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Microtubule organization during the cell cycle of the primitive eukaryote dinoflagellate *Crypthecodinium cohnii*

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SUMMARY

The complete microtubular system of the dinoflagellate *Crypthecodinium cohnii* Biecheler is described, as seen by confocal laser scanning fluorescence microscopy and labelling with anti-β-tubulin antibody. This technique allowed us to observe the organization of the subcortical and internal cytoskeletons and the mitotic microtubular system, and their changes during the cell cycle. These observations are compared with those made in cryosections by light microscopy and in fast-freeze-fixed, cryosubstituted cells by electron microscopy. We show the organization of the cortical microtubules, and in particular of the thick microtubular bundles arranged as a three-pronged fork from which they seem to emanate. This fork emerges from a peculiar cytoplasmic zone at the pole of the cell and is in contact with the region of the kinetosomes, at the cingulum. During the G_1 phase, only a single, radial microtubular bundle (a "desmose") is observable in the inner part of the cytoplasm. One of its ends is near the flagellar bases and the other end is close to the nucleus in the centrosome region. During the S phase, the flagella drop off, the cell encysts and the kinetosomes duplicate. In mitosis, the cortical microtubules and the intracytoplasmic microtubular bundles do not depolymerize. The microtubular fork, desmose and centrosome double and migrate, while the divided kinetosomes stay in the same place. Later, the centrosomes organize the extranuclear spindle, which is connected to the kinetosome region by the microtubular desmose. The convergent end of the three-pronged fork seems to be in contact with the centrosome region. In early and mid-prophase, thick microtubular bundles pass through the nucleus in cytoplasmic channels and converge towards the two poles. Asters were never seen at the spindle poles. The channels and microtubular bundles in the spindle double in number during late prophase and lengthen in early anaphase. The spindle bundles diverge in late anaphase, extend to very near the plasma membrane and depolymerize during telophase. The cleavage furrow in which tubulin and actin are characterized appears in anaphase, formed by invagination of plasma membrane in the kinetosome region. The structure and rearrangements of the *Crypthecodinium cohnii* microtubular system are compared with those of other dinoflagellates and protists and of higher eukaryotes.

Key words: cell cycle, microtubular cytoskeleton, mitosis, confocal laser scanning microscopy, cryofixation, *Crypthecodinium cohnii*, dinoflagellate

INTRODUCTION

In eukaryotic cells, microtubules are involved in many cellular functions and in the cytoarchitecture (Dustin, 1984). These dynamic structures take part in the movements of cells and of intracytoplasmic organelles, and particularly in chromosome segregation during mitosis (Inoué, 1981; Alberts et al., 1989). When animal cells pass from interphase to mitosis, cytoplasmic microtubules depolymerize and reorganize into a dynamic mitotic spindle (for a review, see Karsenti and Maro, 1986). The polarity, orientation and spatial distribution of these microtubular networks are usually regulated by the cell's major microtubule-organizing centre (MTOC), the centrosome, which consists of a pair of centrioles surrounded by amorphous material (McIntosh, 1983; Tucker, 1984; Brinkley, 1985; McIntosh and Koonce, 1989; Sluder, 1989; Bornens, 1992). During cell division in higher plants, microtubules are reorganized repeatedly under the influence of acentriolar MTOCs (Baskin and Candle, 1990).

In the protist kingdom, there is considerable diversity both in the structure of the MTOC, which has variously been called a centrosphere, centrocone, rhizoplast, spindle pole body, kinetosome, attractophore or nuclear-associated organelle, and in the spatial organization and behaviour of microtubules during the cell cycle (for reviews, see Kubai,

1975; Raikov, 1982; Heath, 1986; Dutcher, 1989). Although comparison of conserved rRNA sequences suggested that dinoflagellate protists are phylogenetically close to typical eukaryotes such as yeasts and ciliates (Lenaers et al., 1991), dinoflagellates have several distinctive features, including permanently condensed chromosomes, and chromatin devoid of histones and nucleosomes (Herzog et al., 1984; Sala-Rovira et al., 1991). Moreover, during the distinctive closed mitosis called "dinomitosis" (Chatton, 1920), the microtubular spindle passes through the nucleus in cytoplasmic channels without directly touching the chromosomes, because the nuclear envelope persists throughout the cell cycle (for a review, see Triemer and Fritz (1984)). Immunodetection of tubulin in various dinoflagellates has shown that their microtubular cytoskeletal systems are mainly in the cortex (Netzel and Durr, 1984; Brown et al., 1988; Roberts et al., 1988a; Roberts, 1991), and has clarified relations with other components such as actin and centrin (Schnepf et al., 1990; Roberts and Roberts, 1991). Kubai and Ris (1969) studied the heterotrophic dinoflagellate *Crypthecodinium cohnii* in EM and described its unusual extranuclear mitotic microtubular spindle. Recent molecular data suggest that *Crypthecodinium cohnii* is one of the most primitive of the dinoflagellate Peridiniales (Lenaers et al., 1991). Its complex cell cycle has been described (Bhaud et al., 1991), and the presence of centrosome-like structures and their relations with the microtubular spindle have been reported (Perret et al., 1991). According to these results, in interphase cells, human anticentrosome antibodies labeled structures were located either in the cell periphery corresponding to kinetosomes and in the perinuclear area. Such structures, observed during mitosis at the poles of the nucleus, were designated as centrosome-like structures.

Hitherto, relationships between dinoflagellates' cortical and intracytoplasmic microtubular structures have been difficult to observe, because dinoflagellates have a thick theca and because the cortical microtubules mask the intracytoplasmic ones. By permeabilizing the cells to let antibodies in and using confocal laser scanning microscopy (CLSM), we have been able to see the distribution of all the microtubules, and how they interact and change during the cell cycle. Our observations are compared with findings in other eukaryotic cells (other protists, plants and metazoans), and the significance of such microtubular organization is discussed.

MATERIALS AND METHODS

Cell cultures

Crypthecodinium cohnii (see Fig. 1A,B), strain Whd (Woods Hole), was kindly provided by Dr C. Beam (Brooklyn College, City University of New York). Strains were maintained on 1.5% MLH agar medium according to the method of Tuttle and Loeblich (1975). For intensive cultures (3×10^5 cells ml^{-1}), clones were subcultured in MLH liquid medium and put in the dark at 27°C. The length of the cell cycle determined *in vivo* was 10 h for vegetative cells giving two daughter cells (Bhaud et al., 1991). Axenicity of the cultures was monitored by spreading on MLH agar, Zobell medium (Oppenheimer and Zobell, 1952) and nutrient agar.

Enrichment in mitotic cells

Axenic cultures of *Crypthecodinium cohnii* were removed during exponential growth and deposited on Nunclon culture plates (Poly-Labo). Just before mitosis, cells lose their flagella and encyst, so that division occurs in a nonmotile phase. For our experiments we kept encysted cells, which adhered to the surface of the plates, and discarded any swimming cells. Cysts were then suspended by vigorous agitation in a new medium and centrifuged at 2000 ***g*** for 10 min before fixation.

