Protist tubulins: new arrivals, evolutionary relationships and insights to cytoskeletal function

Keith Gull

The protists exhibit probably the most extravagant expression of microtubule-containing structures found in any organism. These structures – flagella, cilia, axostyles, spindles and a veritable constellation of microtubule bundles and cortical arrays – provide shape, form, motility, anchorage and apparatuses for feeding. The cytoskeletal structures have a precise order (i.e. size, position and number) that must be replicated and segregated with fidelity at each division, some components being inherited conservatively and others semi-conservatively. Intriguingly, it is now apparent that much of the high-order organisation, which was recognised and described by light and electron microscopy during the last century, is a reflection of molecular polarities set by assembly of constituent proteins. Tubulins and microtubules lie at the heart of this morphogenetic pattern.

Addresses
School of Biological Sciences, University of Manchester, 2.205 Stopford Building, Oxford Road, Manchester, M13 9PT, UK; e-mail: K.Gull@man.ac.uk

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Abbreviations
dsRNA double-stranded RNA
PFR paraflagellar rod
RNAi RNA interference

Introduction: tubulins – α to η

The proteins that form the microtubule wall — α and β tubulin — were first characterised over 30 years ago. They were found to assemble with an intrinsic polarity. Some 12 years ago, γ tubulin was first identified in *Aspergillus nidulans* in a genetic screen. It was found to be located in microtubule organising centres (MTOCs), where it plays an essential role in initiating microtubule assembly [1]. These three tubulins appear to be present in all eukaryotes and are the only tubulins in some eukaryotes, indicating that this is the minimal set required to define microtubule function in these organisms. The importance of this 'minimal set' is illustrated by the fact that the reduced genome of the secondary endosymbiont of the cryptomonad *Guillardia theta* contains α, β and γ tubulin genes [2].

Exciting recent developments, however, have shown that eukaryotes contain other members of this tubulin superfamily: a further four members — δ, ε, ζ and η — were identified in the past few years. Two of these, δ and η, were identified by mutational genetics in *Chlamydomonas* [3] and *Paramecium* [4••], respectively, and in both cases, the mutants exhibited defective basal body functions. Mutations in *Chlamydomonas* δ tubulin result in elevated frequencies of uniflagellate cells, and flagellar basal bodies possess doublet rather than triplet microtubules. In *Paramecium*, η tubulin mutations exhibit basal body duplication defects and reduction of the oral apparatus, and a low percentage of basal bodies lack microtubules.

The other tubulins, ε and ζ, were discovered by cloning or genomic approaches in the African trypanosome *Trypanosoma brucei* [5••], and ε tubulin was discovered independently in mammalian cells [6••] by genomic approaches. Immunolocalisation of ε tubulin in mammalian cells shows it to be located to the centrosomal area. ζ tubulin is located by immunofluorescence and immunoelectron microscopy to the basal body region in trypanosomes and at the centriolar region in some animal cells (S Vaughan, K Gull, unpublished data).

This review covers the evolutionary biology, biochemistry and function of the microtubule cytoskeleton. It considers the impact of new gene silencing and RNA interference technologies and points out the role of the microtubule cytoskeleton in segregating cytoplasmic organelles of eukaryotic microbes, and even of some intracellular microbes themselves.

A distant relative: FtsZ

Expression of a microtubule cytoskeleton appears to be the prerogative of eukaryotic cells. However, there exists a prokaryotic protein that seems to be a distant relative of tubulin. FtsZ is a prokaryotic cell division protein that can form protofilament structures and has a weak sequence homology to tubulin. Its recently revealed three-dimensional structure emphasised the protein's remarkable similarity to tubulin [7,8]. The FtsZ superfamily exists in eubacteria, some archaea and chloroplasts, and has also recently been found in some mitochondria [9••]. Beech and colleagues [9••] have described a nuclear-encoded homolog from the alga *Mallomonas splendens*. This protein is most closely related to the FtsZs of the α-proteobacteria but possesses a mitochondrion-targeting signal and its location is suggestive of a role in division of mitochondria in this alga. Thus, in eubacteria, archaea and organelles, evidence is rapidly emerging for internal 'skeletons' that have properties linked to shape and division [10•,11].

