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Cleavage furrow: timing of emergence of contractile ring actin filaments and establishment of the contractile ring by filament bundling in sea urchin eggs

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SUMMARY

Cleavage furrow formation at the first cell division of sea urchin and sand dollar eggs was investigated in detail by fluorescence staining of actin filaments with rhodamine-phalloidin of either whole eggs or isolated egg cortices. Cortical actin filaments were clustered at anaphase and then the clusters became fibrillar at the end of anaphase. The timing when the contractile ring actin filaments appear was precisely determined in the course of mitosis: accumulation of the contractile ring actin filaments at the equatorial cell cortex is first noticed at the beginning of telophase (shortly before furrow formation), when the chromosomal vesicles are fusing with each other. The accumulated actin filaments were not well organized at the early stage but were organized into parallel bundles as the furrowing progressed. The bundles were finally fused into a tightly packed filament belt. Wheat germ agglutinin (WGA)-binding sites were distributed on the surface of the egg in a manner similar to the actin filaments after anaphase. The WGA-binding sites became accumulated in the contractile ring together with the contractile ring actin filaments, indicating an intimate relationship between these sites and actin filament-anchoring sites on the plasma membrane. Myosin also appeared in the contractile ring together with the actin filaments. The 'cleavage stimulus', a signal hypothesized by Rappaport (reviewed by R. Rappaport (1986) *Int. Rev. Cytol.* 105, 245-281) was suggested to induce aggregation or bundling of the actin filaments in the cortical layer.

Key words: sea urchin egg, cleavage furrow, contractile ring, actin filament, surface WGA-binding sites, myosin, cleavage stimulus

INTRODUCTION

Cleavage in animal cells is accomplished by active constriction of the contractile ring (equilateral cleavage) or the contractile arc (unilateral cleavage) formed in the cleavage furrow cortex (for review see Schroeder, 1975; Mabuchi, 1986). The contractile ring or arc is mainly composed of actin filaments of opposite polarities oriented in parallel to the cleavage plane (Sanger and Sanger, 1980; Mabuchi et al., 1988). The contraction is likely to be generated by the interaction between these filaments and myosin (Mabuchi and Okuno, 1977; Cande, 1980; Kiehart et al., 1982; Knecht and Loomis, 1987; De Lozanne and Spudich, 1987). However, the mechanism of formation of the contractile ring (arc) actin filaments has not been clear. It has long been supposed that a signal called cleavage stimulus (for review, see Rappaport, 1986) is transferred from the mitotic apparatus to the cell cortex to induce the cleavage furrow. This cleavage stimulus may have an important role in the contractile ring (arc) actin filament organization. However, both entity and function of the stimulus (signal) are not known. Furthermore, where the contractile ring (arc) actin filaments originate in the cell, how these filaments accumulate in the furrow cortex, and how they are organized into the parallel structure are not known.

Recently, a method to visualize actin filaments in the cell has been developed using fluorescently labelled phallotoxins. Thus, it has become possible to investigate the arrangement of these filaments in the cortical layer isolated on a substratum (Yonemura and Kinoshita, 1986; Yonemura and Mabuchi, 1987; Schroeder and Otto, 1988). In this study, visualization of cortical actin filaments in the whole sea urchin egg is newly devised and the process of cleavage furrow formation in sea urchin eggs is investigated in detail using this technique. The contractile ring actin filaments appeared in the equatorial cortex when chromosomal vesicles were fusing with each other, and were then organized into the contractile ring by bundling. Furthermore, a possibility that either surface lectin-binding sites (Yoshigaki et al., 1989) or myosin (Mabuchi, 1986) are relevant to the cleavage signalling is investigated in this system by using fluorescently labelled WGA or myosin antibodies, based on the idea that signal molecules would accumulate in the anticipated furrow region prior to the assembly of the contractile ring actin filaments. A portion of this study has been published in a preliminary form (Mabuchi, 1990).

MATERIALS AND METHODS

Eggs

Eggs of sea urchins, *Hemicentrotus pulcherrimus*, *Anthocidaris crassispina*, *Strongylocentrotus nudus*, and *Pseudocentrotus depressus*, and of a sand dollar *Clypeaster japonicus* were obtained by acetyl-

choline-induced spawning. Eggs were fertilized and the fertilization membranes were removed by treating the eggs with 10 volumes of 1 M urea, 10 mM $NaHCO_3$ (regular sea urchins) or Ca^{2+}-free artificial sea water (CaFSW) (*C. japonicus*), followed by passage through a nylon mesh. Then they were cultured at 20-21°C in CaFSW until the first cleavage.

Preparation of anti-egg myosin antibodies

Precipitates of crude egg myosin were obtained from unfertilized eggs of *Tripneustes gratilla* according to the method of Kane (1980). They were dissolved in 1 M KCl, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM MOPS buffer, pH 7.4, 1 mM dithiothreitol, 10 μg/ml leupeptin, and re-precipitated by dialysis against 50 mM KCl, 10 mM MOPS buffer, 0.2 mM dithiothreitol, 5 μg/ml leupeptin. Precipitates formed were dissolved in 0.8 M KC1, 10 mM MOPS buffer, 0.2 mM dithiothreitol, 5 μg/ml leupeptin, clarified by centrifugation at 100,000 ***g*** for 30 minutes, supplemented with 5 mM ATP, and applied to a Sepharose 4B column that had been equilibrated with the same buffer solution containing 0.2 mM ATP. A myosin fraction eluted just after a flow-through fraction was then applied to a hydroxylapatite column. Myosin was eluted with 0.2 M phosphate buffer (pH 6.8). This fraction was electrophoresed in the presence of SDS on a 6% polyacrylamide slab gel. The heavy chain band was cut out after brief staining with Coomassie Brilliant Blue, homogenized and dialyzed against a phosphate-buffered saline, emulsified with Freund's complete adjuvant, and injected subcutaneously into a male rabbit. Subsequent injections were performed twice with intervals of 4 and 2 weeks, respectively. Antiserum was obtained 3 weeks after the last injection. Specificity of the antibodies was tested by immunoblotting on a nitrocellulose membrane onto which total *T. gratilla* egg extract proteins had been transferred from a 6% acrylamide SDS-gel as described previously (Mabuchi and Kane, 1987).