Fixation and cryomicrotomy

Crypthecodinium cohnii pellets were fixed in 3% formaldehyde in PBS buffer (0.15 M NaCl, 0.01 M Na_2HPO_4, 0.01 M KH_2PO_4, pH 7.4). After three baths in PBS buffer + 0.1% Tween 20, pellets were incubated overnight in 20% polyvinylpyrrolidone (PVP), 1.7 M sucrose, according to the method of Tokuyasu (1989). After quick freezing in liquid nitrogen, 2 μm thickness cryosections were deposited on coverslips in a drop of 2.3 M sucrose and stored at −20°C. For chromosome staining, PBS-washed cryosections were incubated in 0.1 μg/ml DAPI (Sigma, St Louis, USA) for 5 min, then rinsed with PBS and mounted in Mowiol containing 5% *N*-propyl gallate as an anti-fading agent. A Reichert (Leica) Polyvar optical microscope was used for fluorescence or bright-field microscopy observations.

Preparation of the *Crypthecodinium cohnii* cells for CLSM observations

Removal of the theca

Enriched pellets (cell density <100 000/ml) were rinsed in sea water and then suspended in a 1.1 M solution of sorbitol containing an enzymatic mixture of 1 mg ml^{-1} pectinase, 100 mg ml^{-1} sulphatase, 100 mg ml^{-1} lyticase (Sigma, St Louis, USA) incubated at 30°C for 90 min. Dinoflagellate protoplasts were rinsed in 1.1 M sorbitol and sedimented onto poly-L-lysine-coated coverslips.

Permeabilization of the plasma membrane

Some protoplasts were directly permeabilized and fixed in methanol at −20°C for 10 min and then washed in PBS + 0.1% Tween 20. Others were first fixed in 3% formaldehyde in PBS and then permeabilized with 1% Triton X-100 in PBS for 30 min at room temperature.

Immunocytochemistry

After the cysts had been fixed and washed in PBS, the coverslips were incubated with selected antibodies. First and second antibodies were diluted in PBS, 0.1% Tween 20, 1% BSA.

To visualize all the microtubules, coverslips were incubated with mouse anti-β-tubulin monoclonal antibodies (IgG class, from Amersham, France) (1:1000) overnight. The second antibody incubation was with goat anti-mouse Ig (GAM) coupled with FITC (Amersham, France) (1:100) for 2 h at room temperature. The coverslips were mounted in Mowiol containing 5% antifading *N*-propyl gallate.

To visualize actin, coverslips were incubated with monoclonal anti-actin antibodies (IgM class, from Amersham, France) (1: 500) overnight at 4°C. The second antibody incubation was with goat anti-mouse Ig (GAM) coupled with FITC (Amersham, France) (1:100) for 2 h at room temperature. The coverslips were mounted in glycerol containing 5% *N*-propyl gallate. Control incubations consisted of GAM fluorochrome without the first antibody. To visualize nuclear DNA, cryosections were stained with 0.1 μg/ml DAPI (4′,6-diamidino-2-phenyl indole) from Sigma, St Louis, USA.

Confocal laser scanning microscopy (CLSM)

Fluorescence-labelled cells were imaged on a confocal laser scanning microscope (Leica-Laser Technik, Heidelberg, Germany) with objective 100, zoom × 2. The laser source was an argon class IV laser, Spectra-Physics series 2000 (model 20920-05), excitation wavelength 488.0 nm. After fast scanning of the sample volume to determine the total height for laser sectioning and consequently the section pitch, 16 serial optical sections were acquired to show the whole cell. The pictures were systematically treated with a median filter. For three-dimensional reconstructions the number of sections was adjusted to give the best images of intracytoplasmic microtubular structures. Photographs were taken with a Sony UP3000P printing colour video recorder and a Polaroid video system (Freeze Frame).

EM procedures: fast-freeze fixation and freeze-substitution

Mitotic cells of *Crypthecodinium cohnii* were concentrated from the growth medium by centrifugation. A drop of highly concentrated cells was laid on filter paper (10 mm^2) and mounted on a specimen holder, by the method of Escaig et al. (1977). The sample was slammed onto a metal-mirror block of pure copper cooled with liquid helium at −269°C on a cryovacublock (Reichert-Jung, Leica) (Escaig, 1982), and stored in liquid nitrogen. Then freeze-substitution, in acetone and 2% OsO_4 in the presence of molecular sieves (0.4 nm, Perlform; Merck) to absorb the water extracted from the sample, was carried out in a CryoCool apparatus (RUA) for 3 days at −80°C. The temperature was then gradually raised to −30°C and kept there for 2 h. Finally, the samples were thawed at room temperature for 1 h, washed successively in pure acetone, absolute ethanol, and propylene oxide, and embedded in Epon. Sections, stained with uranyl acetate and lead citrate, were examined on a Hitachi H-600 electron microscope (Hitachi, Tokyo).

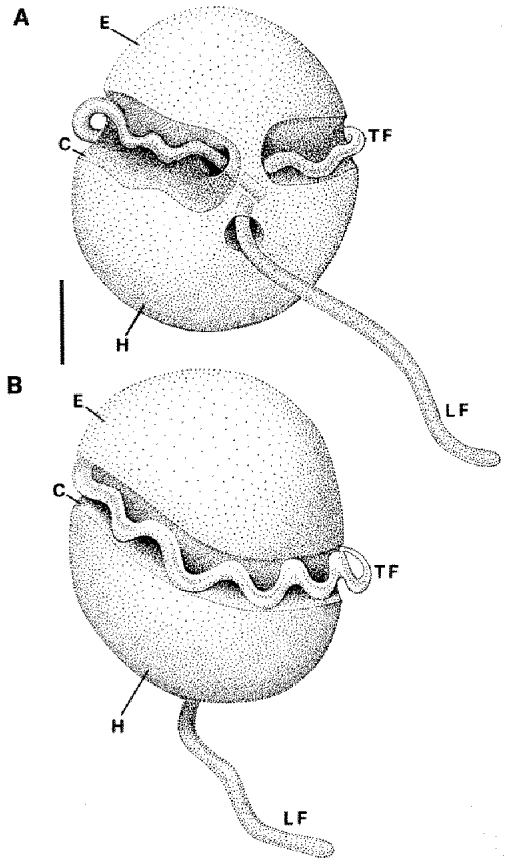

Fig. 1. Schematic representation of the *Crypthecodinium cohnii* cell, drawn from previously published SEM views (Perret et al., 1991). (A) Ventral view. (B) Dorsal view. E, episome, H, hyposome; L.F., longitudinal flagellum; T.F., transverse flagellum; C, cingulum. Bar, 5 μm.

RESULTS

Distribution and rearrangements of microtubules during the cell cycle in nondividing cells

The G_1 phase of *Crypthecodinium cohnii* is easily recognizable from the presence of both a transverse and a longitudinal flagellum (Fig. 1A,B) and from the presence of a roundish nucleolus and large chromosomes (1 μm in diameter) (Fig. 2A).

Cortical distribution

The distribution of microtubules was observed by CLSM in permeabilized whole cells labelled with anti-β-tubulin antibody. Cortical microtubules correspond closely to the external morphology (Figs 1B, 3A) and present several microtubular zones. According to the terminology of Roberts et al. (1988b), the episome is made up of microtubules in tight, oblique rows, and the hyposome, of more-spaced vertical rows. These two regions are separated by the equatorial cingulum, which is edged with numerous rows of vertical microtubules (Fig. 3A). The cingulum almost completely girdles the cell, as can be seen in the ventral view of the dividing cell of Fig. 3B.

