DISTRIBUTION OF TUBULIN-CONTAINING STRUCTURES IN THE EGG OF THE SEA URCHIN *STRONGYLOCENTROTUS PURPURATUS* FROM FERTILIZATION THROUGH FIRST CLEAVAGE

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ABSTRACT

Eggs of the sea urchin *Strongylocentrotus purpuratus* were examined by indirect immunofluorescence microscopy for tubulin-containing structures at intervals from fertilization through first cleavage. The staining revealed that the monaster is made up not only of the sperm aster but also of tubulin-staining fibers originating elsewhere in the egg. The monaster does not divide directly but is broken down first before the amphiaster or interphase asters begin to form. The interphase asters reach a peak of development at the streak stage and are in turn broken down before the formation of the mitotic apparatus. The breakdown of the monaster, interphase asters, as well as the asters of the mitotic apparatus proceeds from the cell center or aster centers to the periphery of the cell and is followed by growth of new asters, also proceeding outward from the aster centers. The pattern suggests a transient wavelike movement of some condition, or factor, which favors microtubule depolymerization.

KEY WORDS microtubules, sea urchin eggs, indirect immunofluorescence, fertilization, mitosis

Although the study of fertilization and early cleavage of sea urchin eggs has a history of well over a hundred years, the application of new or improved techniques often warrants a reexamination of these much-studied events. Recent development of indirect immunofluorescence microscopy has provided a useful means of visualizing tubulin-containing structures in whole cells (for review see reference 15). Although most of the studies on mitosis using this technique have been carried out on relatively flat tissue culture cells (1, 2, 15), antitubulin staining has recently been used successfully to study the meiotic divisions of the larger but clear mammalian egg (14). It thus seemed worthwhile to apply the method to the rapidly dividing and highly synchronous fertilized sea urchin eggs, about which a great deal is already known concerning the cellular ultrastructure and the biochemistry of the cell cycle.

The disadvantage of cell thickness and yolk content resulting in lowered resolution was more than compensated for by the visualization of tubulin distribution in the whole cell—a picture that would have been almost impossible to obtain by serial sectioning of individual cells for electron microscopy. In an initial report (8), we have de-
scribed a transient spiral system of microtubule bands at a depth of 10-15 μm from the cell surface, which appears around the time of pronuclear fusion and disappears by the time of the streak stage. This report is concerned with the distribution of tubulin-containing structures throughout the cell cycle, from the time of sperm entry through first cleavage.

MATERIALS AND METHODS

Sea Urchin Gametes

Sea urchins (Strongylocentrotus purpuratus) were obtained from Pacific Bio-Marine Labs, Inc. (Venice, Calif.) and maintained in artificial seawater (Tropic Marin Neu, Dreieich, W. Germany). For handling of eggs and embryos, an artificial seawater developed especially for marine embryological studies was used (Jamarini-U, Jamarini Laboratory, Osaka, Japan), and is referred to below simply as seawater.

Gametes were obtained by injection of 0.5 M KCl into the body cavity. Eggs were collected by inverting the urchin over a beaker of seawater at 15°C. The eggs were then passed through 90-μm-mesh bolting cloth to remove large debris and washed several times by settling, decanting, and resuspending in fresh seawater. Sperm was collected “dry” in small plastic petri dishes and diluted just before use.

A small amount of diluted sperm was added to an egg suspension containing ~0.1 ml packed eggs/10 ml. Fertilization membranes were removed to allow penetration of antibodies. A neutralized solution of mercaptoethyl gluconamide (Cyto Chemical Division of Travenol Laboratories Inc., Los Angeles, Calif.) in seawater was added ~15-30 s after the addition of sperm, to make a final concentration of 0.1 g/100 ml egg suspension. The resulting fertilization membranes became very thin and distended, and were easily stripped by passing the 75-μm eggs through 80-μm-mesh bolting cloth as early as 5 min after the addition of sperm. The stripped eggs were then washed either by allowing them to settle, before resuspension, in normal seawater or by diluting the egg suspension with calcium-free seawater. The calcium-free seawater was prepared by adding 10 mM EGTA, pH 8.0, to chelate the 10 mM Ca”+ in the Jamarini-U seawater. Fertilized eggs were incubated at 15°C in a single layer in an open dish, and stirred after each sampling. By gently swirling the dish, the eggs could be concentrated rapidly in the center without the necessity of centrifugation and possible distortion or clumping.

