IN VITRO POLYMERIZATION OF MICROTUBULES INTO ASTERS AND SPINDLES IN HOMOGENATES OF SURF CLAM EGGS

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ABSTRACT

The eggs of the surf clam Spisula solidissima were artificially activated, homogenized at various times in cold 0.5 M MES buffer, 1 mM EGTA at pH 6.5, and microtubule polymerization was induced by raising the temperature to 28°C. In homogenates of unactivated eggs few microtubules form and no asters are observed. By 2.5 min after activation microtubules polymerize in association with a dense central cylinder, resulting in the formation of small asterlike structures. By 4.5 min after activation the asters formed in vitro contain a distinct centriole, and microtubules now radiate from a larger volume of granular material which surrounds the centriole. By 15 min (metaphase I) the granular material is more disperse and only loosely associated with the centriole. Microtubules are occasionally observed which appear to radiate directly from one end of the centriole. The organizing center can be partially isolated by centrifugation of homogenates of metaphase eggs and will induce aster formation if mixed with tubulin from either activated or unactivated eggs. Pretreatment of the eggs with colchicine does not prevent the formation of a functional organizing center. Complete spindles can also be obtained under polymerizing conditions by either homogenizing the eggs directly into warm buffer or by adding a warm high-speed supernate to spindles which have been isolated in a microtubule stabilizing medium. Extensive addition of new tubulin occurs onto the isolated spindles, resulting primarily in growth of astral fibers, although there occasionally appears to be growth of chromosomal fibers and of pole-to-pole fibers. Negatively stained aster microtubules have a strong tendency to associate side by side, and under some conditions distinct cross bridges can be observed. However, under other conditions large numbers of 300-400-A particles surround the microtubules; the presence of stain between particles can give the appearance of cross bridges.

Microtubule assembly and breakdown is a major process in the complicated series of events during cell division. Although the assembly of spindle microtubules is only one part of the total cycle of mitosis or meiosis, it involves several separate problems of cellular control. The spindle, of course, forms at a particular time with respect to the other events of mitosis, but its location within the cell is also determined as may be its orientation with respect to the axis of the cell or tissue. It seems probable that microtubule assembly must be regulated by more than a single process.
The formation of microtubules per se may be controlled by soluble cytoplasmic factors such as calcium (4, 7) or cyclic AMP (8). However, it is unlikely that position and orientation of the spindle in the cell could be determined by such diffusible factors alone. A membrane component or an aggregate of tubulin itself could provide a nondiffusible factor for determining position and orientation of microtubule assembly. In the unactivated eggs of the surf clam a fraction of the tubulin, which is about equal to the amount of tubulin which will appear in the spindle, is present as a large aggregate associated with the egg cortex (16). This aggregate breaks down immediately before spindle assembly and is presumably involved in spindle formation. The involvement of a nondiffusible factor in the assembly of microtubules has been demonstrated by the observations of Tucker on microtubule assembly in the ciliate *Nassula* (15). The function of nondiffusible components of tubulin is most likely to aid in determining the correct position and orientation of microtubules within the cell. This would be particularly important in the early cleavage divisions of embryos and may explain the highly developed tubulin aggregate observed in surf clam oocytes.

A major factor which is also clearly involved in microtubule assembly in the living cell is the microtubule organizing center (MTOC). All eukaryotic spindles appear to have organizing centers, although the morphology of these structures can vary tremendously. MTOCs are not confined to the spindle, and have also been demonstrated in other microtubule systems (14, 15). To study the organizing centers in a systematic way it would be useful to have an in vitro system in which the MTOC was present in an active form and from which it could be isolated. In this paper we report on such a system which duplicates in vitro many of the features of aster and spindle assembly. Polymerization of microtubules in homogenates of surf clam eggs can result in the formation of asterlike structures, and in this report we will describe the structure of these asters and the changes which occur during the development of artificially activated eggs. Some of this work was reported in preliminary form earlier (16).

