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Centrosomal Proteins in Termite Symbionts: Gamma-Tubulin and Scleroderma Antibodies Bind Rotation Zone of *Caduceia versatilis*

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Abstract

Caduceia versatilis, a large devescovinid (amitochondriate parabasalid protist) is an intestinal symbiont of the kalotermitid (dry wood-eating termite) *Cryptotermes cavifrons*. Several bacterial symbioses led to the evolution of this parabasalid, composed of at least six once-independent genomes. Its prominent karyomastigont, an organellar system minimally comprised of the nucleus, the undulipodium, and a proteinaceous nuclear connector that joins them, is especially pertinent to studies of early eukaryotic evolution (Dolan et al., 2002). The anterior portion of *C. versatilis* (the karyomastigont that includes the nucleus) incessantly rotates in a counterclockwise direction due to the dynein-tubulin interaction of a conspicuous nucleus-associated microtubule organelle, the axostyle (Bloodgood and Fitzharris, 1974). Study of the mitotic, axostylar nucleus, aids in the reconstruction of the evolutionary history of the origin of nucleated cells. Investigation of this unique polygenomic protist in which the karyomastigont displays a "rotary motor" and other motility proteins reveals centrosomal proteins at the site of rotation.

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Fluorophore-tagged antibodies to the motility proteins γ-tubulin, pericentrin, and other centrosomal proteins were applied to all the intestinal symbionts of *Cryptotermes cavifrons* and *Neotermes mona* in hindgut preparations. Protists and bacteria were viewed using fluorescence light microscopy. A distinct pattern of fluorescent staining was observed in *C. versatilis* cells treated with FITC-conjugated scleroderma antisera and anti-γ-tubulin antibodies. Antibodies in both cases localized to the membrane at the shear zone (the "neck ring" or bacteria-free cell-rotation zone). No such pattern was seen in *C. versatilis* treated with anti-pericentrin antibodies, nor in a non-rotating unidentified large *Caduceia* from *N. mona*. Our observations suggest a novel location (but unknown function) of centrosomal proteins. Clearly motility protein homologies can be assessed *in situ* in amitochondriate protists and accompanying termite hindgut bacterial symbionts.

Keywords: Amitochondriates, eukaryotic rotary motor, origin of the nucleus, parabasalids, pericentrin, protists

1. Introduction

Caduceia versatilis, one of the largest protists in the gut of the kalotermitid *Cryptotermes cavifrons*, is a wood-ingesting parabasalid devescovinid. Each cell is composed of at least six once-independent genomes (d'Ambrosio et al., 1999). Two genomes (that of a *Thermoplasma*-like archaebacterium and that of a spirochaetal eubacterium) putatively merged to form the ancestral protist genome (Margulis, 1993; Margulis et al., 2000). The merger produced the karyomastigont, by hypothesis (Melnitsky et al., 2004). This organellar system, named the "karyomastigont" by Janicki (1915), is minimally comprised of the nucleus, the undulipodium (cilium or "eukaryotic flagellum"), and the proteinaceous band (or rhizoplast) that connects the two. Often, as in devescovinids, the microtubular axostyle and the membranous parabasal body (a kind of Golgi apparatus) are also components of the karyomastigont organellar system. The putative sequence of evolution of the nucleus via the karyomastigont is illustrated in *Eukaryosis*, a seventeen-minute video starring organisms whose ancestors, by hypothesis, figured in the Proterozoic eon scenario 2500–600 million years ago (Margulis, 2003). The other four genomes of *C. versatilis* are those of symbiotic bacteria. Cytoplasmic bacteria comprise the "bacterial cup" which sits below the nucleus. Furthermore, the nucleus itself houses bacteria (Tamm and Tamm, 1974). Flagellated Gram-negative rod bacteria and non-flagellated fusiform bacteria cover the protist's surface in a motility symbiosis (Tamm, 1982; d'Ambrosio et al., 1999).