Antibodies

The antibody to tubulin was raised in rabbits against highly purified tubulin from porcine brain. It was made monospecific by passage over tubulin coupled to Sepharose 4B (17). Its specificity and staining of microtubular structures in cells in culture have recently been summarized (16). In the current studies the second antibody was prepared by conjugating fluorescein to monospecific sheep-anti-rabbit γ-globulins using dichlorotriazinyl-amino-fluorescein (for details, see reference 18). Antibodies were used at a final concentration of 0.05 mg/ml.

Staining Procedure

Eggs were attached to coverslips by the method of Mazia et al. (12). The coverslips were prepared just before use by immersing them in a freshly prepared 1 mg/ml aqueous solution of poly-L-lysine for 5-10 min, rinsing in distilled water, and air drying. At appropriate times after fertilization, a concentrated suspension of cells was removed with a Pasteur pipet and several drops were placed on the dry coverslips. Excess water from the egg suspension was drawn off and the coverslips were immersed in -10°C 90% methanol containing 50 mM EGTA at pH 6.0. After 6 min the coverslips with adhering eggs were rehydrated in phosphate-buffered saline (PBS) and processed for immunofluorescence microscopy by the usual procedures (15). Rabbit monospecific tubulin antibody was added and the cells were incubated for 1.5 h at 37°C. After thorough washing with PBS, fluorescein-labeled sheep-anti-rabbit γ-globulins were added and the cells were again incubated for 1.5 h at 37°C. After further washing with PBS, the coverslips were mounted on slides with Moviol (Hoechst, Frankfurt, W. Germany) and photographed on Kodak Tri-X or Plus-X film, using a Zeiss Photomicroscope equipped with epifluorescence optics and X 40 Zeiss Planapo, Phase, NA 1.0, oil objective.

For comparison with the above method, eggs were also fixed and stained in suspension in small conical centrifuge tubes and subsequently mounted under coverslips. There was a considerable loss of cells because of sticking to glass surfaces; a larger volume of antibody was needed per sample, and, because the staining pattern was practically identical to that of cells on polylysine-coated coverslips, this method was abandoned.

Several fixation procedures were also explored, using metaphase cells as test material, since the appearance of mitotic structures is already well known from other techniques. Glutaraldehyde fixation was unsatisfactory, because the eggs became highly autofluorescent. Formaldehyde was somewhat better in this respect, but the cell structure was badly distorted and aster centers vacuolated. Cold 100% methanol alone without postfixation preserved cell shape, gave low background and no autofluorescence, but aster microtubules often appeared to end as rows of small dots, suggesting breakdown as a result of Ca”+. Rinsing the cells in Ca”+-free seawater before the methanol treatment greatly improved the preservation, as did addition of EGTA to the methanol. The large amount of EGTA necessary for good preservation of microtubules indicated a possible Ca” release from membrane-bounded intracellular stores brought about by the lipid solvent action of the methanol.

The pH used was borrowed from fixation procedures designed for the best visualization of microtubule bundles in glutaraldehyde-fixed whole mounts and stained sections for light microscopy (7) and in osmium tetroxide-fixed cells for electron microscopy (5). Although this may not be the optimum pH for immunofluorescence, the results seemed satisfactory and the pictures matched closely those obtained with stained thick sections of glutaraldehyde-fixed, Epoxy-embedded material. See reference 7 for comparison.

RESULTS

Although several batches of eggs were examined with tubulin antibody at intervals throughout the cell cycle, the illustrations used here are for the most part from a single batch, to eliminate any differences caused by external factors or batch-to-batch variations. Exceptions are Figs. 1 and 2 from a series of experiments examining very early stages, Fig. 7 from one of several studies examining the formation of the cortical spirals, and Figs. 19-
24 from three different experiments in which only mitotic stages were examined. The average division time from sperm entry to cytokinesis was 135 min, with well-known markers pronuclear fusion at 30 min, streak stage at 75 min, beginning prophase at 95 min, and metaphase at 115 min.