Labelling of whole eggs with rhodamine-phalloidin

Eggs were attached onto a protamine (10 mg/ml)-coated glass slide and fixed for 0.5 to 1 hour at room temperature with 5% (v/v) formalin dissolved in F-buffer (0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM MOPS buffer, pH 7.4). They were further incubated for 30 minutes in 5% formalin in F-buffer that contained 0.8 M glucose and 0.2% Nonidet P40. They were then washed with F-buffer containing 0.8 M glycerol (glycerol-F-buffer) and stained with 0.3 μM rhodamine-conjugated phalloidin (Rh-ph; Molecular Probes, Inc., Junction City, OR, USA) and 0.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI) dissolved in glycerol-F-buffer and 0.1 M 2-mercaptoethanol in order to see actin filaments and chromosomes, respectively. The solution between the coverslip and the glass slide was blotted elaborately with pieces of blotting paper to compress the eggs.

Indirect immunofluorescence

Eggs attached on a glass slide were fixed and washed as described above, and incubated with normal goat serum (1/50 dilution) for 30 minutes at room temperature. Then they were incubated with anti-myosin serum (1/500 dilutioin) for 1 hour. They were washed for 6 hours in glycerol-F-buffer and then incubated with rhodamine-conjugated goat anti-rabbit IgG (Cooper Biomed. Inc., Malvern, PA, USA) for 1 hour. After washing overnight, the eggs were stained with 0.3 μM fluorescein-conjugated phalloidin (Fl-ph; Molecular Probes, Inc.) dissolved in glycerol-F-buffer and 0.1 M 2-mercaptoethanol.

Fluorescent labelling of surface WGA-binding sites

Cultured eggs were incubated in CaFSW containing 5 μg/ml fluorescein-conjugated WGA (Fl-WGA, Vector Laboratories, Burlingame, CA, USA) for 1 minute, washed once with CaFSW, stuck onto the protamine-coated glass slide, fixed, and stained with Rh-ph and DAPI as described above.

Flourescent labelling of isolated cortices

Cortices of cleaving eggs were isolated on a protamine-coated glass slide (Vacquier, 1975; Yonemura and Mabuchi, 1987) using F-buffer containing 0.8 M glucose and 10 μg/ml leupeptin. They were immediately fixed with 5% formalin dissolved in glycerol-F-buffer for 1 hour. After washing with glycerol-F-buffer, they were supplemented with Rh-ph plus 0.1 M 2-mercaptoethanol.

Fluorescence microscopy

The fluorescently labelled specimens were examined with an Optiphot microscope (Nikon, Co. Ltd, Tokyo) equipped with EFD epifluorescence optics (Nikon). The objective lenses used were Fluor 20, Fluor 40 and Plan 100 DIC. Photographs were taken on Kodak T-Max films (ASA 400).

Scanning electron microscopy

Eggs were attached onto protamine-coated coverslips and fixed with 2.5% glutaraldehyde in CaFSW for 30 minutes and then with 1% OsO4 in CaFSW for 30 minutes. The samples were dehydrated in ethanol, immersed in isoamyl acetate, critical point dried in CO_2, and coated with gold-palladium. They were examined with a Hitachi S-2500 scanning electron microscope.

RESULTS

Time-course of division of eggs

The time-course of cytokinesis was recorded by photographing eggs every 30 seconds or video-recording after anaphase. The diameter of the egg at the anticipated furrow region (equator), and at the subsequent cleavage furrow was plotted versus time (Fig. 1). The egg slightly elongated at late anaphase to early telophase, and its diameter at the equator was reduced gradually. The indentation of the equatorial cortex (the cleavage furrow) was formed about 90 seconds after the onset of the elongation in *A. crassispina*, 100 seconds in *H. pulcherrimus* and *C. japonicus*, and 3 minutes in *S. nudus.* The diameter at the furrow reduced acceleratedly, and the reduction speed reached a maximum about 60 seconds after the onset of the furrowing in *H. pulcherrimus* and *C. japonicus*, 90 seconds in *A. crassisipina* and 2 minutes in *S. nudus*. The contraction proceeded at this speed until the diameter of the eggs at the furrow reached 10-30% of the original. In the examples shown in the figure, the maximum speed (the length of the circumference of the egg at the furrow/minute) was 54 μm/min for *H. pulcherrimus* egg, 50 μm/min for *C. japonicus* egg, 48 μm/min for *A. crassispina* egg, and 43 μm/min for *S. nudus* egg. The reduction in the equatorial diameter before furrowing lead us to examine whether the contractile ring is already formed at this stage.

Fluorescence localization of actin in whole eggs

In a previous study (Mabuchi, 1990) whole eggs were observed without compression using a spacer between a glass slide and a coverslip. The eggs were compressed after fixation and staining in the present study. The advantage of this method was that both the organization of the cortical actin filaments and the appearance of DNA could be observed in much more detail. The width of the cleaving eggs became relatively larger compared to the length after compression. However, the indentation of cortex at the cleavage furrow, even at an early stage of cytokinesis, did not disappear after the compression.

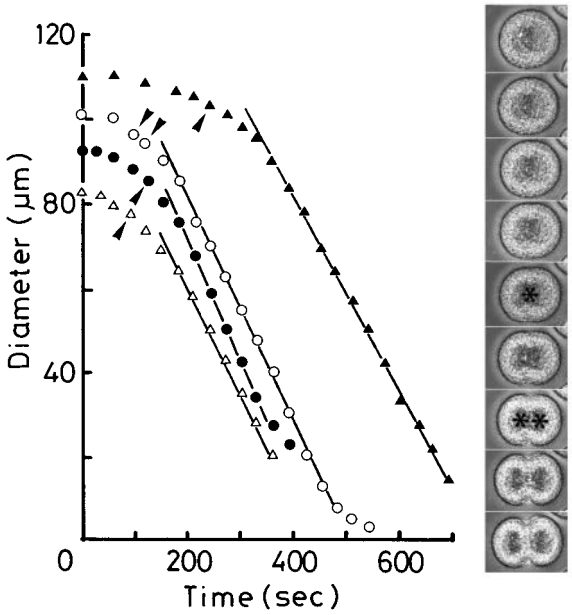

Fig. 1. Time-course of cleavage of sea urchin eggs. The diameter of the egg at the equator is plotted versus time. The solid line indicates that the diameter is reduced in proportion to time during mid-cleavage. △, *A. crassispina*; ●, *H. pulcherrimus*; ○, *C. japonicus*; and ▲, *S. nudus*. The arrowheads indicate the onset of furrow formation. The onset for the *C. japonicus* egg is marked with two arrowheads since this egg cleaved in a slight asymmetric fashion. The right pictures show a *H. pulcherrimus* egg, from which the data in this figure were obtained at times 0, 30, 90, 60, 120, 150, 180, 210 and 240 seconds. An asterisk indicates the onset of furrow formation, and the two asterisks indicate the initiation of contraction at maximal speed.