The many *Caduceia versatilis* cells predictably present in all *Cryptotermes cavifrons* termites are easily distinguished by the continuous clockwise rotation

of their anterior ends. Before the formal species epithet was introduced, following the lead of Sidney Tamm, and still informally, this devescovinid is called "Rubberneckia" (d'Ambrosio et al., 1999). The anterior portion of the cell, which includes the nucleus, is separated from the stationary cell posterior by the bacteria-free shear zone (Fig. 1). The shear or cell-rotation zone is conspicuously devoid of the rod bacteria that cover the anterior surface of *C. versatilis* in transverse rows (Tamm, 1982; d'Ambrosio et al., 1999). The membrane of the cell-rotation shear zone is otherwise continuous with the rest of the plasma membrane and of similar composition (Tamm, 1979; Tamm and Tamm, 1983). Although membranous tubules are evident in transmission electron micrographs of the cell-rotation zone of *C. versatilis*, microtubules are conspicuously absent. These membranous cytoplasmic tubules near the protist's surface do not fuse with the cell membrane (Tamm and Tamm, 1974). The tubules run from the karyomastigont's parabasal body (Golgi apparatus) to the shear zone (Tamm and Tamm, 1983).

We report here microtubule-organizing center (MTOC) proteins in the cell-rotation zone of *C. versatilis*. Proteins such as γ-tubulin and pericentrin, concentrated in mammalian centrosomes, are found in all eukaryotes. These proteins are present even in acentriolar MTOCs of protists, those in which no [9(3) + 0] centrioles are found (Shang et al., 2002; Suh et al., 2002). Gamma-tubulin, a highly conserved 50-kDa protein, nucleates microtubule assembly of both the mitotic spindle fibers as well as the nine pairs and central microtubules of undulipodia (McKean et al., 2003). Gamma-tubulin forms rings 25 nm in diameter that transiently stabilize the minus-end of growing microtubules, as suggested by Moritz et al. (1995). The much larger pericentrin protein (220 kDa) colocalizes with γ-tubulin at the centrosomes, where their assembly is mediated by dynein (Young et al., 2000).

Pericentrin was first discovered by use of antibody preparations extracted from the blood sera of patients with scleroderma, an autoimmune disease (Doxsey et al., 1994). The autoantigens, absent in healthy controls, dramatically target proteins of the centrosome, including pericentrin (Gavenescu et al., 1999). Pericentrin is a major component of mammalian centrosomes which are sites of α, β, γ and other tubulins, as well as several less familiar protein components. Scleroderma antibodies were used here to seek possible centrosomal protein homologies in *C. versatilis*.

2. Materials and Methods

A colony of dry wood-ingesting termites, *Cryptotermes cavifrons*, from central Florida was maintained in the laboratory on dry wood and water. *Neotermes mona* were collected from Lameshur Bay, St. John (U.S. Virgin

Islands), and soldiers were sent to Rudolf Scheffrahn (University of Florida) for identification. The catalogue of termites and their symbionts compiled by Yamin (1979) was used to aid in identification of protists. The *N. mona* symbionts include a large devescovinid that resembles *C. versatilis* but it lacks a rotational shear zone. We identified it as a member of the genus *Caduceia* and used it as a control.

Termite hindguts were removed using fine forceps and teased open in a 5 µl drop of termite Ringer's solution. Their contents were allowed to settle onto 22 × 22 mm amino-silane (Sigma)-coated coverslips. Each coverslip was placed for 5 min in a Columbia jar containing Streck Tissue Fixative (Streck Laboratories), dipped for 10 sec in 4°C acetone, then stored for 5 min in a Columbia jar of cytoskeleton buffer, prepared as described by Mies et al. (1998).