**MATERIALS AND METHODS**

The eggs of the surf clam *Spisula solidissima* were obtained and activated essentially as described previously (16). Briefly, the eggs were dissected from a live animal, washed at least three times in sea water, and then activated by the method of Allen (1) using 7-8 ml of 0.52 M KCl/100 ml of sea water containing the eggs. At the desired time the eggs were washed twice by gentle hand centrifugation in 1.0 M glycerol, 1 mM sodium phosphate at pH 8 (12). The eggs were then homogenized at 0°C in 0.5 M MES buffer (3) normally containing 1 mM EGTA at pH 6.5. Most preparations were performed using 1.5 ml of buffer/ml of packed eggs, and the time after activation when the eggs were homogenized refers to the time when the buffer was added. The suspended eggs were homogenized thoroughly in a glass homogenizer with a Teflon pestle using a motor drive at about 100 rpm. For most experiments the homogenate was centrifuged for 5 min at 4°C at about 2,000 g (2,500 rpm in a Sorvall GLC-1 centrifuge with a HL-4 rotor, Ivan Sorvall, Inc., Newtown, Conn.) and the supernate recovered for experimentation. For collecting the MTOC the supernate was further centrifuged for 15 min at 15,000 rpm in a Sorvall SS-34 rotor also at 4°C. The pellet contained the MTOC and the supernate was used for experiments requiring MTOC-free tubulin. Aster formation was initiated by incubation at 28°C. Asters were observed directly by phase-contrast microscopy with no further treatment, or were diluted with at least 5 vol of 1 M hexylene glycol, 0.01 M sodium phosphate at pH 6.2 (HGL) (7) to stabilize the microtubules. Aster formation can be observed directly by simply placing a drop of cold supernate on a warm slide and covering with a cover slip.

For thin section electron microscopy the polymerized samples were fixed by adding several volumes of 3% glutaraldehyde in 0.5 MES at pH 6.5 directly to the solution at 28°C. After 30 min the particulates were collected by centrifugation, washed in MES buffer, and postfixed in 1% osmium tetroxide. The pellets were dehydrated in ethanol and embedded in Epon. Sections were stained with uranyl acetate and lead citrate and were examined in a Philips model 300 electron microscope equipped with a goniometer stage and a tilt-rotate holder. For negative staining, the asters were either diluted in HGL solution, collected by centrifugation (2,500 rpm for 10 min in the GLC-1), and resuspended in HGL solution, or were simply spun down at room temperature with no additions and the pellet was resuspended in warm MES buffer. A drop of the sample was placed on a Formvar- and carbon-coated grid, replaced by 1% uranyl acetate, and blotted nearly dry. Protein was measured by a modification of the Lowry procedure (5) and colchicine binding was determined as described earlier (16).

**RESULTS**

**Formation and Stability of Asters**

Formation of microtubules and asters in vitro has only been obtained in crude homogenates or low-speed supernates of *Spisula* eggs; no success...
has yet been achieved using more purified systems. Precise conditions cannot be specified, therefore, for the polymerization of microtubules in this system, but some conditions have been found necessary or useful in obtaining polymerization. The two washes of the eggs with 1 M glycerol before homogenization serve two purposes: they remove traces of sea water and soften the eggs to allow easier breakage. Some glycerol which is present in the final egg pellet may aid polymerization, although it is not necessary since the eggs can be washed in buffer and polymerization will still occur. The concentration of ions in the homogenizing medium is important; very little aster formation was observed in 0.1 M MES, and at concentrations below 0.3 M the asters were clearly much smaller than at 0.5 M. This is an unexpected result since brain tubulin will polymerize well at 0.1 M. However, the difference may be explained by the higher internal salt environment of the eggs or by a salt requirement for the functioning of the MTOC. With the crude system no other additions of either ions or nucleotides were required to obtain polymerization. Presumably, the nucleotide (GTP or ATP) which is probably required for polymerization (17) is present at sufficient concentration in the homogenate. Although generally added, a calcium chelator (EGTA) was not necessary in this system, and the Spisula tubulin may be less sensitive to calcium than was observed previously for brain tubulin. On the basis of morphological observations of the asters, a concentration of CaCl₂ above 2 mM was required to obtain significant inhibition. Since the contribution of Ca from the eggs is unknown and MES has some Ca binding activity the actual concentration of Ca could not be determined.