Actin filaments in the cortical layer of *H. pulcherrimus* eggs at the anaphase-telophase transition, where the chromosomes began to be vesiculated, appeared to be clustered as seen by Rh-ph fluorescence staining. The clusters were about 5 μm in diameter and their appearance did not seem to be fibrous (Fig. 2a). The actin appearance before this stage will be described later. Until this stage, no difference in the cortical actin organization was detected between the equatorial and polar regions. The clusters of actin filaments turned out to be fibrous when the chromosomal vesicles began to fuse with each other and the egg elongated slightly in the pole-to-pole direction at telophase (Fig. 2b). This suggests that the clustered actin filaments formed bundles.

The first sign of contractile ring formation was observed at this stage: the density of the actin filaments at the equatorial zone of the cortex increased slightly. The width of such a zone was 15-20 μm in the compressed egg. The equatorial actin filament bundles did not form distinct parallel structures, but were associated with each other into dense meshwork. After fusion of the chromosonal vesicles to form daughter nuclei, the elongation of the eggs proceeded although the cleavage furrow was not recognized yet (Fig. 2c). The cortical actin filament bundles began to disintegrate again except at the equatorial zone. When the cleavage furrow was recognized, the actin filaments in the equatorial cortex were more tightly associated with each other (Fig. 2d). In the advanced furrow, the width of the ring became narrow (3 μm) and the intensity of the rhodamine fluorescence became very strong so that the detailed structure could not be recognized (Fig. 2e). It is known from electron microscopy that the contractile ring is established and the actin filaments are oriented in a parallel fashion in the ring at this stage (Schroeder, 1975). On the other hand, the actin organization in the bulk cortical layer turned out to be uniform.

Scanning electron microscopy of the egg surface

Whether microvilli interfered with the above examination was checked by scanning electron microscopy, since fertilized eggs have numerous microvilli on the surface and each microvillus contains an actin filament bundle (Burgess and Schroeder, 1977). As shown in Fig. 3, the surface of the eggs cultured in CaFSW and then fixed with glutaraldehyde was covered with numerous microvilli. However, most of the microvilli were detached from the eggs treated with 5% formaldehyde and then with 0.2% Nonidet P40. Therefore, the actin patterns observed above were not due to the microvillar actin filaments but due to the cortical actin filaments.

Fluorescence localization of actin in isolated cortices

Four cortices from *C. japonicus* eggs, which contained the contractile ring region, are arranged in Fig. 3 in a possible sequence of the contractile ring formation. Those from *H. pulcherrimus* eggs showed similar patterns. Since these cortices are dumbbell-shaped, they are considered to be derived from eggs of stages between those shown in Fig. 2d and e, respectively.

The actin filament bundles in the cleavage furrow region (Fig. 4a,b) were oriented roughly in parallel with each other. The bundles then seemed to fuse or very closely associate with each other to form a belt (Fig. 4b,c). The density of the actin filaments in the belt was very high compared to that of the bulk cortex. In the advanced furrow (Fig. 4d), the actin filaments were organized into a dense and thin bundle.

It is occasionally observed in normal division of *C. japonicus* eggs that the mitotic apparatus is formed at a slightly eccentric position, which causes an asymmetric furrow formation. In the egg shown in Fig. 5a, which was not compressed, a furrow is seen only in its left side. Staining of actin filaments with Rh-ph in this egg revealed that the filaments seemed to be more concentrated in the side of the egg where cleavage started than in the other side where the cleavage furrow was not seen yet (Fig. 5b). Some of the cortices isolated from *C. japonicus* eggs at this stage show a peculiar pattern reflecting this asymmetric furrow formation: actin filament bundles were densely accumulated in a part of the cortex while the bundles were fusing with each other in the neighboring region (Fig. 5c,d). In other words, the process shown in Fig. 4 is seen in one egg cortex.

Co-localization of surface WGA-binding sites and the actin filaments in the cleavage furrow

Fl-WGA was added to live *H. pulcherrimus* eggs and the localization of surface WGA-binding sites was compared to that of the cortical actin filaments. At metaphase, the actin filaments were uniformly distributed in the cortex, while the WGA-binding sites were distributed on the surface as small dots (Fig. 6a). The density of the dots was 2.0-2.6/μm^2. At mid-anaphase the dots became aggregated and some fibrillar appearances were