Subcortical distribution

At the pole of the episome are located thick microtubular bundles that look like a three-pronged fork, in which each main bundle, or prong, is split in two at the tip (Fig. 3C,D). Each prong is several micrometres long, almost a quarter the circumference of the cell. The polar apical stereo-pair views of Fig. 3E and E′ show the microtubular rows of the episome radiating from this three-pronged structure. The origin and the divergent extremities of the fork penetrate into the cytoplasm and are clearly visible beneath the cortical microtubules (Fig. 3G). In EM, these microtubular bundles are visible under the theca and parallel to it (Fig. 5A). Just beneath the theca is an amorphous substance, which is interrupted at the level of the cortical rows of microtubules (Fig. 5B). The bundles of the three-pronged fork consist of many parallel microtubular rows surrounded by amorphous substance and regularly organized into a basket shape in which mitochondria and tubular structures of undetermined nature are located (Fig. 5B,C).

Intracytoplasmic distribution

After partial three-dimensional reconstruction of the images of the microtubules, a single intracytoplasmic microtubular bundle, or “desmose”, is visible in an interphase cell (Fig. 3F). This desmose originates near one end of the three-pronged fork (Fig. 3D,G) and extends towards the centre

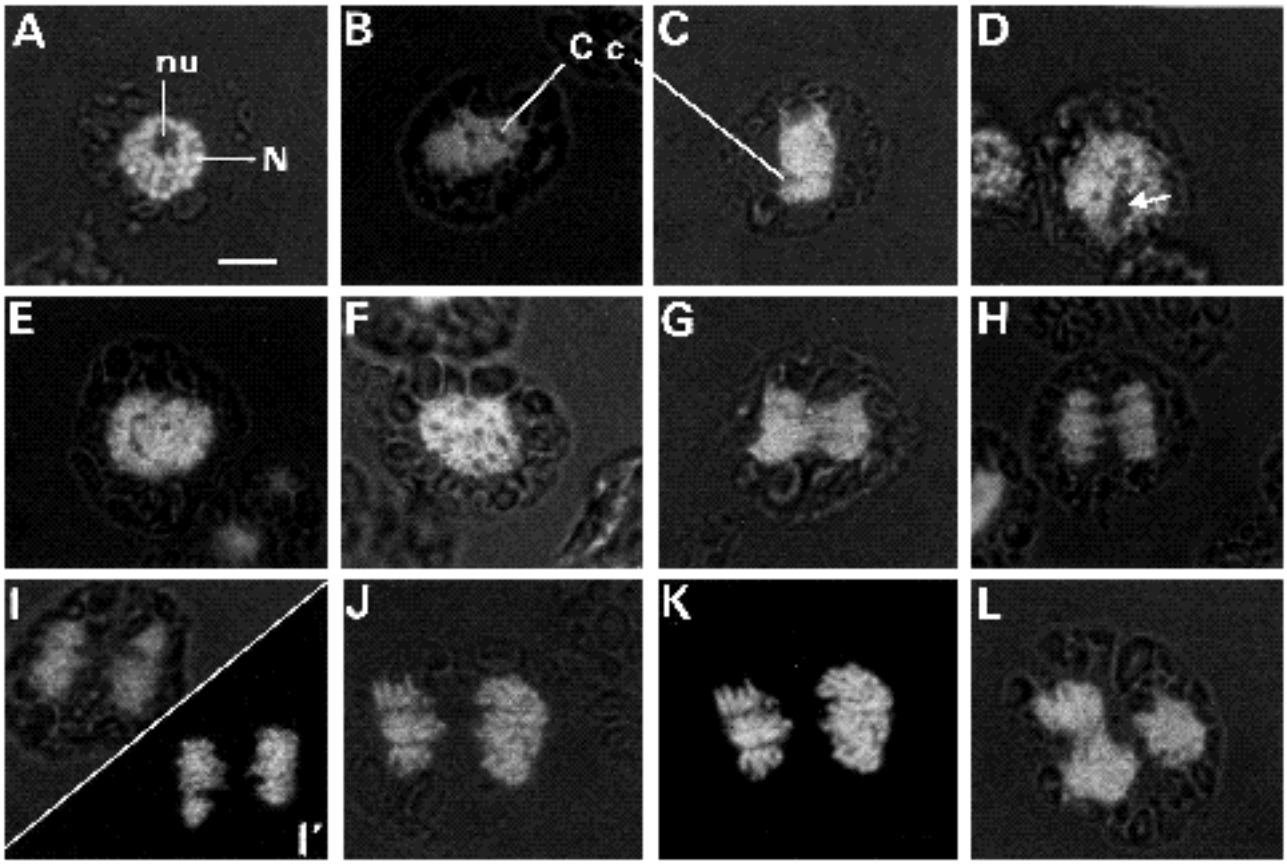

Fig. 2. Course of the *Crypthecodinium cohnii* cell cycle, seen in thick (2 μm) cryosections after DAPI staining of the nuclei. (A) G_1 nucleus (N) with a central nucleolus (nu). (B,C) Early prophase with very few cytoplasmic channels (C c). (D) Mid-prophase with a very large channel (arrow). (E) Mid-prophase, with smaller channels and with chromosomes at right angles to them. (F) Late prophase, with many small channels and with chromosomes at right angles to them. (G) Early anaphase. (H) Late anaphase. (I,I′) Telophase. (J,K) Early prophase of second division. Note that the chromosomes are at right angles to the channels, seen in longitudinal (left) and transverse (right) section. (L) Early anaphase of the second division. (A-L) ×7000. Bar, 1 μm.

of the cell in the region previously shown labelled by anti-centrosome antibodies (Perret et al., 1991). In the EM view of Fig. 6A, where the nucleus is in G_1 phase, the longitudinal flagellum is present and a thin bundle of about twelve microtubules is seen in cross-section close to the clear kinetosome region (Fig. 6B). Near the longitudinal kinetosome can be seen two dense satellites and a link between the two kinetosomes.

Fig. 3. Confocal laser microscope observations (mean 16 sections by image) of permeabilized *Crypthecodinium cohnii* cells and immunofluorescence of anti-β-tubulin during the cell cycle. (A-E′) Cortex views. (A) Dorsal view. Observe the equatorial cingulum (c) (B) Ventral view. The cingulum is nearly complete. The mitotic spindle (MS) is visible beneath the microtubular rows. (C-D) Almost apical views. One microtubular dichotomized three-pronged fork (TpF), or microtubular "basket", is visible here and on the stereo-pair (E, E′), where cortical microtubular rows are seen radiating from this structure. (F) A single radiating intracytoplasmic microtubular bundle (D, arrow) is observable in a nondividing cell. (G-P) Mitosis. (G). During early prophase, the microtubular desmose splits, linking the three-pronged fork (TpF) to the two poles (arrows) of the nucleus. (H) Mid-prophase. The thick microtubular bundles (about 8 to 10) of the mitotic spindle are converging towards the poles and seem linked to the cortex by thin microtubular bundles (arrows). (I) Another orientation of the same stage shows the converging and closed microtubular bundles of the mitotic spindle. No aster is visible. (J-M) Late prophase. (J) The number of the bundles of the mitotic spindle has doubled (to about 18). (K) Here the spindle pole (arrow) is very close to the cell cortex. (L,M) The two spindle poles are associated with the split three-pronged fork (TpF) and a double structure (arrows). The incipient cleavage furrow (cf) in the kinetosome region is labelled with anti-β-tubulin. (N) Early anaphase. The two desmoses (D) link the poles of the spindle to the kinetosome region, and the microtubular spindle is elongated, with the poles very close to the cortex. The cleavage furrow (cf) has finished forming. (O) Late anaphase. The microtubular bundles of the spindle diverge at the poles and are in close contact with the cortex (arrow). (P) In telophase, the microtubular bundles of the spindle depolymerize. (Q) Early prophase of second division. Cross- and oblique-sectioned groups of microtubular bundles are visible in the two dividing daughter nuclei. A, E-I, N-Q, ×10 000. Bar, 1 μm. B-D, J-M, ×13 600. Bar, 1 μm.