Tubulin superfamily: evolutionary distribution

Drawing conclusions about the evolutionary distribution of tubulins is difficult. There is a diverse dataset from completed and partial genomes of a wide range of organisms. Certain themes, however, are emerging and we have noted in a previous paper the interesting evolutionary distribution

of the new tubulins [5••]. So far, the ancient protozoan *T. brucei*, whose genome sequencing project has not yet been completed, has six tubulin superfamily members (α, β, γ, δ, ε and ζ), whereas some organisms whose genome sequencing projects have been completed contain only the minimal set (α, β and γ). Organisms whose genome sequencing projects have been completed include *Saccharomyces cerevisiae*, *Saccharomyces pombe*, *Arabidopsis*, *Caenorhabditis elegans* and *Drosophila* [1,12]. Individual examples of the new tubulins are well represented within the genome databases of a variety of protists and vertebrates such as *Xenopus*. There is a reasonable correlation between the occurrence of the new tubulins (δ, ε, ζ and η) and the possession of a motile axoneme and triplet microtubule basal bodies or centrioles [5••,12]. The yeasts and higher plants are well known for their lack of such structures; *C. elegans* lacks normal triplet microtubule basal bodies or centrioles and, although there are reports of centrioles with nine triplets in *Drosophila*, it is known that variations occur in early development. Moreover, the position in insects in general is unclear [13]. An important additional aspect of the new tubulins may be to endow organisms with probasal body or basal body (or procentriole/centriole) structures linked to cell cycle patterns of duplication, maturation and inheritance.

Modifications of α and β tubulin

Post-translational modifications to tubulin have long been an enigmatic area of cell biology. They occur in many eukaryotes but are not universal [14,15]. Their distribution provides an intriguing evolutionary and cell biological puzzle. Lysine 40 of α tubulin can be reversibly modified by the addition of an acetyl group. *Tetrahymena* mutants engineered to express an α tubulin unable to be acetylated at lysine 40 showed no discernable phenotype, suggesting that acetylated α tubulin is either not important under the conditions tested or alternative events, or proteins can supplant the normal function [16]. The α tubulin tyrosination cycle involves the enzymatic removal of the carboxy-terminal tyrosine residue and subsequent restoration via a tubulin–tyrosine ligase [17]. The functional relevance is not clear but the modification appears to be a reflection of the length of time an individual molecule has spent in a microtubule. Highly stable microtubules, therefore, such as those of the trypanosome sub-pellicular array, are generally detyrosinated [18].

The α and β tubulin carboxy termini can be modified by polyglutamylation and polyglycylation, which are processes involving the attachment of oligoglutamyl and oligoglycyl side chains, respectively, to specific glutamate residues [15]. These side chains can be of considerable length. For instance, axonemal tubulin in *Paramecium* is modified by up to 34 glycyl residues, and the microtubules of *T. brucei* are modified by 15 glutamyl residues per α tubulin subunit.

Polyglutamylation and polyglycylation are particularly associated with stable microtubule structures such as the axonemes of cilia and flagella. *Tetrahymena* has again recently provided the vehicle for addressing the function of one of these modifications. The microtubules of this protozoan are formed from single α and β tubulin isotypes, hence allowing molecular engineering of the proteins such that they cannot accept the modifications. Modifying the multiple polyglycylation sites in α tubulin produced no observable phenotype [19]. In contrast, β tubulin polyglycylation was essential and, moreover, reducing but not eliminating polyglycylation of β tubulin resulted in slow growth, reduced motility and cytokinesis defects. Other experiments involving α or β chimeric tubulins suggested that it is the level of polyglycylation modification that is important. This is an elegant set of experiments but intriguing puzzles remain.

One conundrum is how to explain the evolutionary occurrence of the modifications amongst protozoa that construct complex microtubule cytoskeletons. A successful collaboration between the Weber and Schneider labs [20–24] has mapped the tubulins of three groups — the trichomonads (*Tritrichomonas mobilensis*), the trypanosomatids (*T. brucei)* and the diplomonads (*Giardia lamblia)* — using advanced protein chemistry. Tubulin acetylation and polyglutamylation are present in all three, whereas tyrosylation was only detected in trypanosomes. Conversely, polyglycylation was detected in the diplomonads and not in the other two groups. Therefore, the ancient flagellate *T. brucei* appears to be able to do without polyglycylation, whereas the experimental evidence suggests it is essential in the ciliate *Tetrahymena*.