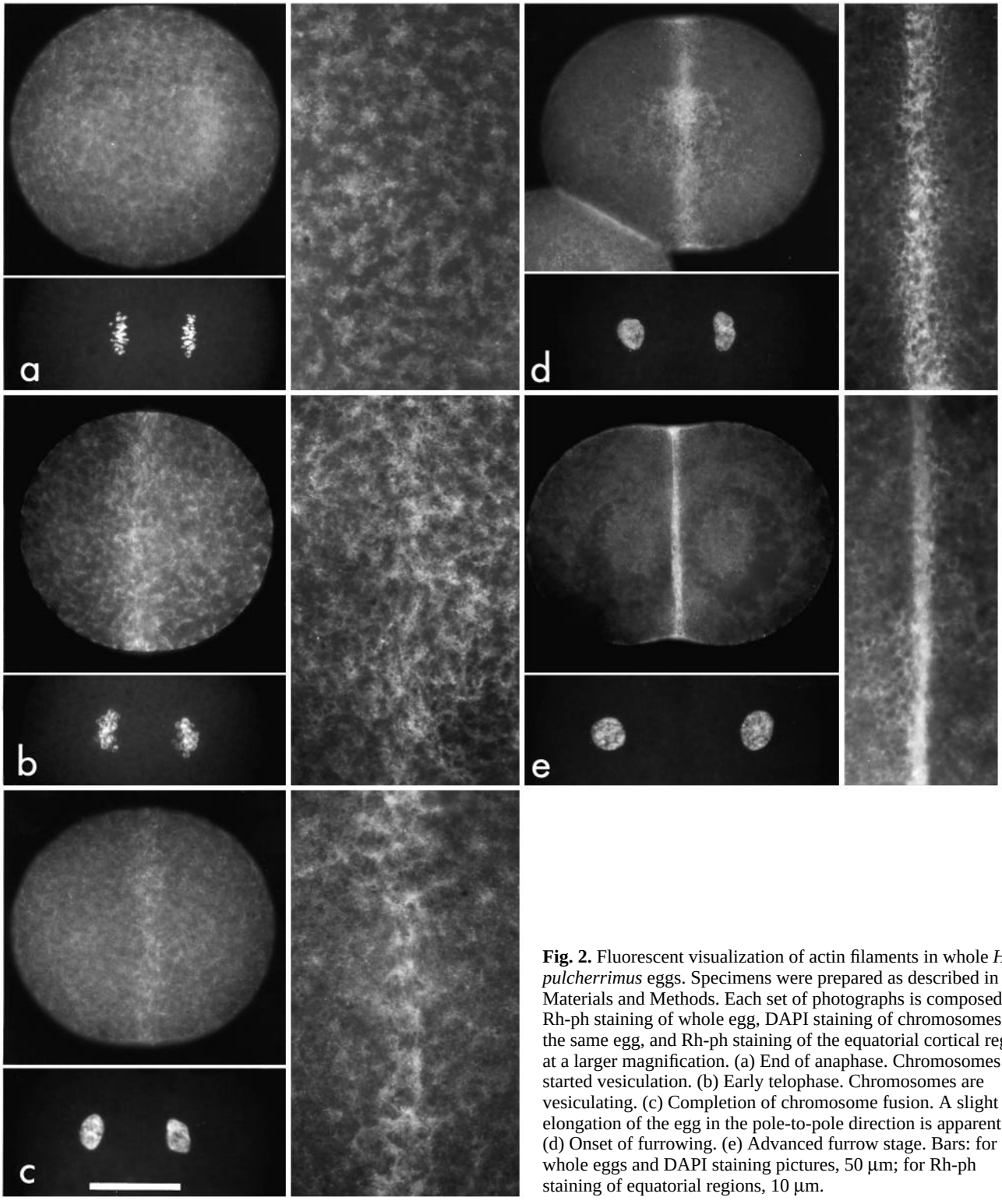

Fig. 2. Fluorescent visualization of actin filaments in whole *H. pulcherrimus* eggs. Specimens were prepared as described in Materials and Methods. Each set of photographs is composed of Rh-ph staining of whole egg, DAPI staining of chromosomes in the same egg, and Rh-ph staining of the equatorial cortical region at a larger magnification. (a) End of anaphase. Chromosomes just started vesiculation. (b) Early telophase. Chromosomes are vesiculating. (c) Completion of chromosome fusion. A slight elongation of the egg in the pole-to-pole direction is apparent. (d) Onset of furrowing. (e) Advanced furrow stage. Bars: for whole eggs and DAPI staining pictures, 50 μm; for Rh-ph staining of equatorial regions, 10 μm.

observed (Fig. 6b). The pattern was similar to that of the actin filaments, but the former was more punctate. The fibrillar appearance of the surface WGA-binding sites was further developed at late anaphase to telophase. At the anaphase-telophase transition when chromosomal vesiculation started, the distribution of the cortical actin filaments and that of the surface WGA-binding sites became very similar (Fig. 6c). The stage of the egg shown in Fig. 6c almost corresponded to that of the egg shown in Fig. 2b but the accumulation of the contractile ring actin filament was not seen in this egg yet. After all the chromosomal vesicles fused with each other, when the actin filament accumulation in the equatorial cortex was obvious, the surface WGA-binding sites also accumulated in the same region and showed a similar pattern (Fig. 6d,e). In the cleaving egg, the

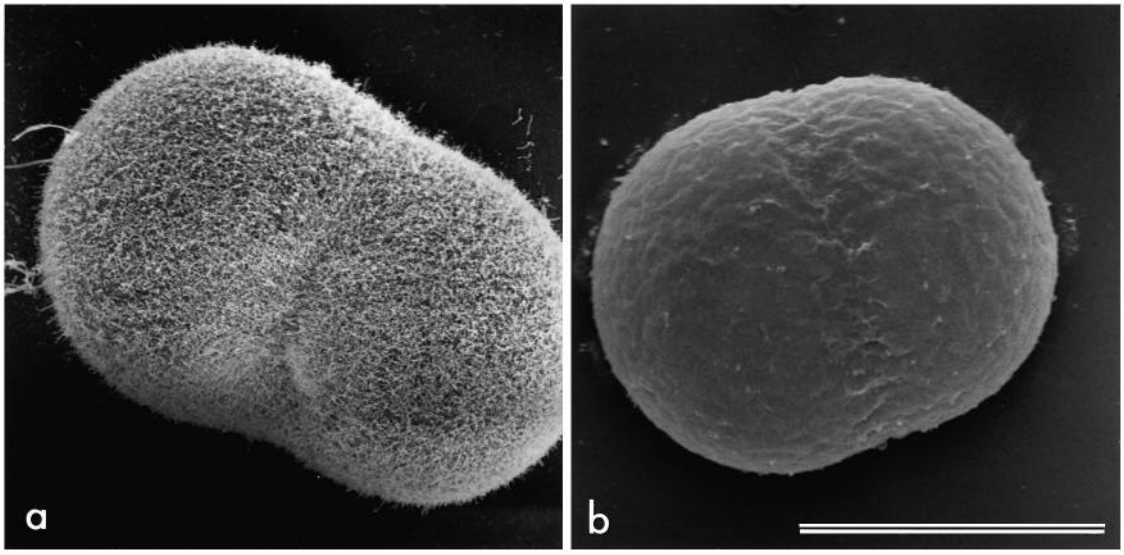

Fig. 3. Scanning electron micrographs of dividing *H. pulcherrimus* eggs. Specimens were examined as described in Materials and Methods. (a) The egg fixed with 2% glutaraldehyde and then with 1% OsO_4. (b) The egg fixed with 5% formalin followed by treatment with 0.2% Nonidet P40 prior to the glutaraldehyde-OsO_4 fixation. Bar, 50 μm.