The assembly of asters is temperature dependent as expected from other in vitro and in vivo investigations. No asters can be detected at 0°C – 4°C but they can be observed at temperatures above 20°C. Although some increase in the size of the asters occurs as the temperature is increased above 30°C the asters which form at higher temperatures reform very poorly after cooling, and at incubation temperatures above 35°C they can become insensitive to cooling and do not break down even after 1 h at 0°C. Most polymerization experiments, therefore, were carried out at a temperature of 28°C as a compromise between the amount of polymerization and the stability and reversibility of the tubules.

Asters can be prevented from forming by the addition of 10⁻⁴ M colchicine before incubation at 28°C. However, asters which had been incubated for 10 min at 28°C broke down only at concentrations of colchicine higher than 10⁻⁴ M, and after incubation for 30 min asters did not break down even in the presence of 10⁻³ M colchicine for an additional 30 min. The sensitivity of the asters to dilution is similar to their sensitivity to colchicine. After 10 min of incubation at 28°C the asters broke down within 5 min after dilution with an equal volume of buffer, but after 30 min of incubation at 28°C asters did not break down after dilution. Sensitivity to low temperature, however, is not lost at the same rate as sensitivity to colchicine and dilution. Asters which have lost sensitivity to colchicine and dilution by incubation at 28°C for 30 min still break down within a few minutes if the temperature is lowered to 0°C. Loss of sensitivity to low temperature will occur within 10 min, however, if the asters are incubated at 35°C.

The ability of asters to reform after depolymerization at 0°C is lost at a rate similar to the rate of loss of sensitivity to colchicine and dilution. After 10 min at 28°C, asters can be depolymerized and will reform when the temperature is returned to 28°C. However, repolymerization results in a clear reduction in the size and number of asters observed, and after two cycles of cooling and warming it is generally not possible to reassemble asters. Incubation at 28°C for 30 min completely eliminates the ability to repolymerize asters.

**Light and Electron Microscopy**

Asters were generally observed by phase-contrast microscopy (Fig. 1), although Nomarski interference and polarization microscopy (Fig. 2) were occasionally used. With phase-contrast microscopy the asters can be identified by a number of characteristics. When formed optimally in homogenates of metaphase eggs they stand out very dramatically and can be easily identified. They consist of a very dark central region surrounded by a relatively clear “halo.” The size of the central dark area is variable and depends upon the stage of the eggs at the time of homogenization and the degree of polymerization. The density and size of the center is to a great extent a result of the high density of microtubules in this region and does not necessarily indicate the presence of a special material in the center. In well-flattened prepara-
FIGURE 1 Phase-contrast micrograph of a field showing four asters formed in vitro. The homogenate was prepared as described in Materials and Methods, and after polymerization at 28°C was observed with no further treatment. The eggs were in metaphase when homogenized. × 350. Inset: A higher magnification view of one of the asters exposed to show the structure of the center. × 900.

FIGURE 2 Polarization micrograph of a field of asters obtained from a homogenate of metaphase eggs. The micrograph was taken with the assistance of Shinya Inoue. × 300.

Electron microscope observations of the asters are generally consistent with the phase-contrast observations. The fully formed asters (Figs. 3, 4) of a metaphase egg homogenate have very nearly the appearance of spindle poles described in sections of artificially activated Spisula eggs (13). The only clear difference is the lack of stained material outside of the center region in the in vitro asters, which occurs because cytoplasmic material not associated with the centriole is removed by homogenization of the eggs. It should be pointed out that only a single centriole has been clearly observed in any aster observed in thin section, which may indicate that the second spot observed by light microscopy is not a centriole or that the centriole has been removed by preparation for electron microscopy. In sections parallel to the centriole the center may have a distinctly asymmetrical appearance and one end of the centriole faces a notch in the granular material. This is particularly clear at earlier times when the center is relatively compact (Figs. 6, 7). The end of the centriole facing the
FIGURES 3 and 4  Electron micrographs of asters prepared from homogenates of metaphase eggs. A distinct centriole is present and is surrounded by an irregular area of stained material in which microtubules are embedded. Fig. 4 inset shows the centriole of Fig. 4 after tilting by 30°. Fig. 3, x 30,000; Fig. 4, x 45,000; Inset, x 150,000.
notch appears to be closed off and microtubules are occasionally observed which appear to grow directly out of the centriole at this end.