Functional analysis using microtubule inhibitors

Microtubule inhibitors have received some attention in recent years as probes for determining function and as avenues for possible therapeutic approaches [25]. During the 1980s, our laboratory mapped the differential drug sensitivity of protists and showed, by purification of tubulin and its assembly *in vitro*, that their sensitivity profile was a direct reflection of the drug activity against constituent microtubules [26,27]. Colchicine and some benzimidazoles, both potent inhibitors of mammalian tubulin polymerisation, had only very slight effects on some protist tubulins. Vinblastine and the maytansinoids are good inhibitors of trypanosome tubulin polymerisation. We were also able to show that the protozoa were sensitive to herbicides such as trifluralin, oryzalin and their analogues that act via microtubule inhibition [28] and, subsequently, these studies were extended by others in the 1990s [29]. More recently, Werbovetz and colleagues [30,31] have purified *Leishmania* tubulin, studied its *in vitro* drug sensitivity profile and related this to cellular effects of the inhibitors. The usefulness of vinblastine in studies of cell cycle and cytokinesis in *Trypanosoma cruzi* has also been demonstrated [32] .

Bell [33] has rehearsed the usefulness of microtubule inhibitors both in studies of malaria and as potential antimalarials. As stated previously, we have long known

that the selective toxicity of microtubule inhibitors on protists and mammalian cells is a direct consequence of the target tubulins. However, tubulin is a complex target for antiprotozoal chemotherapy, given the difficulties of fast, rational screens and the critical importance of the protein in man. Although microtubule inhibitors have proven track records as anticancer agents, enthusiasts for tubulin as a target for antiprotozoal chemotherapy will have to establish compounds with a very wide safety to efficacy margin.

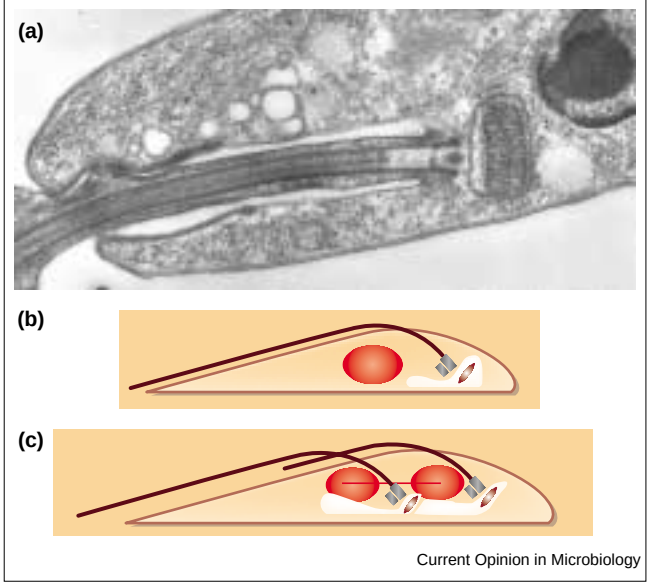
The usefulness of an expanding arsenal of microtubule inhibitors as probes for microtubule functions in protozoal parasites is, however, very clear. Use of microtubule inhibitors [34] has defined the necessity of a small set of microtubules (the f-MAST) in development of merozoites of *Plasmodium falciparum*. In *Toxoplasma*, Shaw *et al.* [35] used taxol and oryzalin to show that microtubules are involved in daughter-cell budding of tachyzoites. Drug treatments stopped parasite replication with extensive morphological consequences. However, taken together with other earlier studies in *Toxoplasma*, the phenotype of the cell cycle block and terminal phenotypes suggests that this and other protozoan parasites appear to vary from higher eukaryotes in their cell-cycle checkpoints. Evidence for this has come from studies on *T. brucei*, in which cell phenotypes obtained using microtubule and DNA inhibitors showed that cytokinesis is not dependent on either mitosis or nuclear DNA synthesis [36,37]. Drug treatments lead to the production of anucleate, flagellated cytoplasts termed zoids. These studies emphasize the importance of basal body segregation as an important factor in defining structural and regulatory aspects of the cell cycle and cytokinesis.

Functional analysis using RNA interference

Cytoskeletal protein expression in trypanosomes can be ablated by a gene silencing or RNA interference (RNAi) approach. Both of the earliest, serendipitous applications [38,39] produced distinct morphological phenotypes (stable ablation of PFRA expression, leading to paralysed cells and transient inhibition of α tubulin expression, leading to misshapen 'FAT' cells). This RNAi technology has now been developed to provide generic, inducible and heritable production of hairpin double-stranded RNA (dsRNA) or alternatively, dsRNA from two opposing promoters [40••,41•,42••,43••].