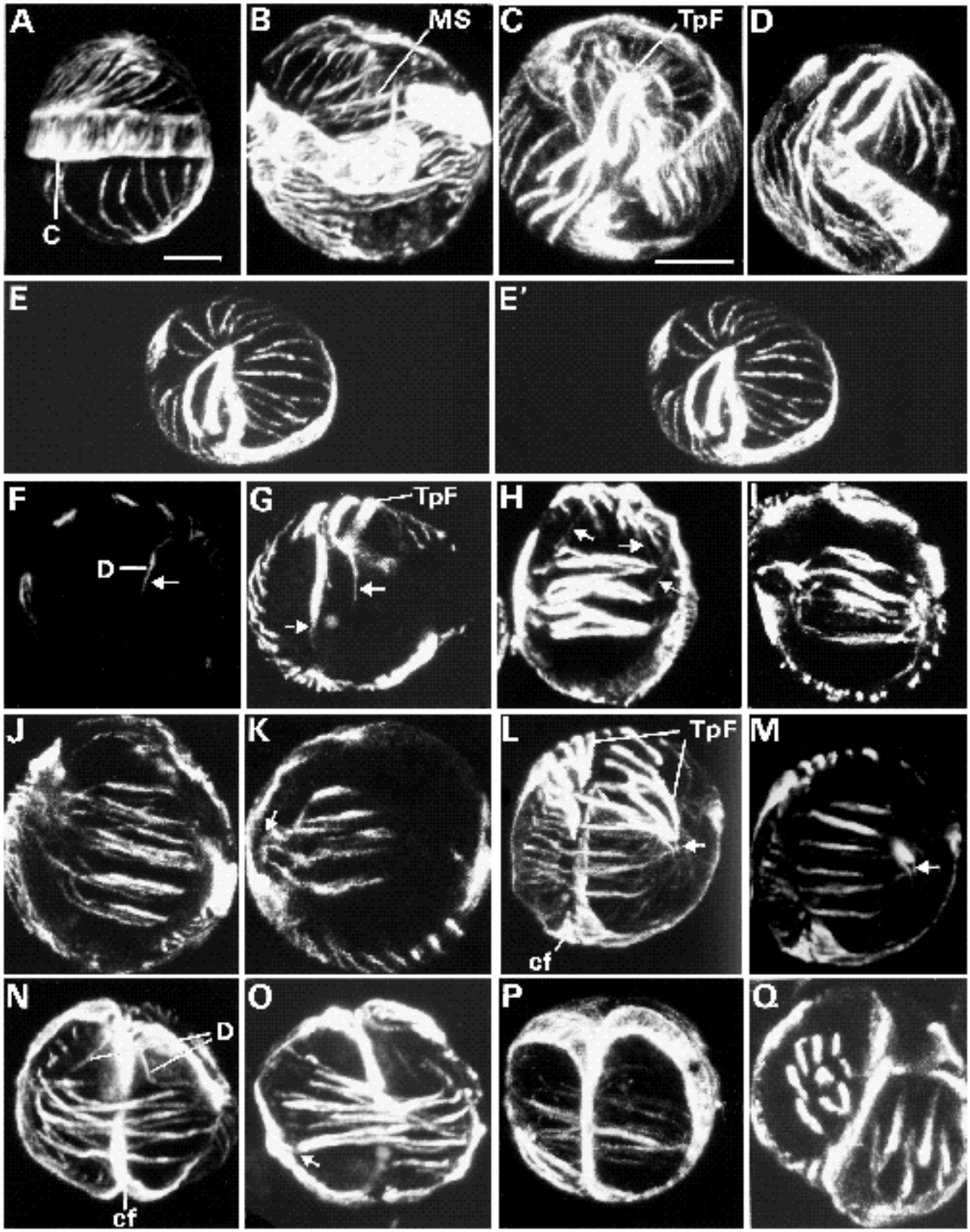

Fig. 3

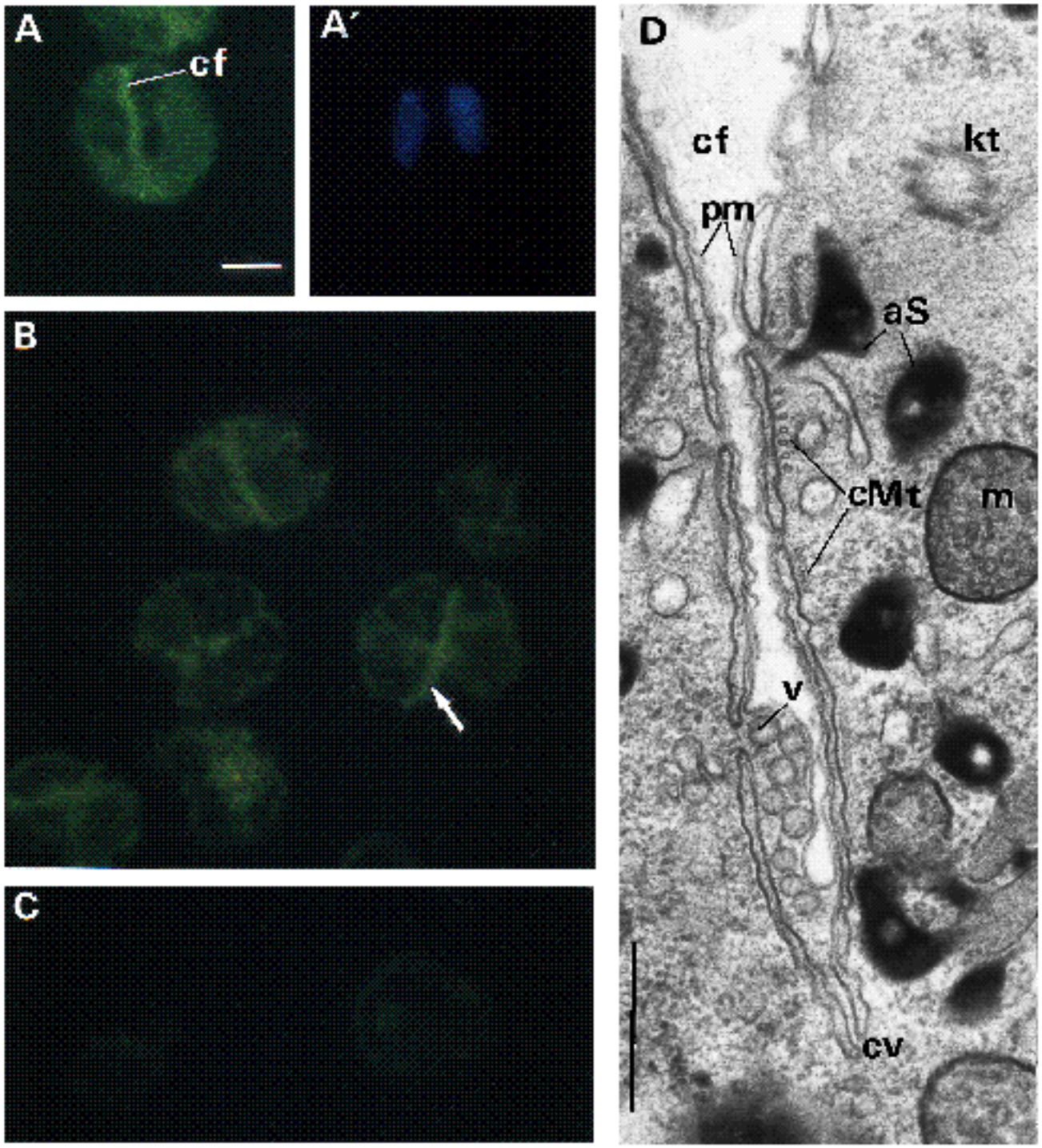

Fig. 4. Organization of the cleavage furrow in *Crypthecodinium cohnii* cells. Evidence for the presence of actin (A-C) and EM after helium cryofixation and cryosubstitution (D). In semi-thin sections (2 μm thickness) of cryofixed cell in telophase (A,B), the cleavage furrow (cf, arrow) is labelled with anti-actin Ab. (C) Control specimen incubated in GAM fluorochrome without anti-actin Ab exhibits no fluorescence. (A′) Nuclear DNA stained with DAPI. ×5,400. Bar, 2 μm. (D) The cleavage furrow (cf) forms in the vicinity of kinetosome (kt). Groups of cortical microtubules (cMt) lie under the numerous and flat cortical vesicles (cv), which are located just beneath the plasma membrane (pm). Amorphous substance (aS) and mitochondria (m) are also distributed in the same cortical region. v, vesicle. ×56,000. Bar, 0.5 μm.

Microtubular cytoskeleton in dividing cells (Fig. 3B,G-P)

The first event of the *Crypthecodinium cohnii* division is the loss of the flagella before the beginning of the S phase (Bhaud et al., 1991) and encystment. Fig. 2 (B-I′) represents thin cryosections of dividing cells in which cytoplas-