**Development of the MTOC**

The structure of the aster varies with the time after activation when the eggs are homogenized. This can be easily seen by comparing sections of asters obtained from eggs 2.5 min after activation (Figs. 8–10), with asters obtained at 4.5 min (Figs. 5–7), and at 15 min (Figs. 3, 4). In 2.5-min homogenates the asters which form are quite different in structure from those forming at later times. These asters do not appear to have a distinct centriole, but do contain what appears to be a "procentriole." This usually appears as a very darkly stained tube (Figs. 7–10) although it sometimes appears as a nearly solid cylinder, but no triplet microtubules have been observed in the center at this early time. The central cylinder is coated by a thin layer of darkly stained granular material, and microtubules radiate directly out of, or insert into, this layer. By 4.5 min after activation the in vitro asters begin to have a more typical appearance. The center at this time contains a definite centriole which contains triplet microtubules. In the 4.5-min asters the granular material is clearly associated with the centriole and has a fairly well-defined outline. By 15 min after activation the volume occupied by the granular material is nearly double that of the 4.5-min asters and the outline is more irregular and poorly defined. The center of the 15-min aster generally includes a large number of clear, unstained regions, and the actual amount of granular material may not be significantly different from that in the 4.5-minute asters.

**Appearance after Negative Staining**

Asters have also been observed in whole mounts negatively stained with uranyl acetate (Figs. 11–14). The center of the aster stains too darkly for any substructure to be observed, and only the microtubules have been studied. The microtubules are very long (free ends are rarely observed) and tend to be associated into bundles of several tubules associated side by side. This side-by-side association occurs even if the microtubules have been broken off from the aster. If the asters have been stabilized in HGL solution (Figs. 11, 12) the microtubules tend to stain "positively," and under these conditions what appear to be cross bridges can be observed between adjacent tubules. The appearance of the HGL-stabilized aster microtubules is identical with that of the microtubules of the isolated Spisula spindle (16). Although these "cross bridges" may be real structures, other observations suggest an alternative explanation. Asters which have been centrifuged down without treatment and resuspended in MES buffer are stable for a short time, and when negatively stained they give a different appearance (Figs. 13, 14). Microtubules are still observed in side-to-side association but are surrounded by large numbers of clear 300–400-A particles. The space between adjacent particles may fill with stain which can give the appearance of a cross bridge. It is possible that at least some of the cross-bridge structures observed in the HGL-treated asters may be a result of the same phenomenon. The possibility that cross bridges between cytoplasmic microtubules may be an artifact caused by gaps in some material surrounding the microtubules was previously suggested by Bhisey and Freed (2). Particles very similar to those observed here have been reported by Rebhun and Sanders in thin sections of rapidly frozen Spisula eggs (11), indicating that the association between particles and microtubules observed in the asters is not an artifact of the preparation.

**Role of the MTOC**

Although homogenates of unactivated eggs do not produce asters, it has been possible to demonstrate that tubulin subunits in these homogenates are competent to form asters and that it is the lack of a functional MTOC which prevents aster formation.

In homogenates prepared 15 min after activation, the ability to form asters is lost in the supernatant fraction after centrifugation for 15 min at 15,000 rpm in a Sorvall SS-34 rotor at 4°C. The ability to form asters is regained, however, if the supernate is remixed with the pellet. Furthermore, normal asters can be formed even if the supernate is obtained from a homogenate of unactivated eggs provided the pellet is obtained from metaphase eggs. The pellet obtained from unactivated eggs cannot induce aster formation, regardless of the source of the supernate. Although the behavior of the organizing center and of the supernatant fraction have not been investigated yet at intermediate times, this experiment indicates that the ability of tubulin to polymerize in this
Figures 5-7  Electron micrographs of asters prepared from eggs homogenized 4.5 min after activation. The centriole is surrounded by a relatively compact mass of stained material as compared to metaphase asters. In Figs. 6 and 7 the centriole is positioned near a "notch" in the organizing material and some microtubules appear to radiate directly from the end of the centriole at this site. The section of Fig. 5 was tilted 46° to show the centriole in cross section. × 50,000.
FIGURES 8-10 Asters formed from homogenates prepared 2.5 min after activation of the eggs. Microtubules radiate from a densely stained central cylinder. No triplet tubules are observed at this time. Both Figs. 9 and 10 were heavily dodged in an attempt to reveal any substructure. Fig. 10 is the same section as Fig. 9 but was tilted 40°. Fig. 8, × 75,000; Figs. 9 and 10, × 50,000.
FIGURES 11 and 12 Negatively stained aster microtubules which have been stabilized in HGL solution. Microtubules are present in bundles, and apparent cross bridges can be observed at a number of locations. Fig. 11, × 40,000; Fig. 12, × 100,000.