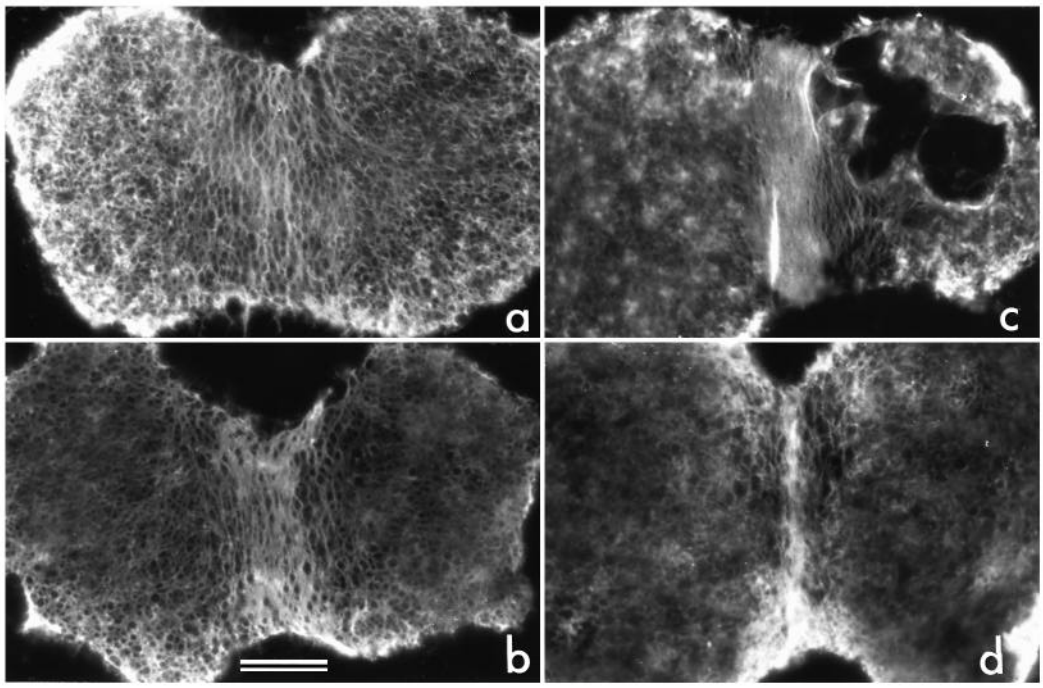

Fig. 4. Isolated cortices of *C. japonicus* eggs stained with Rh-ph. This series of pictures (from a to d) show the possible sequence of the formation of the contractile ring as revealed by Rh-ph staining. Bar, 10 μm.

sites were concentrated in the cleavage furrow (not shown) as reported previously (McCaig and Robinson, 1982; Yoshigaki et al., 1989). The above results were confirmed using cortices isolated from eggs of various division stages.

Next, it was investigated whether cytochalasin B, which has been known to inhibit furrowing (Schroeder, 1972), would interfere with accumulation of the contractile ring actin filaments but would not affect that of the WGA-binding sites. When *P. depressus* eggs between metaphase and late anaphase were incubated for 10 minutes in CaFSW containing 4 μM cytochalasin B prior to the treatment with Fl-WGA, cortical actin filaments aggregated irregularly leaving some dark area where almost no detectable actin filaments were present (Fig. 7). These eggs did not divide. The contractile ring was not formed, or was poorly organized probably because of the irregular aggregation of the accumulated actin filaments (Fig. 7). Even in these eggs, the actin filaments and the WGA-binding sites are co-localized.

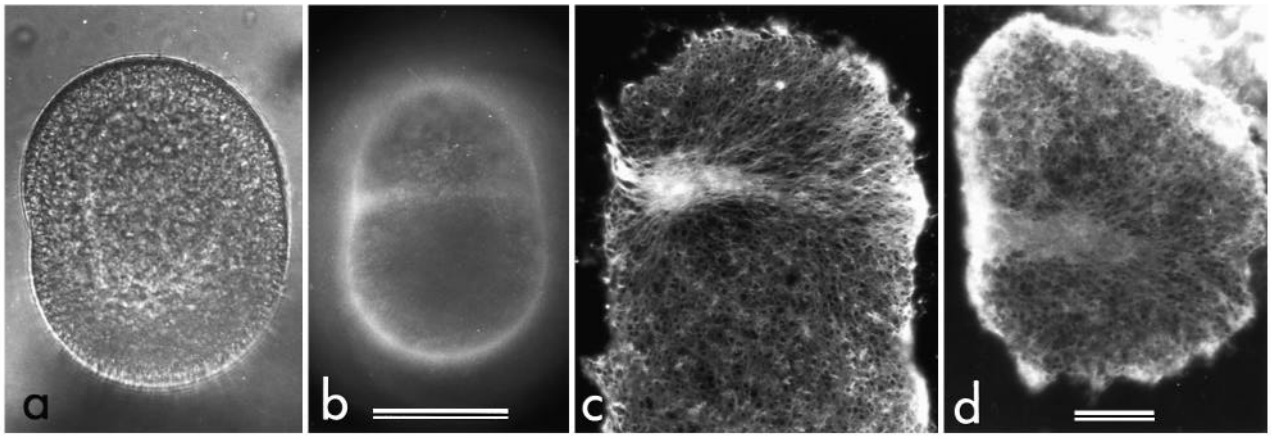

Fig. 5. Fluorescent visualization of actin filaments in *C. japonicus* eggs achieving an asymmetric cleavage. (a) A Nomarski image of an egg prepared without compression showing the cleavage furrow only at the left side. (b) The same egg as in a, stained with Rh-ph. (c and d) Isolated cortices stained with Rh-ph showing the process of the contractile ring organization. Bars: a and b, 50 μm; c and d, 10 μm.

Immunofluorescent detection of myosin in the contractile ring

The antiserum against egg myosin heavy chain used in this study was shown to be specific to the heavy chain by immunoblotting (Fig. 8). Whether myosin appears in the anticipated furrow region prior to appearance of the contractile ring actin filaments was studied by imunofluorescence microscopy using *C. japonicus* eggs forming an asymmetric contractile ring. When the leading edge of the contractile ring was examined, the fluorescent phalloidin staining and myosin immunofluorescence completely coincided with each other.

