges of these bases. Below these and to the right, nuclei are in focus and little fluorescence is present in these regions as they lie immediately below the epicortical band of myosin.

After this stage cellularization of the blastema begins. Figs. 4A and B show a cross-section of the cortex shortly after this process has begun. The furrow canals have reached a level just below the tops of the nuclei in the cortex. These
Fig. 2. Sections of stage-10 embryos either in cross-section (A, B) or in oblique section (C, D). A and C decorated with anti-myosin IgG 2 mg/ml. B and D are phase-contrast images of the fields shown in A and C. Scale bar = 10 µm.

nuclei have already begun to elongate. By this stage the nuclear caps are no longer distinct entities. Scanning electron micrographs of this stage show an increased number of microvilli which mesh together between the forming cells with no evidence of any smoother membrane between them (Turner & Mahowald, 1976). The fluorescence associated with the epicortical region is still present and appears to have thickened somewhat, although the surface is somewhat irregular in Fig. 4(A, B), which makes the band seem wider. In addition a very thin line of fluorescence runs down with the new plasmalemma to what appears to be a right 'dot'. In fact this fluorescence surrounds the cavity of the furrow canal but due to light coming from below the plane of focus the cavity is more or less obscured. Fig. 4C and D shows an oblique
Fig. 3. Sections of stage-14 embryos either in cross section (A, B) or in oblique section (C, D). A and C decorated with anti-myosin IgG 2 mg/ml. B and D are phase-contrast images of the fields shown in A and C. Scale bars = 10 \( \mu m \).

section through the cortex of a Drosophila embryo at a slightly earlier stage than Fig. 4A and B. At lower left the fluorescence due to myosin in the superficial cortex is visible. This runs into a pattern of hexagonal fluorescence which surrounds the tops of the nuclei. Bottom right appears to be below the level of the furrow canals and the hexagonal pattern has more or less vanished.

Growth of the plasmalemmas occurs in two stages: a slow first phase that proceeds until the furrow canals reach the bases of the nuclei, and a fast second phase requiring only 5–10 min for growth to be completed to the bases of the cells (Mahowald, 1963). Figs. 5A–D show cross-sections and oblique sections across an embryo at a stage towards the end of the second phase of plasmalemma growth. In this embryo the furrow canals have probably reached the maximum lengths of the cells from which they move inwards to eventually cut off the bases of the cells from the underlying yolk. In cross-section (Fig. 5A, B) the fluorescence appears as triangles around the ‘holes’ of the canal furrows. Figure 5C shows a cut across the bases of the furrow canals revealing a bright honeycomb of fluorescence. Below the honeycomb there is little fluorescence whilst above it for a short distance fluorescence appears to be present mainly at the corners of the rough hexagons. Only a small amount of fluorescence is now visible in association with the apical surfaces of the cells in Fig. 5A.
DISCUSSION

In order to maintain the structure of the nuclear caps of the blastema some supporting system is required. This study locates myosin both in the surface of the caps and in regions between them. Myosin is known to be associated with microfilaments in non-muscle cells (Weber & Groeschel-Stewart, 1974; Fujiwara & Pollard, 1976, 1978) and it is therefore very possible that microfilaments play a major role in the maintenance of cap structure. However, this study does not give any evidence as to how much of the myosin is associated with microfilaments or how. Electron microscopy of these stages has yet to reveal micro-
filaments in and between caps but it is to be noted that Rickoll (1976) comments on the difficulties in finding microfilaments in this material. If we assume that much of the myosin visualized in our study is associated with microfilaments, then the following organization would fit the observed distribution. Microfilaments may surround the caps underneath the smoother intervening membrane. If these were under tension they would help maintain the caps in a raised position. In addition, for further support, particularly of the microvilli, a second, thinner meshwork may run across the undersurface of the plasmalemmas of the caps. At each blastema division the caps could be divided by
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shallow contractile bands. These observations fit with experiments where permeabilized blastema-stage embryos were treated with cytochalasin B (Zalokar & Erk, 1976). As a result the orderly division of nuclei was disturbed with anaphases frequently coming into contact as though a microfilament network which maintained the organization of the blastema surface had been disrupted.

The discovery of myosin around the furrow canals forming a hexagonal arrangement, probably throughout the embryo, strongly supports the concept first advanced by Fullilove & Jacobson (1971) that the cellularization of the blastema depends on the synchronous contraction of bands of microfilaments all joined together over the surface of the embryo. These bands would act as a series of many contractile rings both across and along the main axis of the egg, all joined together to form a single unit. They would continue to act as a single unit until the canal furrow had reached to the base of the forming cell. At this time the hexagonal contractile networks might become separated and might then contract independently to pinch off the bases of each cell. Rickoll (1976) has found that, at least in some areas, cytoplasmic connexions still exist between the cells of the blastoderm and the primitive yolk sac during early gastrulation, so the process of final closure of the cells is fairly slow. He finds circumferential bands of microfilaments around the connexions and also microfilaments below what are probably the remains of the canal furrows. This distribution of microfilaments fits well with the arrangement of myosin described here towards the end of the blastoderm stage.

Further evidence for a role of microfilaments in the cellularization of the blastoderm comes from experiments where permeabilized embryos were treated with cytochalasin B just before the final blastema cleavage (Zalokar & Erk, 1976). No cell membranes formed although the cell nuclei elongated, suggesting that membrane extension is coupled with contraction of bands of microfilaments.

The layer of myosin just beneath the plasmalemma of the forming blastoderm cells may well be a part of a band of microfilaments covering the undersurface of the caps. Such a band may have several functions. The downward movement of the ends of the plasmalemmmas would put pressure on the surface membrane which is thrown up into many microvilli during the first phase of growth. An apical band of microfilaments would support the surface and counteract the downward tension. It would maintain the dimensions of the cell as the furrow canals move downwards. During the first phase of membrane extension there is a significant amount of myosin associated with the apical plasmalemmmas of the forming cells. However, by the time the furrow canals have reached what will become the bases of the cells much of the specific fluorescence has disappeared leaving general background fluorescence. Turner & Mahowald (1976) found that during the second phase of plasmalemma growth the apical surfaces of the cells become smooth. They speculated that this phase occurs as a result of a straightening out and pulling down of the microvilli. If this is so a
subplasmalemma band of microfilaments is a possible agent for controlling the
tautening process. When this was completed it might be expected that the
microfilaments would largely disintegrate.

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