Coverslips were inverted onto 15 µl of diluted antibody and incubated for 30 min in the dark in a humidity chamber. Antibodies were diluted with Tris buffer solution, prepared as indicated by Mies et al. (1998). Control mammalian PtK cells, grown on coverslips in Dulbecco's Modified Eagle's Medium with nonessential amino acids and Earle's salts (Gibco), received the same antibody as treated hindgut cells. Solution was added drop-wise to coverslips to treat them with polyclonal rabbit anti-pericentrin antibodies diluted 1:400. Coverslips treated with monoclonal mouse IgG anti-γ-tubulin antibodies varied in dilutions of the antibody to which they were subjected (1:10,000 or 1:1,000). Those treated with human scleroderma antibodies were treated at three dilutions (1:500, 1:250, or 1:100). All coverslips were washed with cytoskeleton buffer twice prior to incubation for 30 min in 15 µl of secondary antibody (FITC-conjugated anti-rabbit in the case of pericentrin, FITC-conjugated goat anti-mouse IgG in the case of γ-tubulin, and FITC-conjugated anti-human in the case of scleroderma antibodies).

See figure on next page.

Figure 1. Structure of *Caduceia versatilis*. Epibiotic bacteria coat the surface of the cell except at the bacteria-free cell rotation shear zone. The asterisk (*) indicates location of rotary motor and its membranous tubules at the cell's "neck region". Cytoplasmic bacteria are especially concentrated along the prominent axostyle directly below the nucleus, this structure is the "bacterial cup". Emerging from the intracellular kinetosome-centrioles are the four undulipodia: au=anterior undulipodium, pu=posterior undulipodium. The motility symbionts are primarily the flagellated prokaryotes (rbact=rod bacterium) associated with the tightly packed fusiform bacteria (fbact). (See Tamm, 1982 and d'Ambrosio et al., 1999). The bacteria-studded cortex is shown in the center inset whereas "golf-tee" relation of nucleus to the pelta, an extension of the axostylar microtubules, is diagramed in the upper right inset. Drawing by Christie Lyons.