DISCUSSION

Emergence and organization of the contractile ring

The contractile ring formation in the first cleavage of sea urchin and sand dollar eggs was examined by staining actin filaments using Rh-ph, and the stage where it occurs was determined precisely for the first time. The accumulation of the actin filaments in the equatorial cell cortex was first detected at the fusion of chromosonal vesicles, that is, early telophase in *H. pulcherrimus* eggs and a little later in *C. japonicus* eggs. Schroeder (1972) first found the contractile ring actin filaments in sea urchin eggs (*Arbacia punctulata*) by electron microscopy, but he could recognize these filaments only after the cleavage furrow was formed. The reason for this may be that the actin filaments that accumulate in the anticipated cleavage furrow region would not be well organized in *A. punctulata* as well *H. pulcherrimus* and might be difficult to detect by electron microscopy in thin sections. On the other hand, Usui and Yoneda (1982) reported with *Toxopneustes pileolus* and *H. pulcherrimus* eggs that the actin filament meshwork appeared all over the entire cortex at anaphase and that a denser meshwork appeared in the anticipated furrow region about 7 minutes before the onset of cleavage. The latter denser meshwork seems to correspond to the accumulation of actin filaments in the anticipated furrow region found in this study. However, the accumulation was first seen when the eggs became slightly elongated or about 100 seconds before the onset of furrowing in the present study. The reason for the large discrepancy is not clear; it may be due to the difference in the methods used.

The accumulated actin filaments in the equatorial zone at the early stage were not highly organized as well as those in the bulk cortex, and the width of the accumulated filament zone was wide. The zone became narrow and the density of the filaments became high as the division progressed. It is clearly demonstrated with either whole eggs or isolated cortices that packing of the actin filament bundles is involved in this process. In newt eggs, which undergo unilateral cleavage, the contractile arc is continuously formed at the tips of the advancing furrow from pre-existent cortical actin filaments through bundling (Mabuchi et al., 1988). Therefore, it is shown that both the contractile ring and the contractile arc are organized by bundling of the cortical actin filaments. The bundling of the contractile ring actin filaments may at least partly be caused by actin crosslinking proteins, such as alpha-actinin (Mabuchi et al., 1985), spectrin (Kuramochi et al., 1986; Fishkind et al., 1987) and 255 kDa protein (Mabuchi and Kane, 1987; Maekawa et al., 1987). In the contractile arc of newt eggs, some actin-crosslinking strands were actually recognized by electron microscopy (Mabuchi et al., 1988).

Contractile ring formation and contraction

As seen in Fig. 1, eggs become elongated in the pole-to-pole direction before furrowing, suggesting that the anticipated furrow region may already elicit contractile force. Actually, the actin filaments emerge at the equator and form a meshwork of bundles at this stage. Thus, it is plausible that the meshwork is already contracting. The elongation in *H. pulcherrimus* eggs starts about 2 minutes before attainment of the maximum rate of contraction of the cleavage furrow. It is likely that the maximum rate of contraction is obtained by the tightly packed contractile ring actin filaments. In other words, the contractile ring is established as it contracts. The early contraction would help with the compaction of the actin filaments in the equatorial cortex. Unfortunately, there has been no direct measurement of the force exerted at the equatorial cortex at the early stages, although for late furrows the force was measured (Rappaport, 1967; Hiramoto, 1975). Nevertheless, Yoneda and Dan (1972) and Hiramoto (1968) calculated the constricting