FIGURES 13 and 14 Negatively stained aster microtubules prepared with no HGL treatment. Microtubules are still observed in bundles but are surrounded by large numbers of 300-400-Å particles. In Fig. 13, several examples can be seen where stain appears to have filled the space between microtubules, giving the impression of a cross bridge. Figs. 13 and 14, × 100,000.
system can be effected by a particulate factor which is probably the MTOC.

The apparent ability to separate the MTOC from the soluble fraction of the homogenates has made it possible to carry out experiments which test the activity of the MTOC after various treatments. The effect of colchicine on the development of the MTOC has been studied by incubating eggs in 0.5 mM colchicine for 1 h before activation. These eggs undergo a normal nuclear breakdown but fail to form any spindle. If these eggs are homogenized 15 min after activation and centrifuged to bring down the MTOC, the pellet which is obtained will induce aster formation if remixed with a supernate from untreated eggs. Although no electron microscopy has been performed on such asters yet, it is clear from this experiment that formation of a functioning MTOC is not inhibited by colchicine.

**Growth of Spindles In Vitro**

In all of the experiments reported above, the eggs were homogenized in cold buffer and only asters were formed upon inducing polymerization by warming. If the eggs are homogenized in warm buffer complete spindles will be released from the eggs, but if the solution is cooled only asters will reform upon warming. This may be a result of the MTOCs drifting apart when the spindle breaks down, or possibly may be due to failure of the chromosomes to function normally in this system. In neither light nor electron micrographs of in vitro asters have structures been observed which could be identified as chromosomes with microtubules radiating from the kinetochore. In this system the kinetochores have not been observed to have microtubule organizing activity, although it is not known whether they are even stable under the conditions of these experiments. In an attempt to obtain large numbers of reasonably clean intact spindles under approximately physiological conditions, we have added a high-speed egg supernate (containing active tubulin subunits) to a pellet of metaphase spindles isolated in HGL solution (11).

It was hoped that the tubulin in the supernatant fraction would prevent breakdown of the spindles after removal of the stabilizing solution by reversible association with the spindle microtubules. What was observed was a rapid and dramatic growth of the spindles (Figs. 15-18). From preliminary observations with this system, it appears that the pole-to-pole distance generally remains about the same as it is in the original isolated spindles, although some possible examples of pole-to-pole growth have been observed (Fig. 16). Some evidence for growth (Fig. 17) and shortening (Fig. 18) of chromosomal fibers has been observed, but, since it was not possible to observe the growth of each spindle as it occurred, no definite statement can be made about what changes actually took place in individual spindles. While this work was in preparation, similar results have been reported by Rehbun et al. (10).

**DISCUSSION**

The results presented here represent the first report of organized assembly of microtubules in an in vitro system. The ability to form asters in vitro has allowed a more systematic study of the formation and function of the MTOC. The structure of the asters in vitro appears identical in the important aspects (the presence of a centriole and associated granular material which appears to have the actual MTOC activity) with the structure of spindle asters in vivo, and it is probable that the results obtained with this system should be applicable to the living cell. This system is also valuable because it allows the development of the centriole and associated structures to be studied more systematically. Although it should be emphasized that this work is still in an early stage, the results obtained allow some conclusions to be reached and suggest some possible explanations for various aspects of spindle structure and function.