**Early Stages**

In a sample taken at 9 min after the addition of sperm, a small bright star near the surface of the egg, often with the sperm tail clearly visible, identifies the forming sperm aster (Figs. 1 and 2). The sperm aster grows rapidly and by 13 min it has formed a cone-shaped structure pointed toward the egg center. The exact location of the sperm nucleus is hard to determine, but the egg nucleus can be seen to the left of the bright sperm aster in Fig. 3. Besides the sperm aster, a tubulin-staining network of fibers prominent in the cortical region and extending some distance into the cell is usually visible (Fig. 4).

**Pronuclear Fusion and Formation of the Interphase Aster**

By 25 min after sperm entry the monaster has formed and already two brightly staining spots on either side of the zygote nucleus identify the future spindle poles. Tubulin-staining fibers are arranged radially around the nucleus, but the region of the original sperm aster remains brighter than the rest of the monaster (Figs. 5 and 6). In a sample taken at 30 min (Fig. 7) there appears to be a rearrangement of the radially oriented fibers. They no longer penetrate into the interior of the cell, where the tiny interphase asters are clearly visible. The fate of these fibers as they move closer and more parallel to the cell surface and eventually form a rather dramatic spiral structure in the cortex (Fig. 8) has been described elsewhere (2). They have been shown by electron microscopy to be made up of large aggregates of microtubules.

The interphase asters continue to grow, apparently quite independent of the cortical microtubule bands. During the next 20 min there is a gradual loss of cortical microtubules as the interphase asters increase in size, and by 65 min any that remain are difficult to distinguish from the aster fibers. By the time of the streak stage the cells are literally packed with microtubules. Fig. 9 shows a somewhat flattened cell at 76 min after fertilization, displaying great numbers of microtubules and the characteristic brightly staining spots in the nucleus. Although the intranuclear staining did not occur in control samples using only second antibody or nonimmune rabbit γ-globulins, it cannot be ruled out that this staining is nonspecific. Fig. 10 shows another cell from the same sample in which a few cortical microtubules are still present.

**Preparations for Division**

After the streak stage there is a loss of microtubules from the interphase asters that progresses in a very characteristic fashion. In cells that are oriented so that the eccentric position of the nucleus can be seen, as in Fig. 11, a loss of microtubules is apparent in the region just below the nucleus. The large bulk of microtubules is thus lost progressively from the center of the cell outward (Fig. 12), while microtubules remaining attached to the aster centers become very straight, as if under tension. Compare Fig. 13 with Fig. 9.

In a sample taken 10 min later, practically all the cells have lost their large interphase asters. There is an increase of tubulin-staining material at the aster centers, which are now surrounded by relatively few, and very short astral rays (Fig. 14). From these small centers the mitotic asters begin to grow, accumulating more material in the aster centers and sending out very stiff and straight astral rays, entirely different in appearance from...
the streak stage asters. At this time all intranuclear tubulin staining disappears and the nucleus itself appears dark against the generally bright cytoplasmic background (Fig. 15). Figs. 16–18 show progressive stages of late prophase, prometaphase, and what is probably beginning anaphase. The brightness of the aster center is exaggerated because these regions are clear of yolk granules. Furthermore, the figures are over-exposed so as to show details of the outer part of the asters.

Separation of the Centers and Cytokinesis

In another batch of cells, the mitotic apparatus is seen at metaphase (Fig. 19), anaphase (Fig. 20), and telophase (Fig. 21). The bright dots in the aster centers probably represent the centrioles with their pericentriolar material. In metaphase they appear single, but in the anaphase cells they are clearly double. At telophase the centriolar dots, as well as the brightly staining rim around the aster center or centrosphere, have practically disappeared, but the astral rays continue to grow and fill the space of the daughter blastomeres. Fig. 22 shows an anaphase tetrapolar figure in a polyspermic cell.

Shortly after division the interphase asters of the daughter blastomeres have a fan-shaped appearance, spreading widely in a direction away from the furrow (Fig. 23). The remaining connection between the two cells is filled with microtubules, but the midbody itself does not stain. This is similar to what is seen in mammalian cells, in which it is known, from electron microscope studies, that the microtubules traverse the midbody and are surrounded by very electron-dense material, which presumably prevents the antibody from reaching them. Later, as the blastomeresc prepare for second division, the interphase microtubules are lost and new asters begin to form (Fig. 24). There is no evidence from these experiments of recurring cortical microtubule spirals during the second division.