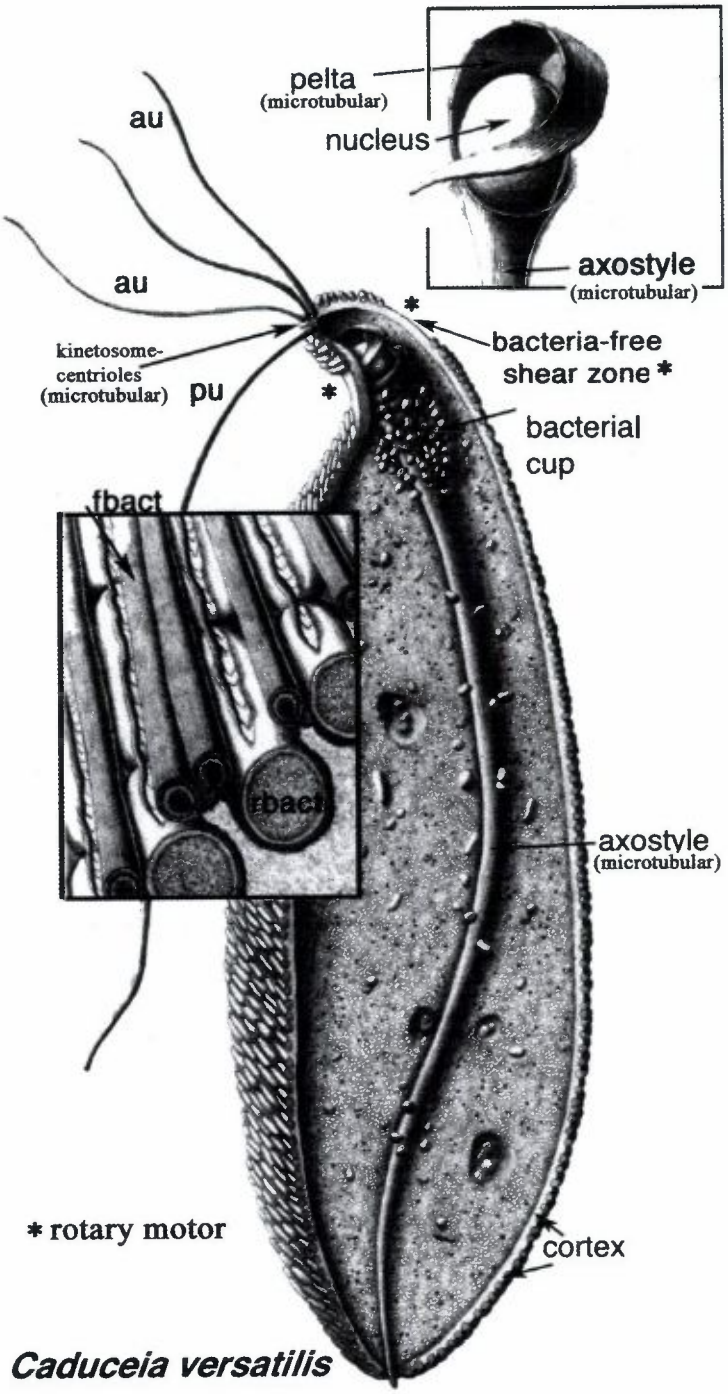

Figure 1. Structure of *Caduceia versatilis*. See legend on previous page.

Controls to detect nonspecific fluorescence were treated only with the secondary antibodies. Controls to estimate the pattern of fluorescence intrinsic to wood were treated only with primary antibody. Coverslips were washed twice with cytoskeleton buffer, mounted on slides, and viewed with an Optronix camera mounted on a Nikon Optiphot microscope fitted with epifluorescence, Nomarski differential interference, and phase contrast microscopy. This apparatus was used for videomicroscopy and photography. The video images were stored on 3/4-inch Sony U-matic 60-min tapes. Photographs were taken with 160 ASA 35 mm Ektachrome film.

3. Results

When treated with FITC-conjugated anti-γ-tubulin a distinctive pattern of fluorescence at *C. versatilis*' cell-rotation zone membrane resulted (Fig. 2). This "neck ring" pattern was seen in all *Caduceia versatilis* cells in at least seven different preparations made from different *Cryptotermes cavifrons* termites, both workers and soldiers. The same pattern was seen in the protists treated with FITC-conjugated scleroderma antibodies in all *Caduceia versatilis* cells in five different preparations (different termites, different days; Fig. 3). Each interpretable preparation contained at least five *C. versatilis* cells that all showed the same pattern. At the highest resolution a discontinuous punctate band of fluorescence was seen that resembled a pearl necklace apparently at the cell membrane. The constituent "pearls" ranged in shape from circles to ovals.

Both the antipericentrin antibody preparation and the non-rotating protist gave negative results. The "neck ring" pattern was not seen in any of the large unidentified devescovinids from the St. John termites. The anterior region of the *N. mona* protist does not rotate, nor is there a distinctive bacteria-free shear zone. Unlike the scleroderma and γ-tubulin positive fluorescence results, treatment with anti-pericentrin antibodies showed no discernible pattern. Anti-pericentrin fluorescence was difficult to distinguish from the sporadic distribution of autofluorescent wood. A few uninterpretable fluorescent dots were present in some cells. The two dilutions of γ-tubulin produced indisputably similar positive results. The optimal, most interpretable preparations made with the scleroderma antibodies were at buffer dilutions of 1:250.

The mammalian cell controls were unsatisfactory because the fluorescence was inconsistent from preparation to preparation and from cell to cell within a preparation for both antibodies. Although the fluorescence pattern is consistent with pericentrin and γ-tubulin antibody binding to centrosomes, no clearly fluorescent centrosomes were seen. In other experiments with α- and β-tubulin antibodies the protist preparations were more interpretable than the

mammalian controls: fluorescence of known microtubular structures (i.e., axostyles, axonemes) was consistently brighter and localized (Melnitsky et al., 2004). Controls for structure and wood fluorescence exhibited no such fluorescence pattern.