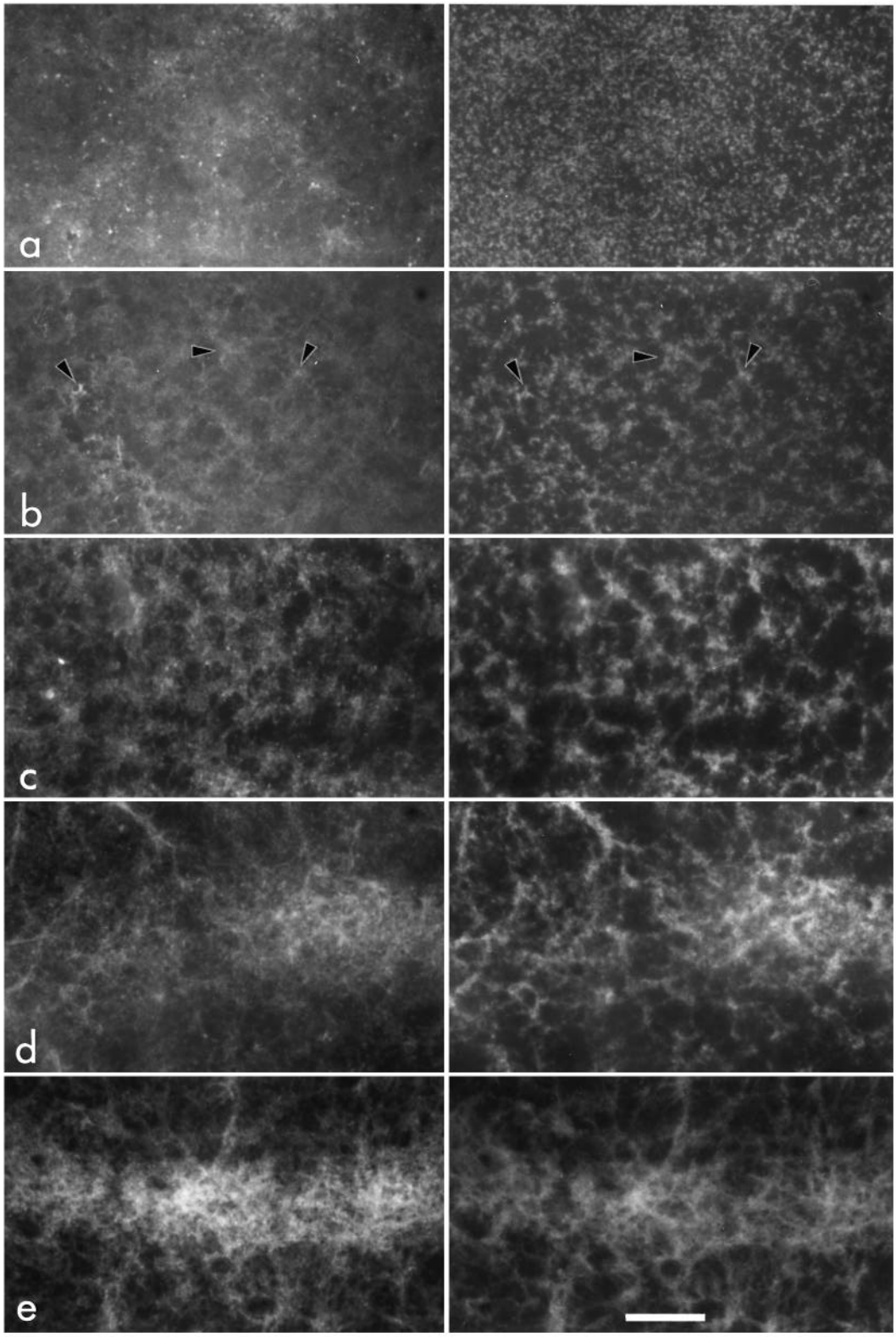

Fig. 6. Distribution of WGA-binding sites on the equatorial surface of *H. pulcherrimus* eggs. Eggs treated with Fl-WGA were fixed and processed as described in Materials and Methods. The stages of the eggs were confirmed by examination of chromosomes with DAPI staining (not shown). Photographs are arranged in such a way that the cleavage plane is horizontal. (a) Metaphase; (b) mid-anaphase; (c) start of chromosome vesiculation. Actin accumulation in the equatorial cortex is not apparent yet in this egg. (d) After chromosome fusion. Contractile ring actin filaments are apparent. (e) After chromosome fusion. This egg is more advanced than the egg in d, while the cleavage furrow was not seen yet. Left panels show Rh-ph staining; right panels show Fl-WGA staining of the same regions. Arrowheads indicate typical structures shared by the left and right images. Bar, 10 μm.

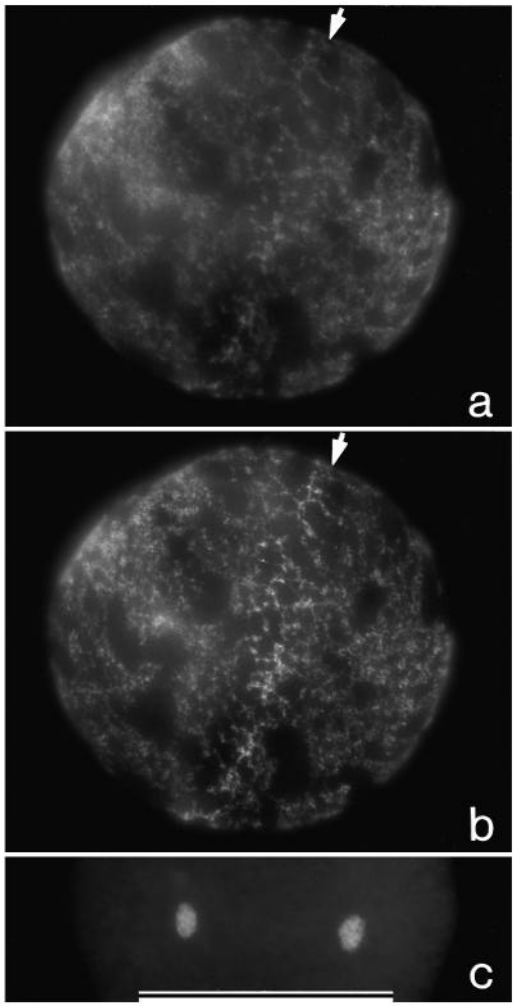

Fig. 7. Distribution of WGA-binding sites in a cytochalasin B-treated *P. depressus* egg. The egg was treated with 4 μM cytochalasin B dissolved in CaFSW at late anaphase for 10 minutes, then with Fl-WGA, and processed as described in Materials and Methods. (a) Rh-ph staining. (b) Fl-WGA staining. (c) DAPI staining. The arrows indicate poorly organized contractile ring. Bar, 100 μm.

force at the equatorial cell cortex from the shape of the egg in the absence of extracellularly applied force to be very small at early stages of furrow formation. Such a force may be able to be exerted by the poorly oriented meshwork of bundles.

Induction of actin filament changes after anaphase and 'cleavage stimulus'

It has been hypothesized that a signal called 'cleavage stimulus' is transduced from the two asters of the mitotic apparatus via astral rays to the cortical layer (Rappaport, 1986) and the level of the stimulus may be sufficient to cause furrowing only at the equator (Devore et al., 1989). The entity of the signal is not known, except for a suggestion that protein phosphorylation including myosin light chain phosphorylation (Mabuchi and Takano-Ohmuro, 1990; Tosuji et al., 1992; Satterwhite et al., 1992; Mabuchi, 1993; Yamakita at al., 1994), and a small G-protein rho (Kishi et al., 1993; Mabuchi et al., 1993) may be relevant to this process. The asters of the mitotic apparatus, which are small and do not seem to reach the cortical layer at metaphase, grow up after anaphase to fill up the cytoplasm at telophase (e.g. see Henson et al., 1989). Therefore, it is likely that the signal is transferred all over the cortical layer after anaphase if all the astral microtubules are able to transfer the signal. In the present study, it was observed that the cortical actin filaments aggregated after anaphase and the aggregation advanced to form bundles until telophase. This change occurred all over the cortical layer. It may be induced by the signal elicited by the astral microtubules. In other words, the signal may be able to induce the aggregation or bundling of the cortical actin filaments. The filament bundling advances only at the equatorial cortical layer after telophase so that they are arranged in parallel. Is this caused by the high concentration of the signal at the equator as speculated by Devore et al. (1989)? Furthermore, it is interesting that the aggregation of the cortical actin filaments was reversed after formation of the daughter nuclei except for the equatorial region. Further studies are required to explain these phenomena at a molecular level.