DISCUSSION

The technique of indirect immunofluorescence microscopy provides an excellent and perhaps the only practical method of evaluating tubulin distribution in whole cells of dimensions of the sea urchin egg. In this study especially, where large numbers of cells are examined at frequent time intervals and where different batches of eggs must be sampled, serial sectioning of individual cells for electron microscopy simply is not feasible. Furthermore, fixed and stained sections for ordinary light microscopy do not distinguish tubulin-containing structures, for example microtubule bundles, from elongated cisternae or other fibrous elements in the mitotic apparatus.

Immunofluorescence microscopy alone is not enough, however. Although individual microtubules can be resolved in extremely flattened cytoskeleton preparations of mammalian cells (13), they are difficult to visualize in large cells such as sea urchin eggs, except where they exist in large numbers. It is thus necessary to combine various techniques to show the details in representative cells, and this is especially true in studying the very early stages after sperm entry.

Monaster Formation

Although there are many descriptions from early light microscopy of the sperm monaster

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**FIGURE 7** F + 30 min. The monaster microtubules have disappeared from the central part of the cell, but remnants are still present near the cell periphery. × 700.

**FIGURE 8** F + 32 min. Monaster remnants become oriented nearly parallel to the cell surface, often forming elaborate spirals. Sections for light and electron microscopy show that they are 10–15 μm from the surface and are made up of bundles of microtubules. × 700.

**FIGURE 9** F + 76 min. The main changes from the 30-min sample (Fig. 7) are the loss of the peripheral monaster microtubules and the extensive growth of the interphase asters. × 700.

**FIGURE 10** Another cell at F + 76 min. Streak stage. × 700.

**FIGURE 11** F + 90 min. Microtubule loss is beginning in the central region of the cell. × 700.

**FIGURE 12** F + 100 min. Microtubule loss progresses outward, while some microtubules remain attached to the aster centers. × 700.
which forms soon after fertilization (see reference 4 for recent review), and from fine-structure studies primarily concerned with the changes in the pronuclei (9, 10), the overall structural changes during the early stages of fertilization are not at all clear. The propulsion of the sperm nucleus by the growth of the sperm aster is generally assumed, but the female nucleus also moves to the center of the egg, even in parthenogenetically activated eggs, without any interaction with a sperm nucleus or aster. Because this latter movement is also stopped by colchicine (11), it can be assumed that microtubules are also involved with the movement of the female nucleus.

In this study, an network of tubulin-staining fibers, separate from the sperm aster, can be seen in a large proportion of the cells in the earliest stages (Fig. 4). Although Figs. 1 and 2 do not show the network, many of the cells in the same preparation did. Whether this material is actually in the form of microtubule bundles and whether it originates in the cortex or in the region of the egg nucleus is not known, and here electron microscopy along with other techniques will be necessary to elucidate the details. This tubulin network appears to contribute, along with the sperm aster, to the formation of the monaster seen in Figs. 5 and 6. At this stage the radially arranged monaster fibers extend from the fusion nucleus to the cell periphery, and the two centers derived from the sperm centrioles are seen as clearly separated brightly staining spots.

Two Asters from One

There is a large body of literature describing the division of the centers and the astral system, reviewed in detail by Wilson (20). The early assumption that an amphiaster (or pair of asters) was derived by the direct splitting of the mother aster was later found to be erroneous. As Wilson notes, "In most cases the old aster degenerates and the amphiaster appears as a new formation within its remains, even while the old aster is at the height of its development" (page 680 of reference 20).

Until now, the division of the sperm aster in the fertilization of the egg was believed to be a rare exception to this rule. Indirect immunofluorescence microscopy clearly shows that the monaster does in fact break down and that the newly forming pair of interphase asters grow independent of the former structure.

Significance of the Spirals

The reorientation of the remnants of the monaster after pronuclear fusion to form a spiral in the subcortical region has been described in more detail elsewhere (7, 8). It was originally supposed that the spiral axis might coincide with the egg axis and thus play some role in the orientation of the spindle for the first cleavage, which is always meridional. However, if this were the case, one would expect to see the same structures orienting the second division, which is also meridional with respect to the egg axis. In the studies described here there was no indication of recurring spirals, although the second division cycle was not studied in detail.