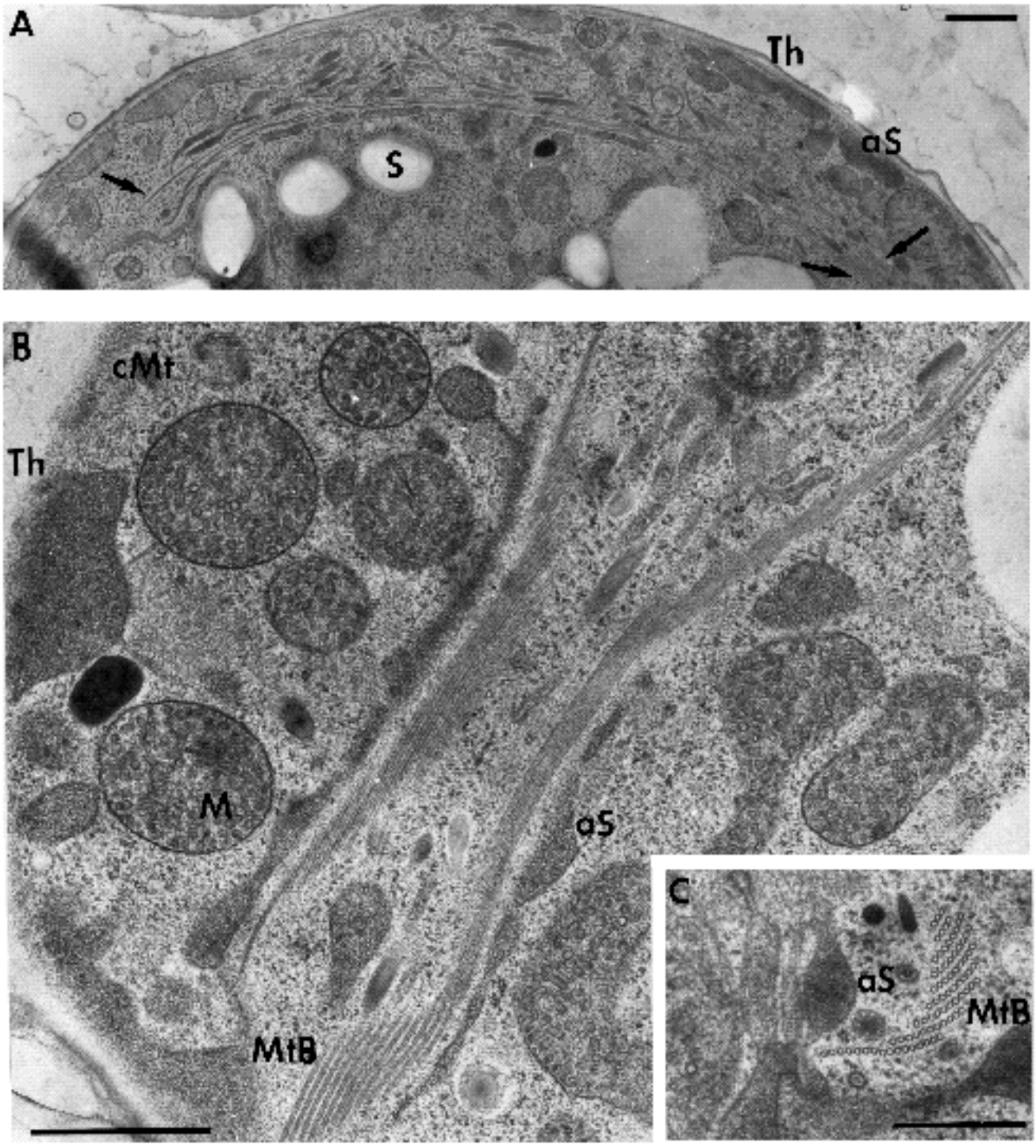

Fig. 5. EM views of helium cryofixation and cryosubstitution of a part of the microtubular "basket" (MtB). (A) Longitudinally sectioned microtubule bundle (arrows) several micrometres long with numerous associated tubular organelles parallel to the theca (Th) in the subcortical region. An amorphous dense substance (aS) is located just beneath the plasma membrane and all along the microtubular basket (MtB), as can be seen in the longitudinal section of B. cMt, cortical microtubules; M, mitochondrion. (C) Cross-sectioned microtubular rows of the microtubular basket (MtB) surrounded by amorphous substance (aS). A, ×12 000. Bar, 1 μm. B, ×30 000. Bar, 1 μm. C, ×44 000. Bar, 0.5 μm.

mic channels are clearly visible in prophase nuclei (B-F). No metaphase plate is observable, but in Fig. 2F thin duplicated chromosomes are visible perpendicularly oriented to the cytoplasmic channels. All these phenomena are characteristic of dinomitosis.