The paraflagellar rod (PFR) is a massive structure that runs alongside the axoneme in flagella of trypanosomes and euglenoids and is composed of two major proteins, PFRA and PFRC. When expression of PFRA dsRNA is induced in trypanosomes, PFRA protein synthesis ceases, and cells lack the normal PFR structure and are viable but paralysed [38,40••]. Ablation of α tubulin expression in *T. brucei* is obviously lethal. However, the early phenotype showed that lowering the α tubulin content of the cell dramatically affected morphology [39]. The trypanosomes lost their vermiform appearance and became spheroid, cytokinesis failed, and the trypanosomes exhibited abnormalities in their microtubule arrays. A recent study has confirmed these phenotypes but a γ tubulin RNAi construct produced no phenotype [41•]. Using a slightly different approach and an inducible RNAi system, we have been able to show that γ tubulin is essential in *T. brucei* and the mutant has major abnormalities in motility and cytokinesis (P McKean, K Gull, unpublished data). This result extends a previous study on *Paramecium* that shows that basal body duplication requires γ tubulin. Interrogation of protein function in *Paramecium* is achievable by a post-transcriptional gene-silencing phenomenon that bears similarities to the RNAi approach [44]. Even though there is still doubt that RNAi technologies will work in every context, they offer an exciting opportunity to reveal cellular functions of proteins in a range of protists.

