The *SM19* gene, required for duplication of basal bodies in *Paramecium*, encodes a novel tubulin, η-tubulin

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The discovery of δ-tubulin, the fourth member of the tubulin superfamily, in *Chlamydomonas* [1] has led to the identification in the genomes of vertebrates and protozoa of putative δ homologues and of additional tubulins, ε and ζ [2–4]. These discoveries raise questions concerning the functions of these novel tubulins, their interactions with microtubule arrays and microtubule-organising centres, and their evolutionary status. The *sm19-1* mutation of *Paramecium* specifically inhibits basal body duplication [5] and causes delocalisation of γ-tubulin, which is also required for basal body duplication [6]. We have cloned the *SM19* gene by functional complementation and found that it encodes another new member of the tubulin superfamily. SM19p, provisionally called eta-tubulin (η-tubulin), shows low sequence identity with the tubulins previously identified in *Paramecium*, namely, α [7], β [8], γ [6], δ (this work) and ε (P. Dupuis-Williams, personal communication). Phylogenetic analysis indicated that SM19p is not consistently grouped with any phylogenetic entity.

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Received: **15 August 2000**
Revised: **18 September 2000**
Accepted: **18 September 2000**

Published: **3 November 2000**

Current Biology 2000, 10:1451–1454

0960-9822/00/$ – see front matter

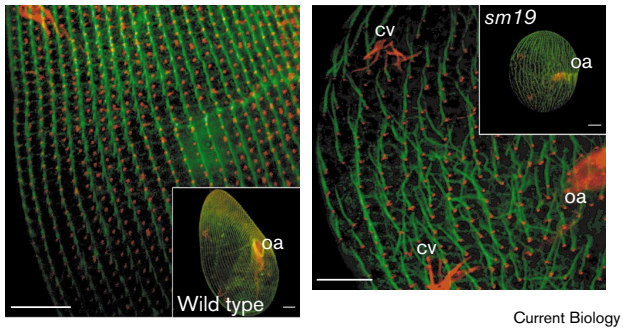
Results and discussion

sm19-1 [5] is a thermosensitive recessive mutation that causes, at the non-permissive temperature (35°C), a progressive reduction in the number of basal bodies, accompanied by reduced cell length and modified cell shape. These defects do not impair the cell cycle, which proceeds like that of wild-type cells at the same temperature. However, *sm19* cells eventually die after a few divisions, because of disorganisation of the oral apparatus caused by the reduced number of its basal bodies. Figure 1 illustrates the mutant phenotype: altered shape, marked reduction of the oral apparatus, rarefaction of basal bodies and secondary disorders in the cortical cytoskeleton. Ultrastructural observations have revealed a rare defect in the basal bodies themselves: missing microtubules in a single triplet, found in 3% (35/1,143) of the cross-sections [5]. This defect occurs generally in the anterior right quadrant of the basal body, which corresponds to the site where, according to Dippell [9], the first microtubules of the initial ring of nine singlets appear in the pro-basal body.

As γ-tubulin is required for basal body duplication [6], an interaction between γ-tubulin and the product of the *SM19* gene was considered possible. Immunolabelling by anti-γ-tubulin antibodies revealed abnormal localisation of γ-tubulin in mutant cells at the non-permissive temperature. Figure 2 shows that, in the mutant, the staining of basal bodies was more diffuse than in the wild type and, most strikingly, there was an accumulation of brightly stained tubule-like aggregates, indicating an abnormal localisation or concentration of γ-tubulin in the cytosol.