We describe the microtubular organization in a cell seen

oriented at a right angle to the episome-hyposome axis of Fig. 3A, so that the kinetosomes are at the top of the pictures. Fig. 3A exactly corresponds to the schematic drawing of Fig. 1B, with the episome located above the cingulum and the hyposome located below.

Cortical distribution

All the cortical microtubules persist during division, as can be seen in Fig. 3B, where the internal mitotic spindle is also visible.

Subcortical distribution

The three-pronged fork doubles during mitosis and its origins can be seen at each mitotic pole (Fig. 3L).

Intracytoplasmic distribution

The radiating microtubular desmose (Fig. 3F) splits (Fig. 3G) during early prophase (Fig. 2B,C). The two desmoses arise close to the microtubular bundles of the three-pronged fork, near the kinetosome region, and end at the two poles of the dividing nucleus. This is confirmed by the EM images of Fig. 6C-F. In early prophase of Fig. 6C, chromosomes are beginning to duplicate and only one cytoplasmic channel is visible; the microtubular desmose can be seen in longitudinal section passing through the Golgi bodies (seen also at higher magnification in Fig. 6F). The same microtubular desmose (arrows) is seen in cross-section in Fig. 6D,E. In the micrograph Fig. 6D, the Golgi bodies are polarized, forming an open "archoplasmic" sphere close to the nucleus. In mid-prophase, the cytoplasmic channels passing through the nucleus are large, as seen in the thick section of Fig. 2D. At this stage (Fig. 3H), thick bundles of microtubules (about 8-10) converge towards the mitotic poles (Fig. 3I). Asters were not seen in any stages of the mitosis.

In late prophase (Fig. 2E,F), where chromosomes are seen at a right angles to the cross-sectioned channels, there are more cytoplasmic channels than in mid-prophase. There are also twice as many bundles (about 18) in the mitotic spindle, and they are thinner and longer (Fig. 3J,K). At this stage the spindle pole is very close to the cortex (Fig. 3K).

In early anaphase (Fig. 2G) the microtubular bundles of the spindle are even longer (Fig. 3N). During these division stages the poles of the spindle are close to the split three-pronged fork (Fig. 3L,M) and remain linked with the kinetosome region by a double desmose structure (Fig. 3H,N). The cleavage furrow, also labelled with anti-β-tubulin (Fig. 3L-P) and anti-actin (Fig. 4A-C) begins to form during anaphase, by invagination of the plasma membrane (Fig. 4D), close to the kinetosomes. The growing plasma membrane is underlined by numerous flat cortical vesicles, under which groups of five or six microtubules are located. More or less amorphous substance and mitochondria are distributed in the same cortical region as shown at high magnification in the EM micrograph of Fig. 4D.

In late anaphase (Fig. 2H) the microtubular bundles of the mitotic spindle elongate, diverge at the poles and seem to be in contact with the cortex (Fig. 3O).

In telophase (Fig. 2I,I′) the microtubular spindle depolymerizes (Fig. 3P), and the cleavage furrow and the splitting cingulum merge in the region of the kinetosomes. In some cases, immediately after telophase the second division occurs, in which the orientations of the two nuclei, either in prophase (Fig. 2J,K) or in telophase (Fig. 2L), are at right angles, and so are their microtubular spindles (Fig. 3Q).

Fig. 6. EM after helium cryofixation and cryosubstitution of the kinetosome region and microtubular desmose in *Crypthecodinium cohnii* cells. (A) Overview of a G_1 motile cell where chromosomes are not dividing and a flagellum is present. ×12 300. (B) Detail of the same cell. The two orthogonal kinetosomes (kt) are linked (arrowhead) and are surrounded by a clear, thinly granular region containing two dense satellites (dS) linked to the longitudinal flagellum (Fl). A small bundle of 12 microtubules lies close to the kt region (arrow). Observe at the right the longitudinally sectioned cortical microtubules (cMt). ×36 800. Bar, 0.5 μm. (C) Overview of a *Crypthecodinium cohnii* cell in early prophase. One cytoplasmic channel (Cc) is visible in the nucleus as well as slightly decompacted arch-shaped chromosomes (Ch). The Golgi region (G) close to the nucleus is crossed by a microtubule (arrow). S, starch. ×12 300. Bar, 1 μm. (D) Passing through the spherical Golgi (G) region, close to the nucleus (N), a small microtubular bundle is seen in transverse section (arrows). (E) Higher magnification of D in which this desmose (D) is observed to be composed of about 8 microtubules. (F) Longitudinal section of one microtubule of the desmose (D) corresponding to higher magnification of C. D, ×33 000. Bar, 1 μm. E, ×66 000. Bar, 0.1 μm. F, ×32 000. Bar, 0.5 μm.

Relations between organelles during mitosis

The drawing in Fig. 7 summarizes CLSM and EM observations on a dividing cell (in anaphase) of *Crypthecodinium cohnii* in which the cortical microtubular rows and microtubular desmoses are not depolymerized and the Golgi bodies remain structured surrounding the centrosome-like zones. The kinetosome region is closely linked with the polar, centrosome-like zones, both by the microtubular desmoses and by the three-pronged forks. The latter run just beneath the cortex and plunge into the cytoplasm at each end, near the centrosomes and the kinetosomes. Throughout the division, the kinetosomes do not migrate, though they do double. Joining the two centrosome-like zones, the extranuclear mitotic spindle is not in contact with the divided chromosomes. Thus, the kinetosome region, microtubular desmoses, centrosome-like zones, three-pronged forks and microtubular mitotic spindle seem to constitute a system with close links in interphase and during mitosis.

DISCUSSION AND CONCLUSIONS

Specific preparation methods

Enrichment in mitotic cells

This technique produced numerous dividing cells in any phase of mitosis, for observation by CLSM or for cryosectioning.

Permeabilization techniques

Once the strong enzyme cocktail had broken down the theca and the thick cyst wall of *Crypthecodinium cohnii*, we were able to use standard methods of permeabilizing the proto-