Figure 1

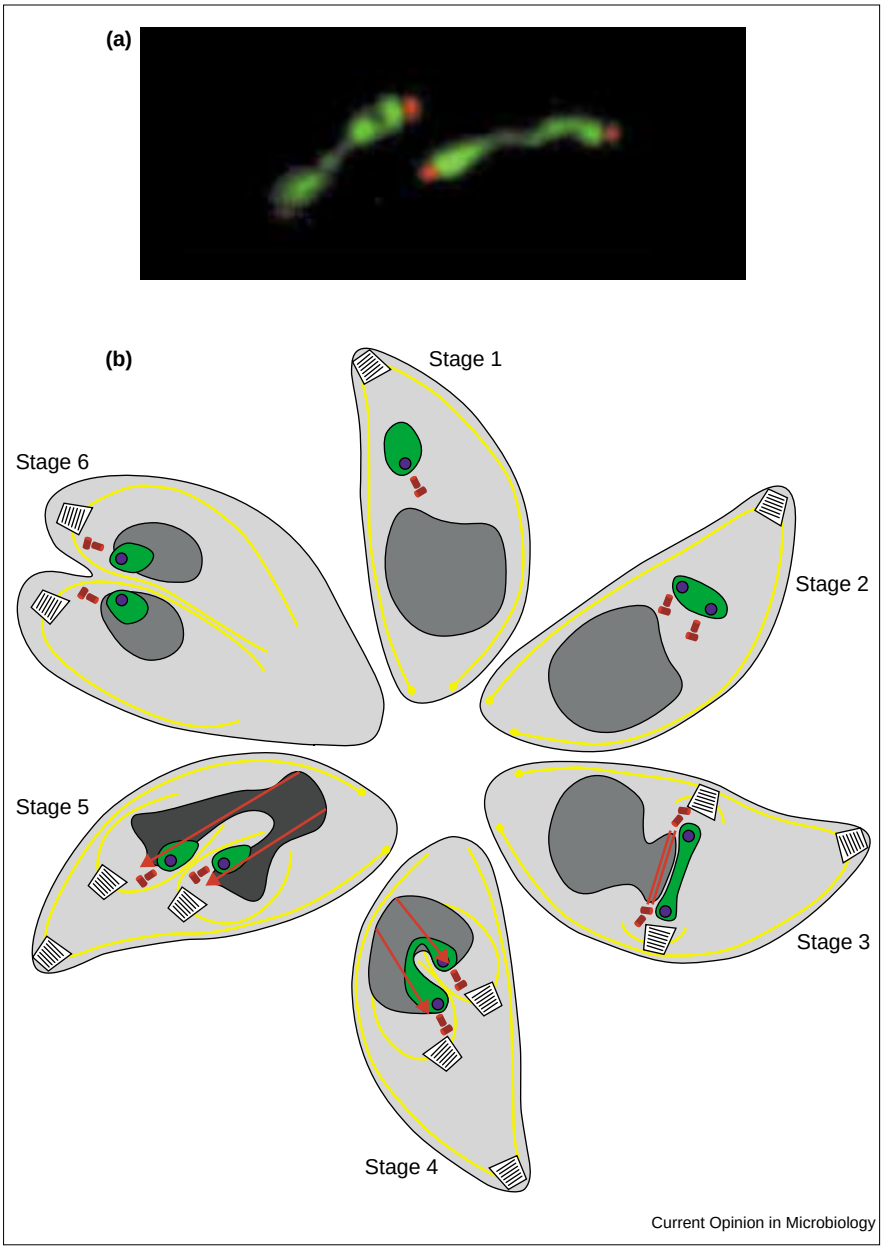


Mitochondrial genome segregation in trypanosomes occurs via attachment of the mitochondrion and the kinetoplast to the flagellum basal bodies [45]. (a) Electron micrograph of *Crithidia* showing the filaments of the attachment complex joining kinetoplast, mitochondrial membranes and basal body [46]. (b) A cartoon of *T. brucei* in the early stage of the cell cycle, showing the position of the mitochondrion and kinetoplast close to the flagellum basal body. (c) A cartoon of *T. brucei* in the mitotic stage of the cell cycle, showing segregation of the mitochondrion and kinetoplast by the moving apart of the old and new flagellar basal bodies [45,46].

Mitochondrial, plastid and parasite segregation: the hitchhikers guide

A number of protists contain single-copy, genome-containing organelles whose replication and segregation are intimately associated with nuclear and/or cytoplasmic events in the cell cycle. These organelles attach to the unique centrosome or flagellar basal body to achieve and ensure division and fidelity of segregation — a little like hitchhiking. Given that unique MTOCs have precise duplication and segregation

Figure 2



Division of the apicoplast in *Toxoplasma* is achieved by attachment to the centrosomal area. **(a)** The ends of the dividing *Toxoplasma* apicoplast are associated with the cell centrosomes. The apicoplast is stained green by immunofluorescence of a tagged protein and the centrosomes red by immunolocalisation of centrin. **(b)** A cartoon of division of the apicoplast (green) associated with the centrosome (red cylinders) duplication cycle. In stage 2, the apicoplast DNA (blue) has replicated and is associated with the centrioles, which locate to the poles of the mitotic spindle at stage 3. Later, movements of the centrosome segregate the apicoplast and the daughter nuclei (dark grey) into the budding daughter parasites. See [47••] for details.

patterns in the cell cycle, the organelles hitch a ride and ensure their own segregation fidelity. In trypanosomes, the mass of mitochondrial DNA in the kinetoplast of the single mitochondrion is attached by fibers to the inner mitochondrial membrane and then by other fibers to the flagellum basal body (Figure 1). Robinson and Gull [45,46] were able to show that segregation of this mitochondrion and its genome was accomplished by the moving apart of the basal bodies of the old and new flagella (Figure 1). Recently, Striepen *et al.* [47••] studied how the apicoplast of *Toxoplasma* duplicates and segregates. The apicoplast of *Toxoplasma* and *Plasmodium* species is a fascinating, essential organelle that contains a characteristic genome. The apicoplast appears to be the remnant of a eukaryotic algal plastid that was acquired by secondary endosymbiosis. In an elegant series of experiments, these authors demonstrate that the apicoplast is tightly associated with the centrosome and, by this association, uses the mitotic apparatus for segregation, each end of the dividing apicoplast being associated with the centrosome at each mitotic pole (Figure 2).

This hitchhiking approach to ensuring fidelity of segregation appears to be deeply embedded in the cell biology of organelles of ancient endosymbiotic origin. Moreover, this hitchhiking approach is also used by intracellular parasites [48] to ensure segregation and therefore vertical transmission to daughter host cells. It is likely that both *Theileria parva*, microsporidial parasites and bacteria harboured in the cells

of *Drosophila* and other organisms use this mechanism. One can predict that a unifying cell biology underlies the mechanism of interaction of genome-containing organelles and intracellular parasites or symbionts with cytoplasmic or spindle microtubules, or basal bodies or centrosomes.

Conclusions

Defining the diversity and structural complexities of the microtubule cytoskeletons of eukaryotic microbes was a cottage industry that boomed in the 1960s and 1970s and continues today. The high level order found in these microbial cytoskeletons is an important feature. However, this mapping of the cellular architectural landscape now provides a fascinating data set against which the functions and evolutionary relationships of the structures can be questioned and interpreted. Advances in microbial cell biology and information from the genome projects have combined to reveal many of the constituent proteins. New gene silencing technologies are enabling real insights to assembly mechanisms and novel functions of the microtubule arrays. We are poised to learn much more about 'how to build a cell', and also much more about the important role of the cytoskeleton in the evolution of microbial cell form.

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