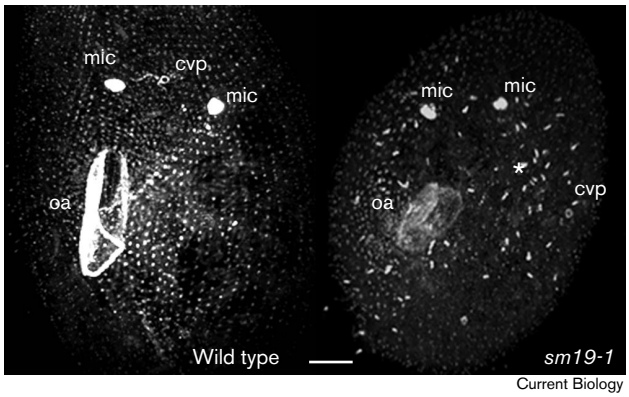
A preliminary attempt to clone the *SM19* gene by functional complementation using described procedures [10,11] showed that wild-type genomic DNA digested with *Eco*RV, *Xba*I, or *Hind*III was able to rescue the mutant after microinjection into its macronucleus, whereas *Bgl*II

Figure 1



Wild-type and *sm19* cells after 3–4 divisions at 35°C (non-permissive-temperature): mutant cells have become smaller and rounder. Immunolabelling protocols were those described by Ruiz *et al.* [6]. The monoclonal anti-tubulin ID5 antibody [17] labels (red) the basal bodies, on the cortex and in the oral apparatus (oa), and the contractile vacuole microtubule rootlets (cv). Each basal body is flanked by a ciliary rootlet decorated (green) by a rabbit antiserum prepared against the purified structures [18]. These appendages, well aligned in the wild-type cells, were dishevelled in the mutant because of the reduced density of the basal bodies. The scale bars represent 10 μm.

Figure 2



The *sm19–1* mutation affects γ-tubulin localisation. After 3–4 divisions at 35°C, as in Figure 1, wild-type and mutant cells were immunolabelled with affinity-purified anti-γ-tubulin antibodies from a rabbit immunised against the carboxy-terminal part of the *Paramecium* γ-tubulin (C.K., F.R., P. Dupuis-Williams, M. Wright and J.B., unpublished work). In both the wild type and the mutant, the same organelles were decorated: micronuclei (mic), pores of the contractile vacuoles (cvp) on the dorsal surface and basal bodies on the cortex, and in the oral apparatus (oa) on the ventral surface; in addition, γ-tubulin was present in the cytoplasm of the mutant where it formed tubule-like aggregates (asterisk), never observed in wild-type cells. Each confocal image corresponds to projections of 15 sections (0.3 μm thick) throughout the cell. The scale bar represents 10 μm.