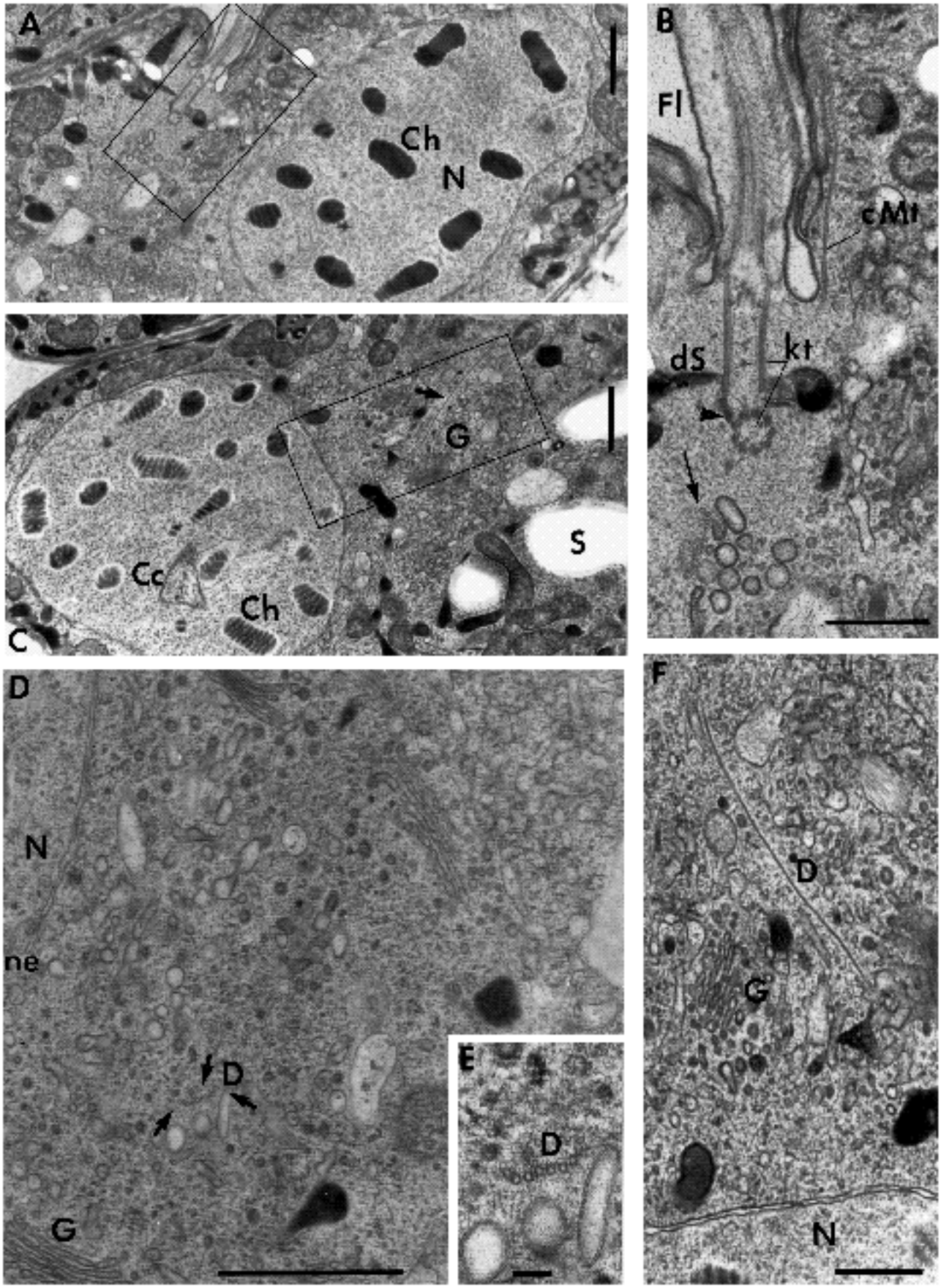

Fig. 6

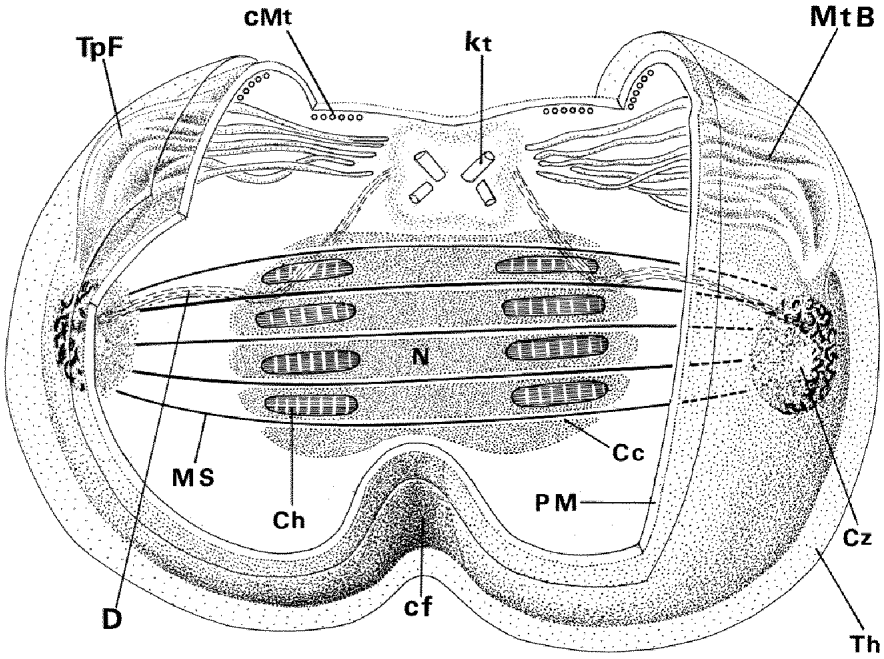

Fig. 7. Schematic representation (drawn by Mrs M. J. Bodiou) based on CLSM and EM observations of a dividing cell of *Crypthecodinium cohnii*, showing the four kinetosomes (kt), the dedoubled microtubular desmoses (D) linking the kinetosome region to the two centrosome-like zones (Cz), themselves closely connected with the microtubular mitotic spindle (MS) and with the proximal regions of the three-pronged forks (TpF) of the microtubular baskets (MtB), while their distal regions are close to the kinetosome areas. The cleavage furrow (cf) has begun in the region of the kinetosomes during anaphase. N, nucleus; Cc, cytoplasmic channel; Th, theca; cMt, cortical microtubules; PM, plasma membrane; Ch, chromosome.

plasts. Use of the universal anti-β-tubulin antibody made it possible to label all the polymerized microtubules, whether labile or not. In all cases, control preparations were made omitting the first antibody and using only the second (GAM) coupled with FITC. About 20% of the antibody penetrated.

CLSM

This technique provided three-dimensional visualization of most of the microtubular structures; in particular, allowing observation of the intracytoplasmic microtubules (mitotic spindle and desmose) after removal of the optical sections corresponding to those of the microtubular cortex. Moreover, EM observations of the microtubules are in agreement with the immunofluorescence observations**.**

Fast-freeze EM techniques

Fast-freeze fixation and freeze-substitution preserved all cell structures, especially microtubules and Golgi bodies, better than conventional preparation does, avoiding any depolymerization of labile structures. The better preservation is due to the rapidity of the freeze-fixation (2 ns) and to the freeze-substitution, which dehydrates the specimen as it thaws and thus prevents the cell structures from collapsing. Use of the technique made possible the first description in *Crypthecodinium cohnii* of the thin, fragile microtubular desmose seen by EM. This preparation method had previously allowed the first description of a similar desmose structure in *Prorocentrum micans* (Soyer, 1977).

Microtubule and centrosome organization during cell cycle of *Crypthecodinium cohnii*

Nondividing cells

Swimming cells are always nondividing (in G_1 phase), and cells just losing their flagella and encysting are in S phase and then begin to divide (Bhaud et al., 1991). In the intracytoplasmic compartment, a single microtubular desmose composed of about ten microtubules is present in G_1 phase: it could be assimilated to the "transverse microtubular root extension", described by Farmer and Roberts (1989) in the dinoflagellate *Amphidinium rhynchocephalum*. This "transverse microtubular root extension" arises in the vicinity of the basal bodies and extends into the cytoplasm where its actual termination is unknown. For a review, see Roberts and Roberts (1991). In *Prorocentrum micans* (Soyer, 1977), such a microtubular bundle extends into the centre of the archoplasmic sphere, near the nucleus, as shown in our present EM observations.