Surface WGA-binding sites, myosin, and the contractile ring actin filaments

It has been reported that concanavalin A-binding sites accumulate in the cleavage furrow in cultured macrophages (Berlin et al., 1978). This accumulation preceded the appearance of the cleavage furrow (Koppel et al., 1982). In sea urchin eggs, it has been found that surface concanavalin A-binding sites or WGA-binding sites accumulate in the furrow (McCaig and Robinson, 1982; Yoshigaki et al., 1989). The WGA-binding sites also accumulate in the calyculin A-induced furrow of the eggs (Tosuji et al., 1992). These accumulations were seen either when the cells had been labelled with fluorescent lectins several minutes before cleavage (Koppel et al., 1982; McCaig and Robinson, 1982; Yoshigaki et al., 1989) or when the cleaving cells were first fixed with formaldehyde and then labelled (McCaig and Robinson, 1982). Therefore, the accumulations of the lectin-binding sites are not induced by the lectins. Yoshigaki et al. (1989) noted that this accumulation occurred before furrowing and it seemed that the position of the accumulation is determined by the asters of the mitotic apparatus. Furthermore, colcemid was capable of dispersing the accumulation when the egg was treated by the drug at an early stage of cytokinesis. Thus the lectin-binding sites might somehow be controlled by astral microtubules and might have a role in determination of the position of the contractile ring. This hypothesis was tested by comparing the times of appearance of the surface WGA-binding sites and contractile ring actin filaments in the anticipated furrow region. As a result, no time difference was detected in their appearances. It was also anticipated that in the presence of cytochalasin B, the WGA-binding sites would accumulate normally in the equatorial membrane while the contractile ring actin filaments would not be organized. However, this was not the case: the localization of surface WGA-binding sites coincided well with that of the cortical actin filaments in the absence of as well as in the presence of cytochalasin B, which induced abnormal aggregation of these filaments as in the case of mammalian cultured cells (Norberg et al., 1975; Weber et al., 1976). This indicates that the WGA-binding sites have some intimate relationship with the actin filament-anchoring points on the plasma membrane. It is likely that the actin filaments and the surface WGA-binding sites are linked at

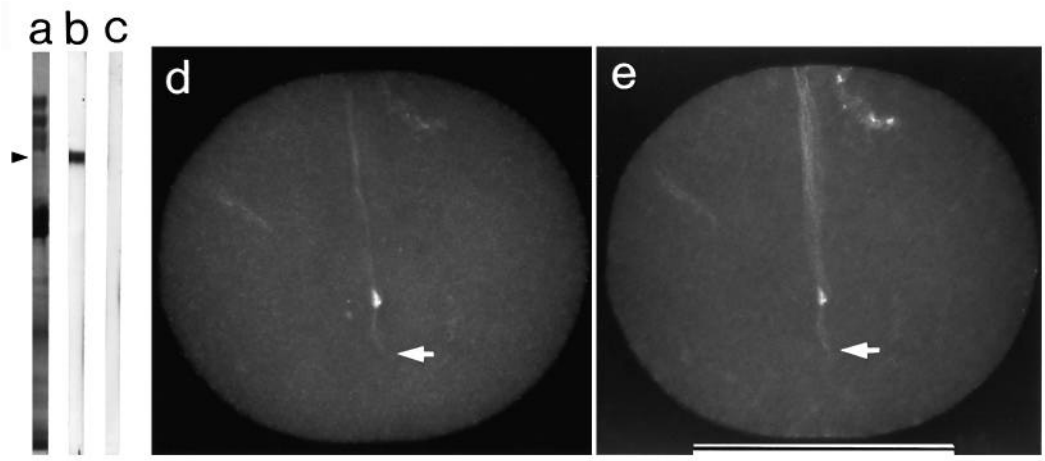

Fig. 8. Anti-myosin immunofluorescence of a whole egg. (a) *T. gratilla* egg extract proteins (20 μg protein) electrophoresed on a 6% acrylamide gel in the presence of SDS, transferred onto a nitrocellulose membrane and then stained with Amido Black 10B. Myosin heavy chain is marked by the arrowhead. (b) The membrane was immunoblotted with the anti-myosin heavy chain serum (1/1,000 dilution). (c) The membrane was immunoblotted with the preimmune serum (1/1,000 dilution). (d) Immunofluorescent staining of myosin in a dividing *C. japonicus* egg. (e) Staining of actin filaments with Fl-ph in the same egg as in d. The arrows indicate the leading edge of the asymmetrically formed contractile ring revealed by the respective stainings. Bar, 100 μm.

least after anaphase and move together into the cleavage plane. Furthermore, the fact that extracellularly applied Fl-WGA showed a fibrillar localization like the actin filaments after anaphase indicates that the actin filaments are anchored to the plasma membrane at lateral sites on the filaments. It is known that the contractile arc actin filaments of newt eggs are anchored to the plasma membrane at least at their barbed ends (Mabuchi et al., 1988). Therefore, the contractile ring or arc actin filaments will attach to the plasma membrane in both the end-on and lateral fashions.

Myosin has also been considered to be a candidate for involvement in cleavage signalling (Mabuchi, 1986), since myosin filaments are able to assemble actin filaments of opposite polarities in vitro (Hayashi et al., 1977). Myosin has been localized in the cleavage furrow of sea urchin (*Strongylocentrotus purpuratus*) blastomeres, but not in the anticipated furrow region before initiation of furrowing (Schroeder, 1987). On the other hand, fluorescently labelled myosin light chains microinjected into Gerbil fibroma cells are concentrated in the anticipated furrow region before furrowing was apparent (Mittal et al., 1987). The difference in the behaviour of myosin in these cells cannot be discussed without comparing the timing of appearance of the contractile ring actin filaments and myosin in these cells as in the case of the WGA-binding sites. It was found in the present study that these proteins appear simultaneously to form the contractile ring. The present results do not exclude the possibility that the WGA-binding component or myosin is somehow involved in the determination of the contractile ring formation site. But it is at least not plausible that the surface WGA-binding component or myosin accumulates at first in the anticipated furrow region and then induces following assembly of the contractile ring actin filaments.

These results showing that actin filaments, myosin and WGA-binding components are simultaneously organized into the contractile ring indicate that the assembly of the ring structure is a highly concerted process. It has been shown that an inhibitor of myosin light chain kinase inhibited the formation of the contractile ring (Mabuchi and Takano-Ohmuro, 1990). It has also been found that a mutation in a gene encoding the myosin regulatory light chain disrupts cytokinesis in *Drosophila* larvae (Karess et al., 1991). It could be that a lack or an alteration of an element of the ring integrity, such as the myosin molecule, would result in the failure in ring formation.

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