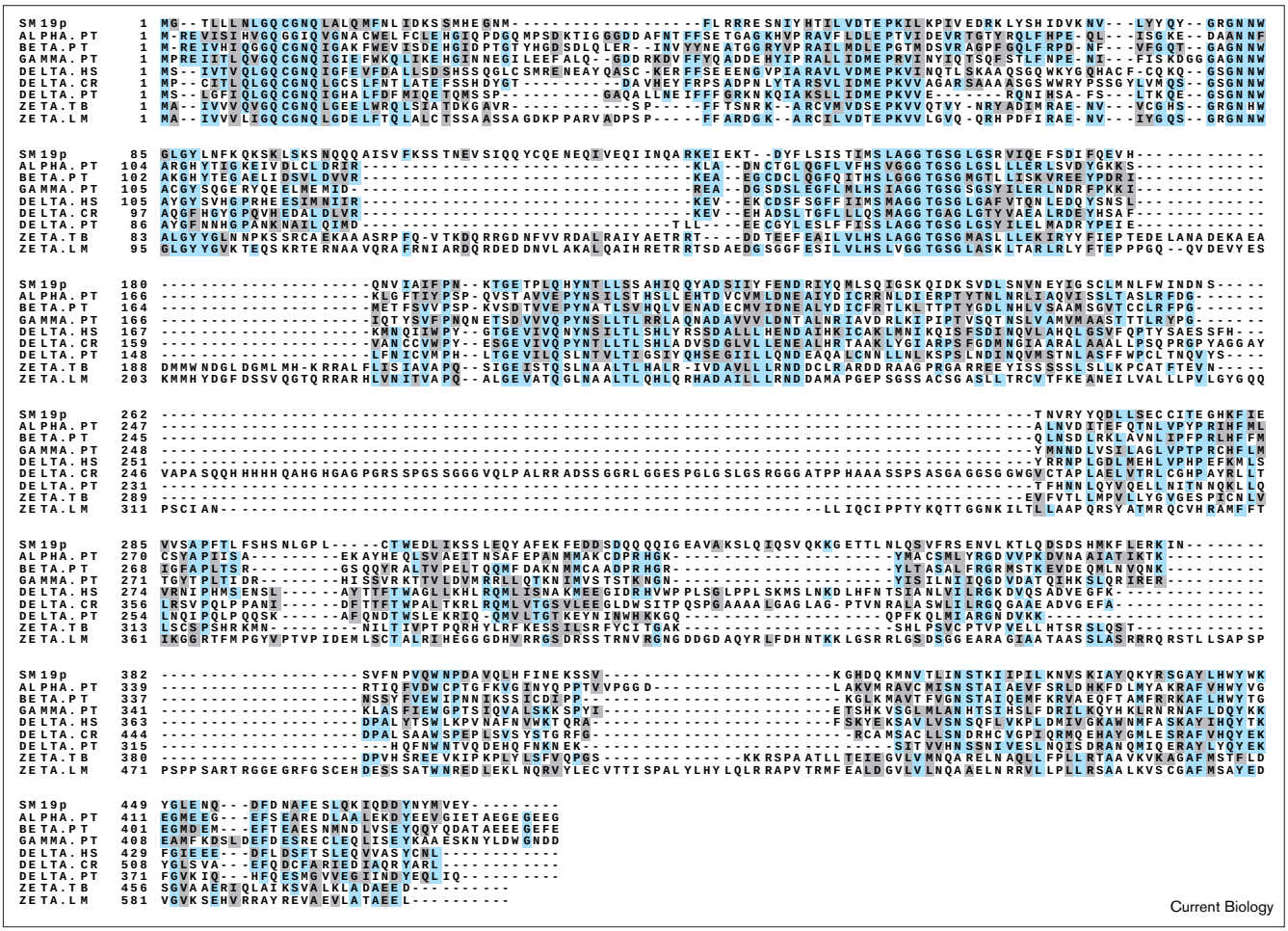
digests could not. A *Bgl*II site was therefore likely to be found in the *SM19* gene. To clone the gene, the recently described indexed library of *Paramecium* [12] was used. Successive microinjections of smaller and smaller subpools of the 60,000 clones of the library led, in six steps, to the identification of a single rescuing plasmid called p158g04. At each step, the rescuing activity was assessed by observation of the offspring of microinjected cells: clones with normal or subnormal cell size and density after 48 hours at 35°C were scored as rescued. The rescuing plasmid contained a 7.2 kb insert with a single *Bgl*II site. The sequencing of the region surrounding this diagnostic site revealed a 1459 bp open reading frame, displaying the features of *Paramecium* coding regions in GC content and codon usage [13] and interrupted by a small 29 bp intron, typical of *Paramecium* [8,14].

DNA prepared by PCR amplification of this open reading frame with its 5′ and 3′ flanking sequences (see Supplementary material) was microinjected into mutant cells and rescued them efficiently. Further evidence that this open reading frame coded for the *SM19* gene was provided by sequencing two mutant alleles, *sm19-1* and *sm19-2*. Both had mutations within the coding sequence: an insertion of 66 nucleotides at position 573 in *sm19-1*, and a point mutation (A→G transition) at position 1437, resulting in a Y470C change in *sm19-2*. The 66 nucleotide insertion in *sm19-1* corresponds to retention of an internal eliminated sequence (IES), normally 'spliced' during the development of a new macronucleus from the germinal micronucleus. Sequencing of the corresponding region of wild-type micronuclear DNA showed that the *sm19-1* mutation is due to a T→C change in the 3′ consensus [15] TA terminal repeat of the IES. Southern blots (data not shown) indicated that *SM19* is a unique gene. Northern blots (see Supplementary material) revealed a 1.5 kb mRNA species of equivalent abundance in the mutant and the wild type, at both 27°C and 35°C.