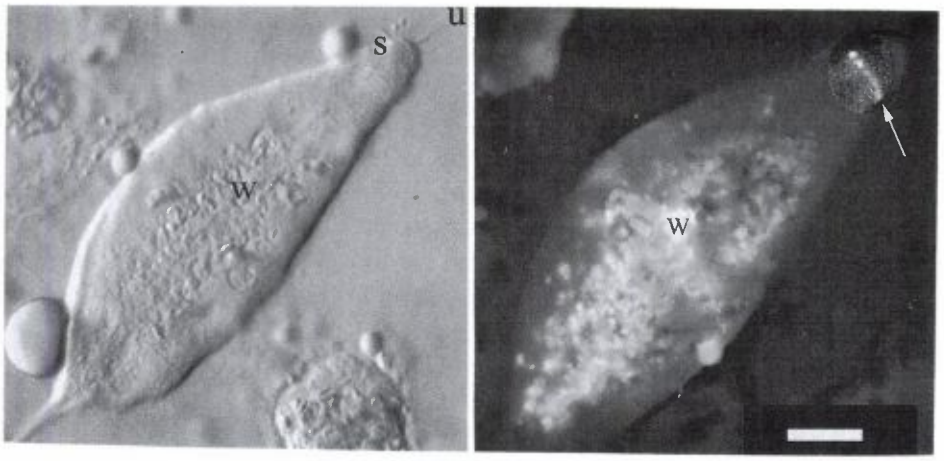

Figure 2. *Caduceia versatilis* treated with mouse anti-γ-tubulin antibodies and goat anti-mouse IgG FITC-conjugated secondary antibody. The corresponding darkfield image (right) shows a fluorescence pattern at the bacteria-free shear zone (arrow) that indicates the presence of γ-tubulin. s=shear zone; u=undulipodia; w=wood. Bar = 10 μm.

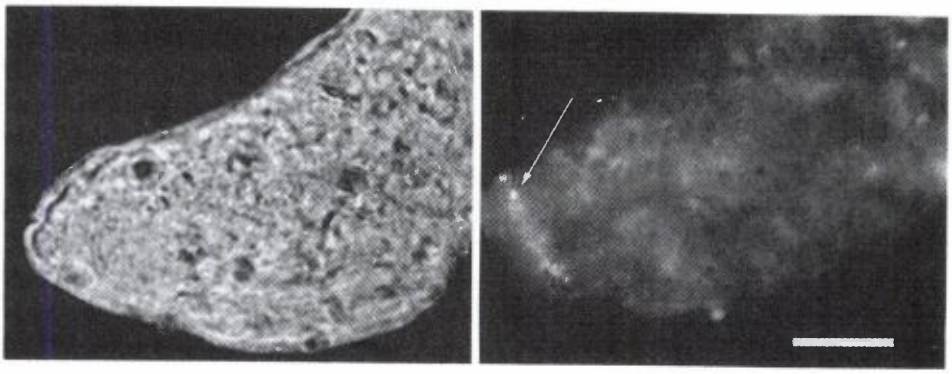

Figure 3. Anterior end of a *Caduceia versatilis* cell treated with human scleroderma antisera and human FITC-conjugated secondary antibody. The fluorescence at the bacteria-free shear zone (arrow) is the same as the "neck ring" pattern of γ-tubulin as seen in Fig. 2. Bar = 10 μm.

4. Discussion

Preliminary findings suggest a novel location, based on observance of the "neck ring" pattern, for two well-established mammalian centrosomal proteins in the parabasalid *Caduceia versatilis*. The fact that a similar devescovinid lacking a cell-rotation zone failed to exhibit the distinct "neck ring" fluorescence pattern suggests that these proteins are highly concentrated in the membranes of the cell-rotation zone. These two proteins, γ-tubulin and whatever protein is bound by the scleroderma antibodies, are likely present in this large devescovinid in some amount, since the proteins have so far been shown to be ubiquitous in eukaryotes. Although probably present in smaller quantities they do not produce the strong signal seen in *C. versatilis* with its highly conspicuous cell-rotation "neck ring". Pericentrin and γ-tubulin may be at the "neck region" of the unidentified *Caduceia* at concentrations too low to detect. The inconsistency of the results of the Ptk cell controls remains unexplained but the use of microtubule-stabilizing buffer instead of Tris in future experiments might improve the immunolabeling in the mammalian tissue culture cells.