More likely, the spiral origin coincides with the point of sperm entry. While this may play a role in determining the division plane, there is still disagreement in this regard, and Horstadius (cited in reference 20) has shown by vital staining that the first cleavage furrow does not necessarily pass through the point of sperm entry. The spiralling does suggest cytoplasmic movement or streaming, possibly movement of the cortical region with respect to the endoplasm, causing an orientation of the microtubules at the interface. But this is only speculation, and there is still no clue—at least, no recognized clue—as to how the egg is able to orient its first division plane so precisely with respect to its animal-vegetal axis.

Pattern of Aster Degeneration

The course of aster breakdown, proceeding from the aster center to its periphery, has been well established in innumerable studies of meiosis and cleavage in a wide variety of invertebrate eggs.
Thus, it is not surprising to find that the pattern is repeated in the breakdown of the monaster and later at the time of interphase aster breakdown. In the latter case, not all the microtubules are lost immediately from the central part of the cell. Some remain attached to the aster centers, and these progressively become very straight and appear stiff compared to appearing rather limp at the streak stage. Eventually these also shorten and disappear, as the cell enters the "pause" stage before beginning to form the mitotic asters.

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**Figure 25** A diagrammatic representation of the results from the immunofluorescence study comparing the formation and breakdown of the monaster, interphase asters and the mitotic apparatus. White areas represent regions where microtubules are stable or increasing in size. Shaded areas are regions where microtubules either are absent or are in the process of breaking down. The central figure of each row shows the transition from one structure to the next as a wave of microtubule breakdown moves from the center to the periphery and is followed by a region supporting microtubule polymerization.

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**Figures 19–21** Metaphase, anaphase, and telophase in somewhat more flattened cells. A bright-staining spot in the center of the metaphase asters is clearly double at anaphase and becomes diffuse or disappears at telophase. These spots probably represent the centrioles with pericentriolar material. × 700.

**Figure 22** Anaphase tetrapolar figure in a polyspermic cell. × 700.

**Figure 23** Two daughter blastomeres just after division, with extensive fan-shaped interphase asters. The intercellular bridge does not stain in the region of the midbody. × 700.

**Figure 24** Blastomeres preparing for second division. Asters are again reduced in size. × 700.
The stiff appearance of the astral rays just before the “pause” and again as they increase in length to form the mitotic asters may result from the association of other structural proteins with the microtubules as the cells approach mitosis. An alternative explanation is that the microtubules are under tension and are being pulled into the aster center by combined interaction with contractile proteins in the aster center and subsequent depolymerization. While this explanation may seem reasonable for the reduction of aster size, it is more difficult to apply to a growing aster unless one supposes that growth at the distal ends of the astral rays is faster than their breakdown in the aster center. A model has been proposed for chromosome movement based on just such a balance of reactions (6). It predicts the pattern of aster breakdown from the center outwards as resulting from trigger waves of Ca++ release and resequestering initiated at the aster centers and mediated by the accumulation of smooth endoplasmic reticulum which is intimately associated with the growing mitotic apparatus (5, 9).

Fig. 25 is a diagrammatic representation of the results from the immunofluorescence studies, comparing the formation and breakdown of the monaster, interphase asters and the mitotic apparatus. White areas represent regions where microtubules are stable or growing. Shaded areas are regions where microtubules are either absent or in the process of breaking down. The central figure in each row shows the transition from one structure to the next, as a “wave” of microtubule breakdown moves from the center to the periphery and is followed by a region supporting microtubule polymerization. The sensitivity of microtubule polymerization to Ca++, first reported by Weisenberg (19), and the demonstration of a transient free calcium wave in the fertilization of fish eggs by Gilkey et al. (3), suggest but do not prove that a similar mechanism may be operating here in the formation and breakdown of microtubular structures.

Although the loss of interphase asters at the time of the pause has long been known and has been described in many invertebrate eggs, the magnitude of this loss has never been demonstrated so dramatically as with the fluorescent antibodies. The similarity between this loss and that described in a variety of tissue culture cells (1, 15) indicates that the basic mechanisms of the cell cycle in these divergent cell types are essentially the same. Additionally, the interphase asters of the sea urchin egg probably correspond to the cytoplasmic microtubule complex of interphase tissue culture cells. Thus, the application of indirect immunofluorescence microscopy to sea urchin eggs and embryos provides another valuable tool for the study of mitosis and early development. Its greatest value, however, will be in combination with studies on fine structure and biochemistry.

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