The deduced polypeptide sequence of SM19p is 476 amino acids long, with a predicted molecular mass of 54.9 kDa and an isoelectric point of 5.79. Comparison with the protein sequence databases showed that SM19p belongs to the tubulin superfamily. However, SM19p shares less than 20% identity with the tubulins characterised so far in *Paramecium*, namely, α [7], β [8], γ [6] and ε (P. Dupuis-Williams, personal communication). Although in BLAST alignments, the best two scores were 23 and 21% for human and mouse δ-tubulins, respectively, SM19p was also distinct from a recently identified *Paramecium* δ- or δ-like tubulin (see Figure 3), with which it shares only 25% identity. Figure 3 compares the predicted SM19p sequence with different δ-tubulins (from man, *Chlamydomonas* and *Paramecium*), the two known ζ-tubulins and the *Paramecium* α-PT1, β-PT1 and γ-PT1, as representatives of the α, β and γ subfamilies. The alignment showed that, aside from the motifs common to all tubulins, there was no other significant similarity. We therefore designate SM19p provisionally as η-tubulin.

To evaluate the phylogenetic status of η-tubulin, phylogenetic trees were constructed (Figure 4 and Supplementary material) using sequences from the same three organisms (man, *Chlamydomonas* and *Paramecium*) for the well-established α-, β- and γ-tubulin subfamilies and all available sequences for the other tubulins. Figure 4 shows the unrooted tree obtained by the maximum likelihood method. Within the α- and β-tubulin subfamilies, orthologues showed a high level of sequence identity, in likely relation to functional constraints. In contrast, the γ-tubulin subfamily, although coherent on phylogenetic trees [16], appeared more rapidly evolving: percentages of identity among presumed orthologues could be as low as ~30%. With the new tubulins δ, ε, and ζ, there are still too few members to appreciate the range of sequence variability and their function is not known. The percentage identity between the *Chlamydomonas* δ-tubulin and its likely homologues in mammals, *Trypanosoma* and *Paramecium* ranges from 29–43%. The *Chlamydomonas* δ plays a role in basal body assembly or maturation [1]; however, a similar role in other systems remains to be established even though immunochemical localisation of δ-tubulin in centrosomes of mouse and man is consistent with a role in

Figure 3



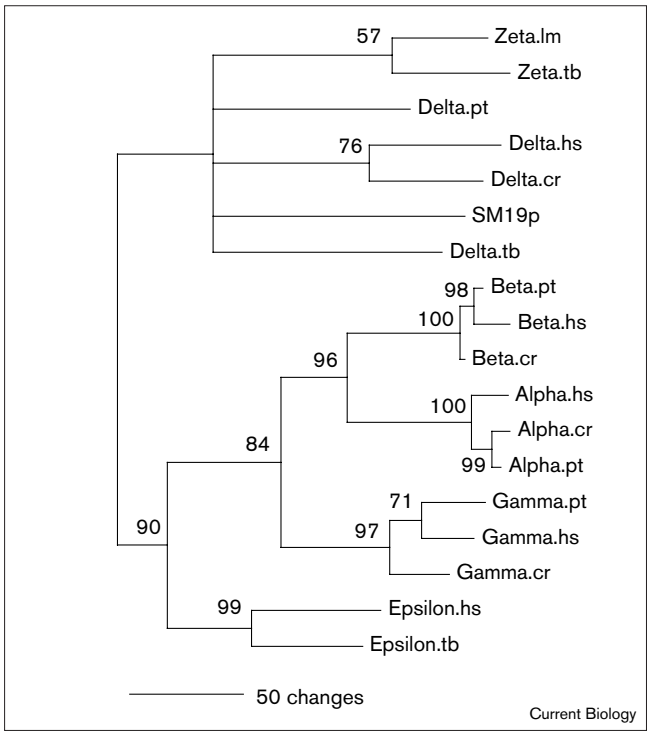
Comparison of the predicted SM19p sequence with *Paramecium* α-, β- and γ-tubulins, and with δ-tubulins from human, *Chlamydomonas reinhardtii* (CR) and *Paramecium tetraurelia* (PT), and ζ-tubulins from *Trypanosome brucei* (TB) and *Leishmania major* (LM). The sequences were aligned using the Clustal W1.8 [19] and Dialign [20] programs and adjusted manually. The boxing threshold was 30% of the aligned sequences. Blue, identical residues; grey, conservative substitutions. The sequences used for alignments in Figures 3 and 4 are: *H. sapiens* α1-tubulin, I 77403; *H. sapiens* β-tubulin, CAA 56071; *H. sapiens* γ1-tubulin, NP001061; *H. sapiens* δ-tubulin, NP07345C; *H. sapiens* ε-tubulin, NP 057346; *C. reinhardtii* α-tubulin, AAA 33095; *C. reinhardtii* β-tubulin, M 10964; *C. reinhardtii* γ-tubulin, AAB 71841; *C. reinhardtii* δ-tubulin, AAB 71840; *P. tetraurelia* α-tubulin, CAA67847; *P. tetraurelia* β-tubulin, CAA 47663; *P. tetraurelia* γ-tubulin, CAA09991; *P. tetraurelia* δ-tubulin, AJ401299; *P. tetraurelia* η-tubulin, AJ272425; *T. brucei* δ-tubulin, AAF 32301; *T. brucei* ε-tubulin, AF 216743; *T. brucei* ζ-tubulin, AF241275; *L. major* ζ-tubulin, AL133468.