Membranous tubules abundant at the cell-rotation zone lend credence to the observation that γ-tubulin was detected at the cell surface, for γ-tubulin binds to α- and β-tubulins to form membrane-associated complexes in plants (Dryková et al., 2003). The membranes of both the macro- and micronucleus of *Tetrahymena* contain γ-tubulin (Shang et al., 2002). Other tubulins (α and β) have been found in mitochondrial membranes (Carré et al., 2002). The evolutionary context that leads us to seek centrosomal proteins in amitochondriate protists is discussed in Melnitsky et al. (2004).

Our karyomastigont model of eukaryosis is detailed in that chapter. The earliest eukaryotes, in an anoxic sulfur-rich environment, began by symbiogenesis with both archaebacterial and eubacterial partners in a syntrophic consortium. The postulated initial step from which evolved the earliest swimming protists was a prokaryotic conjugation that led to permanent genetic fusion in the formation of the nucleus. Sulfur syntrophies prevalent in intertidal marine and estuarine environments of the Proterozoic Eon (2500–541 million years ago) were comparable to the extant "Thiodendron" consortium found today at many locations associated with the brown alga *Fucus*. Mergers between spirochete eubacteria (comparable to *Spirochaeta* that comprise today's Thiodendron) and sulfidogenic archaebacteria with biological features similar to extant *Thermoplasma* (Searcy, 2001; Searcy and Lee, 1998) led to the first nucleated cells. The ancestral spirochete provided the motility system that became the dynamic cytoskeleton including the mitotic apparatus. Thus ubiquitous motility proteins of eukaryotic cell cytoskeleton and mitosis (e.g., of pericentriolar material, axonemal and mitotic microtubules) are predicted to be

more related to pertinent modern spirochete proteins than to those of any other arbitrarily selected prokaryote. From the archaebacterium originated most of the protein synthetic apparatus, sulfidogenesis, chromatin (histone-studded DNA) and the endoplasmic reticulum and its glycopeptide metabolism. The mitotic apparatus including their centriole-kinetosomes, the ATP and GTPases of the cytoskeleton and the golgi in all its guises (e.g., parabasal bodies, golgi apparatus, dictyosomes) by this reckoning evolved from the motile eubacterium by internalization of its motility and attachment structures (Dolan et al., 2002).

The colocalization of both γ-tubulin and the scleroderma antibodies to the bacteria-free cell-rotation zone of this devescovinid suggests a function related to that most widely known: i.e., of a microtubule organizer. Microtubule nucleation, from membrane sites, may simply not have been detected. Alternatively these proteins may either seal or lubricate the underside of the cell-rotation zone. Gamma-tubulin and pericentrin nucleate the minus-end of microtubules, and dynein/dynactin transports membrane fragments along microtubules in the retrograde direction. Thus γ-tubulin and pericentrin may be enriched at the "neck ring" because of incessant membrane transport to this region. Sterol-specific membrane complexes were detected in this same "neck ring" pattern by the use of filipin and digitonin as cytochemical probes (Tamm and Tamm, 1983). Cholesterol and other 3-α hydroxyl sterols were detected and visualized as membrane lesions in high resolution electron micrographs (Tamm and Tamm, 1983). The fact that membrane tubules abound but α-β-tubulin microtubules have not yet been seen at the bacteria-free shear zone does not preclude the presence of γ-tubulin as a membrane-bound microtubule organizer (Khodjakov and Rieder, 1999). The regional hypertrophy of γ-tubulin-membrane-associated rings ought to be carefully sought by electron microscopy of the shear zone before any of these hypotheses can be accepted.

More experimental work must be done to determine the significance of these centrosomal proteins in the cell-rotation zone of *Caduceia versatilis*. Gold- and radioactivity-labeling with immunocytochemical techniques and correlated transmission electron microscopy with autoradiography may illuminate the precise location of these proteins. Further study should extend to other centrosomal and MTOC proteins, such as those listed in Table 1, page 295 of Dolan et al. (2002). Higher resolution and biochemical specificity should clarify the relationship of these centrosomal proteins in a non-centrosomal location.

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