A particulate fraction can be obtained which can induce aster formation in a solution which is known to contain tubulin but which by itself cannot make asters. This indicates that the MTOC is a real structure in the sense that it is stable in solution and can be isolated. It does not appear to be simply a special region of the cytoplasm which is different in some local property (such as a lowered calcium concentration) which can initiate microtubule assembly. It is not yet clear to what extent the ability of tubulin to polymerize is affected by the MTOC. Electron microscope examination of the asters indicates that more microtubules polymerize in homogenates of eggs at later times after activation. Preliminary experiments to be presented elsewhere, attempting to quantitate the amount of polymerized tubulin by measuring particulate protein and colchicine-binding activity, are consistent with this interpretation. Since previous work (15) indicates no increase in the amount of tubulin in the eggs during this time, the change in microtubule polymerization appears
FIGURE 15  An isolated meiotic spindle from *Spisula* before the addition of a warm high-speed supernate to induce further polymerization. × 400.

FIGURES 16–18  Examples of giant spindles obtained by addition of a warm 20,000 rpm supernate, prepared in 0.5 M MES buffer, to a pellet of isolated spindles. Fig. 16 shows an example in which the pole-to-pole distance is significantly greater than that of the isolated spindles. Fig. 17 shows an example which may indicate growth of a pair of chromosomal fibers. Fig. 18 shows an anaphase configuration probably obtained from addition onto a spindle which had been in anaphase when isolated. × 400.

to be a result of the development of the MTOC. It should be pointed out that the particulate fraction which can induce aster formation may have other components which may affect polymerization, and this possibility could affect some of these conclusions.

The change in the appearance of the organizing center after activation suggests a process in which
the centriole "spins out" the organizing material. The decrease in the density of the darkly staining material associated with the centriole may be related to the increase in the size of the organizing center. The amount of organizing material may remain relatively constant but the material may become more disperse with time and thereby expose more organizing sites to microtubule subunits. The alternative explanation, that the organizing material accumulates around the centriole, does not explain as directly the apparent decrease in the density of organizing material with time after activation.

The presence of organizing activity before the formation of the triplet tubules of the centriole indicates that at least this aspect of the ultrastructure of the centriole is not required for organizing activity. This observation is consistent with the many observations that MTOC need not contain a centriole. The failure of colchicine to inhibit formation of a functional organizing center indicates that the center is not composed of tubulin, or that it is composed of tubulin in a state which is insensitive to colchicine. A possible problem with these observations is the intense staining of the center, particularly at early times. It is possible that this stained material could "hide" the triplet tubules, which could only be demonstrated if a procedure could be found to remove this material.

Association of microtubules side by side is a phenomenon which can be duplicated by microtubules assembled in vitro, but the existence of cross bridges as distinct structures has been neither confirmed nor disproven. The association of tubules routinely observed in repolymerized asters is not normally seen in repolymerized mammalian brain microtubules, which indicates that the side-by-side alignment of the aster tubules is not a simple preparation artifact. The cross bridges observed in sectioned spindles (6, 19) do not have any obvious similarities to the structures observed in the negatively stained asters or isolated spindles (16) and do not necessarily represent the same phenomenon.

The presence of large numbers of particles surrounding microtubules in rapidly frozen Spisula eggs (11) is supported by our observations of negatively stained asters. Although the particles reported here are larger than those observed by Rehbun and Sanders (250 A) (11), this may be a result of the differences in preparation. It remains to be determined whether such structures are generally associated with microtubules in other systems and what function they might have. Particles such as those found in Spisula could explain the clear region which has been observed to be a common feature of cytoplasmic microtubules. The particles observed by Rehbun and Sanders (11) were tentatively identified as glycogen, and this is probably consistent with the observations of Lane and Treherne (9) that lanthanum stained the clear zone around neurotubules.

The spindle isolated in HGL solution maintains at least some of its functional activities. It will allow further growth in the presence of additional subunits, and there is some evidence for an increase in spindle length and for growth of chromosomal microtubules. This system should be potentially valuable for experiments on spindle organization and function because it is possible to obtain large numbers of spindles in a known starting condition and the isolated spindles can be experimentally manipulated before the addition of fresh subunits.

The first signs of aster formation in vitro can be obtained at a time when the tubulin-containing structure present in the unactivated egg is still present. The breakdown of the tubulin-containing structure and the formation of an active organizing center occur over nearly the same period of time. By 5 min after activation the organizing center is well developed and contains a distinct centriole, and by this time the tubulin-containing structure has completely broken down (16). It is not clear what the relationship is between these two events but a possible explanation is that the tubulin which is polymerizing onto the newly formed organizing center is obtained from the tubulin-containing structure.

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