centrosome/centriole function [2,3]. As for ζ-tubulin, its function and phylogenetic distribution are not yet known.

On the basis of presently available data, ε-tubulin, so far represented by only two published sequences from distant organisms (man and trypanosome) seems to constitute a monophyletic tubulin subfamily. In contrast, for the δ-, ζ- and η-tubulins, the tree presented in Figure 4, as well as other types of trees (see Supplementary material) fail to resolve their relationships. Only additional sequences and functional characterisation will help to ascertain whether δ-, ζ- and η-tubulins are members of a rapidly evolving family or independently acquired divergent tubulins.

An intriguing property of the newly identified tubulins, including η-tubulin, is their absence from *Saccharomyces cerevisiae*, and probably from *Caenorhabditis elegans* and *Drosophila*. Yeast lacks centriolar structures, *C. elegans* lacks flagella and motile cilia, while the fly does not assemble cilia and its centrioles may be peculiar. This suggests that these tubulins might be involved in specific properties of the centriolar structures, such as duplication, positioning or nucleation of appendages, which are involved in their function as basal bodies. This seems indeed to be the case for SM19p. All the cytological and physiological observations show that the *sm19* mutation specifically inhibits basal body duplication and most

Figure 4



Phylogenetic relationships of SM19p with the other members of the tubulin superfamily. For the α-, β- and γ-tubulins, sequences from the same three organisms, *Homo sapiens* (hs), *C. reinhardtii* (cr) and *P. tetraurelia* (pt) have been used as representatives of their respective subfamilies. The δ-tubulins include sequences from the same three species plus that of *T. brucei* (tb). The ε- and ζ-tubulin classes include the available sequences, from man and *T. brucei* and from *T. brucei* and *L. major*, respectively. The tree has been calculated using Puzzle 4.0.1 [21]. Reliability indices (RI) are shown at the nodes. Only the nodes with more than 90% RI are deemed to be 'strongly supported'.

likely an early stage of the process [5]. As γ-tubulin is also necessary to initiate basal body duplication [6] and appears delocalised in the *sm19* mutant at the non-permissive temperature (Figure 2), we speculate that this novel tubulin might contribute to tether γ-tubulin or γ-tubulin complexes to basal bodies.

Supplementary material

Supplementary material including methodological detail, a figure depicting the characterisation and expression of the *SM19* locus, and additional information on the sequence alignment and the trees is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

We thank Linda Sperling, Roger Karess and Carl Creutz for critical reading of the manuscript, Pascale Dupuis-Williams for sharing unpublished data on *Paramecium* ε-tubulin, Keith Gull for communicating data on new *Trypanosome* tubulins before publication, Jean-Richard Prat and Spencer Brown for confocal imaging, and Jörgen Wehland for the gift of the ID5 anti-tubulin monoclonal antibody. This work was supported by the Centre National de la Recherche Scientifique and the Association pour la Recherche contre le Cancer (contract 5425 to J.B.).

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