Dividing cells

Persistent structures

The kinetosomes are too small to be observed doubling and individualized in immunofluorescence light microscopy. In EM, it was extremely difficult to find all four kinetosomes in the same plane section (data not shown). In an early EM study, Kubai and Ris (1969) described for the first time the cortical microtubules and the subcortical microtubular basket, which are the equivalent of the three-pronged fork; they reported that the fork depolymerized during mitosis. By labelling these structures with anti-β-tubulin antibody followed by CLSM and fast-freeze EM preparation, we have found that the cortex microtubules do not depolymerize even during mitosis, nor do the microtubular basket (three-pronged fork) and the microtubular desmose, which duplicate during division. To account for this duplication, and for the formation of the mitotic spindle, it must be assumed that during the cell cycle either tubulin is synthesized or free cytoplasmic tubulin is mobilized. A close association between all the microtubules and an amorphous substance is observable only after fast-freeze preparation.

The mechanism by which cortical microtubules are maintained and duplicated is similar to that described in *Trypanosoma brucei*, in which the subpellicular microtubule

cytoskeleton remains intact throughout the cell cycle (Sherwin and Gull, 1989a,b) and quite similar to that of Hypotrich ciliates (*Euplotes*) in which two classes of microtubular arrays were described: one composed of stable microtubules, located superficially and partially originating from basal body-associated microtubular fibres, the other composed of dynamic cytoplasmic microtubular network (Fleury, 1991a,b). In *Crypthecodinium cohnii* (present data), the dynamic cytoplasmic microtubules are reduced to the mitotic spindle, while cortex and desmose microtubules are stable. In our present model, the microtubules do not depolymerize before mitosis, so that doubling of the persistent microtubules must precede cell division. This behaviour is different from that of other eukaryotic cells, such as animal cells, in which the interphase cytoplasmic network of microtubules disassembles before division while the mitotic spindle begins to appear, and such as higher plants in which the cortical network depolymerizes in the G_2 phase and gives the preprophase band of microtubules presaging the division plates. After prophase, the mitotic spindle forms and then the cell separates, under the control of the microtubular phragmoplast (Zhang et al., 1990; Murata and Wada, 1992).

Various organelles have been identified in the vicinity of the spindle poles, but the classic centrosome structures with a pair of centrioles near the nuclear envelope have been found only in the Syndiniales (parasitic dinoflagellates) (Hollande, 1972; Ris and Kubai, 1974; Soyer, 1974). In the other dinoflagellates, the spindle pole body is a structure called the archoplasmic sphere. This peculiar cytoplasmic zone, which is surrounded by Golgi bodies and is generally located in a depression of the nondividing nucleus, has been observed by light microscopy in parasitic and free-living dinoflagellates (Chatton, 1920) and by EM in *Blastodinium* (Soyer, 1970, 1971), *Noctiluca scintillans* (Soyer, 1969, 1972), *Prorocentrum micans* (Soyer, 1977), *Oodinium* sp. (Cachon and Cachon, 1977), *Prorocentrum minimum* (Fritz and Triemer, 1983) and *Amphidinium carterae* (Barlow and Triemer, 1988), among others. This structure had never been previously described in *Crypthecodinium cohnii*. The two microtubular desmoses, which originate near the kinetosome region, reach the centre of the archoplasmic spheres at the spindle poles, as observed by CLSM and EM (this work). We think this is the first kinetic description of this process. A similar structure has been seen in *Prorocentrum micans* by light microscopy after treatment with anti-β-tubulin antibodies (Schnepf et al., 1990, Figs 10-12) and in EM (Soyer, 1977).

Non-persistent structures

Combined use of cryosections and CLSM after anti-β-tubulin antibody penetration has afforded new observations of the prophase nucleus, and in particular has revealed the structure of the cytoplasmic channels in early, mid and late prophase and their relations with the microtubular bundles. We observed that the number of channels doubled, and that the microtubular spindle bundles decreased in diameter and doubled in number between early (short, thick bundles) and late prophase (long, thin bundles). We therefore conclude that the duplication and segregation of chromosomes (which are halved in diameter) takes place in late prophase, in what corresponds to an equilibrium phase before their migration. As in *Amphidinium carterae* (Barlow and Triemer, 1988), the mitotic spindle is closed at its two poles and it depolymerizes during telophase. However, the *Crypthecodinium cohnii* cell does not contain kinetochores as *Amphidinium* does, and the structure of the spindle is quite different. In *Crypthecodinium cohnii*, continuous thick microtubular bundles are organized from each pole (Fig. 3H). In other protists, plants and higher eukaryotes, most spindles appear to be composed of two classes of microtubules: kinetochore microtubules, extending from the chromosomes to one of the spindle poles, and nonkinetochore microtubules, extending from each pole and interdigitating with the half-spindle from the opposite pole, as is also the case in *Amphidinium carterae* (Barlow and Triemer, 1988).

A last, transitory structure is the tubulin-actin-rich cleavage furrow, which appears very early, in anaphase, arising from the kinetosome region. As in *Prorocentrum micans* (Schnepf et al., 1990), formation of the cleavage furrow of *Crypthecodinium cohnii* implies the presence of actin, which is also present during the cytodieresis process in animal cells. In this case, the cleavage furrow is a contractile ring composed of actin (Schroeder, 1973) and myosin (Mabuchi and Okuno, 1977). Moreover, in our material, it forms by invagination of the plasma membrane, inducing the participation of the nondepolymerizing microtubules of the cingulum in the cytodieresis process.

Our present observations confirm and synthesize the previous observations on the dinoflagellate cytoskeleton (Brown et al., 1988; Roberts and Roberts, 1991). In addition, we have followed the changes in the microtubular cytoskeleton and the relations among the various microtubular compartments during the life cycle. Microtubule distribution has been extensively studied in various protists, including the slime mold *Physarum* (Wright et al., 1988; Salles-Passador et al., 1991), trypanosomes (Bramblett et al., 1987; Seebeck et al., 1988; Sherwin and Gull, 1989a,b), yeasts (Jacobs et al., 1988; Pillus and Solomon, 1986), the flagellates *Chlamydomonas* (LeDizet and Piperno, 1986) and *Ochromonas* (Bouck and Brown, 1973), and hypermastigotes (Brugerolle, 1975), and of coarse ciliates (Cohen et al., 1982; Olins et al., 1989; Delgado et al., 1988, 1990; Fleury, 1991a,b). As previously shown, some trypanosomes can be compared to *Crypthecodinium cohnii* in having persistent cortical microtubules, and hypermastigotes resemble them in having an extranuclear mitotic spindle and a connection between the spindle poles and the kinetosomes. The most striking feature emerging from cytoskeleton analysis among several Protists is the fact that only three main groups have representatives in which stable microtubular cortex can be characterized: dinoflagellates (present work), trypanosomes (Sherwin and Gull, 1989a,b), and hypotrich ciliates (Fleury, 1991a,b). Predominance of a stable cortical microtubular network could be an indication of a primitive evolutionary stage, which is corroborated by molecular phylogenic data (Lenaers et al., 1991; Schlegel, 1991; Schlegel et al., 1991; Fleury et al., 1992). In their recent work, Schlegel et al. (1991) and Fleury et al. (1992) clearly demonstrated that among ciliates, those with superficial, longitudinal microtubules (i.e. hypotrich Oxytrichidae) are the most primitive. All these data confirm the primitivity

and the close relationship between kinetoplastida, dinoflagellates and ciliates.

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