Structural and Functional Implications of an Unusual Foraminiferal β-Tubulin

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We have obtained sequence data for β-tubulin genes from eight species of Foraminifera (forams) and α-tubulin sequences from four species, sampling major taxonomic groups from a wide range of environments. Analysis of the β-tubulin sequences demonstrates that foram β-tubulins possess the highest degree of divergence of any tubulin gene sequenced to date and represent a novel form of the protein. In contrast, foram α-tubulin genes resemble the conventional α-tubulins seen in other organisms. Partition homogeneity analysis shows that the foraminiferal β-tubulin gene has followed an evolutionary path that is distinct from that of all other organisms. Our findings indicate that positive selective pressure occurred on the β-tubulin subunit in ancestral forams prior to their diversification. The specific substitutions observed have implications for microtubule (MT) assembly dynamics. The regions most strongly affected are implicated in lateral contacts between protofilaments and in taxol binding. We predict that these changes strengthen lateral contacts between adjacent dimers in a manner similar to that induced by taxol binding, thus allowing the formation of the tubulin "helical filaments" observed in forams by electron microscopy. Our results also indicate that substantial changes to these portions of the β-tubulin molecule can be made without sacrificing essential MT functions.

Introduction

Foraminifera (forams) are an ancient group of protists that are a major component of benthic ecosystems and play an important role in biogeochemical cycles (reviewed in Sen Gupta 1999; Lee et al. 2000). The signature morphological feature of the group is their distinctive pseudopodia, which form intricately branched and anastomosed networks. These "reticulopodia" provide the major means by which forams interact with their environment (Lee et al. 2000; Bowser and Travis 2002). Reticulopodia often extend several centimeters from the cell body, with individual pseudopodia extending or withdrawing at rates in excess of 20 μm/s (Allen 1964; Rinaldi and Jahn 1964). Experimental studies have demonstrated that the structural and functional integrity of reticulopodia depends on an extensive cytoskeletal network of motile microtubules (MTs) (Travis and Bowser 1986; Orokos, Cole, and Travis 2000). Forams appear to have evolved a novel assembly mechanism, distinct from that found in other eukaryotes, that allows them to modify their reticulopodial MTs at rates necessary to support the observed speed of pseudopodial extension and withdrawal (Chen and Schliwa 1990; Welnhofer and Travis 1996). However, the molecular basis for this mechanism has not been explored.

The current understanding of MT assembly/disassembly is based primarily on biochemical studies of vertebrate brain tubulin. Current models propose that MT disassembly is initiated by loss of a guanosine triphosphate (GTP)/structural cap (e.g., Arnal, Karsenti, and Hyman 2000), which triggers a series of cooperative conformational changes, leading to the splaying of MT ends into an array of loops, spirals, and stubby filaments (E. M. Mandelkow, E. Mandelkow, and Milligan 1991). These 5- to 6-nm–wide chains of globular subunits are interpreted as individual tubulin protofilaments in a curved guanosine diphosphate conformational state (Ravelli et al. 2004).

Key words: M-loop, microtubule, tubulin polymorph, helical filament, Foraminifera.

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Previous studies on foraminiferal MTs indicate that the conventional view of MT disassembly may not hold in these organisms. Foraminiferan tubulin can exist in two distinct assembled forms: conventional 13-protofilament MTs and helical filaments (HFs), which are two globular subunits thick and 10.7 ± 0.7 nm wide (HFs, see fig. 1) (Hauser and Schwab 1974; Rupp et al. 1986; Welnhofer and Travis 1998). The tubulin lattices undergo rapid transitions between these two states, which in vitro are induced by exposure to 10 mM Ca^{2+} or 50 mM Mg^{2+}; removal of these ions regenerate intact MTs (Welnhofer and Travis 1998). It has been hypothesized that HFs form when ribbons of laterally associated tubulin heterodimers "unwind" from the MT lattice; conversely, MTs form as HFs wind or rewind (Hauser and Schwab 1974; Welnhofer and Travis 1998). HFs have been seen in electron micrographs of every foram species examined to date (Travis and Bowser 1991; Bowser and Travis 2002), and the MT-to-HF transition has been experimentally induced in vivo in three species (McGee-Russell 1974; Bowser, McGee-Russell, and Rieder 1984; Travis and Bowser 1986; Brueker and Hauser 1997), suggesting that these lattice state transitions are a common feature of foraminiferan MT assembly/disassembly dynamics. In contrast to the conventional disassembly model, in which the lateral bonds between protofilaments break first, the formation of HFs by the Hauser and Schwab (1974) model would require the initial fragmentation of protofilaments through the breakage of longitudinal interdimer contacts.

The unique MT-to-HF transition displayed by foraminiferans suggests that their tubulin genes could be modified relative to the eukaryotic consensus. The first analysis of foram tubulin, from the species *Reticulomyxa filosa* (Linder, Schliwa, and Kube-Granderath 1997), identified two isoforms each of α- and β-tubulin. The reported α-isoforms are very similar to one another and are typical of α-tubulin sequences in general. However, the two β-tubulin sequences are clearly dissimilar, with one of the two ("Type 2") representing the most highly divergent β-tubulin sequence in GenBank. The authors speculated that at least some of the numerous amino acid substitutions detected might be responsible for the unusual MT dynamics

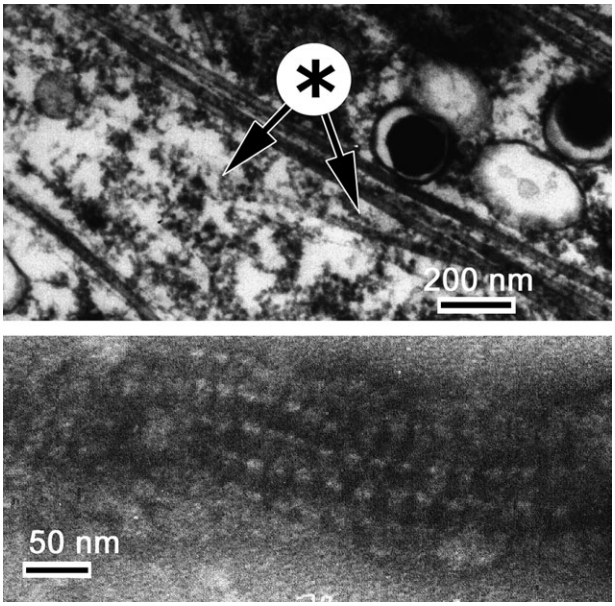


FIG. 1.—HF formation in Foraminifera. Top: transmission electron micrograph showing both intact MTs and HFs; the starred MT is transitioning between the two states. Bottom: negative-stained image of HFs. For details of preparation methods, see Bowser and Travis (2000).

observed. Since that initial report, the same group has deposited an additional highly divergent β-tubulin sequence obtained from another foraminiferan, *Allogromia laticollaris* (accession number Y16168). However, at the time of this previous report, the molecular structures of tubulin and MTs were not available, so the authors were unable to map the substitutions to functionally important areas of the tubulin molecule. The extensive sequence differences between the two β-tubulin isoforms also made it difficult to identify potentially important residues.

In the present study, we report the sequences of α- and β-tubulins from several foram species and describe the patterns of substitution in the group's highly divergent β-tubulins. We explore the possible functional implications of these changes in the context of established structural models of the tubulin heterodimer (Nogales et al. 1999) and assembled MTs (Nogales, Wolf, and Downing 1998; Li et al. 2002). Our analysis shows that areas of the β-tubulin molecule implicated in the formation of contacts between protofilaments are strongly modified in forams, and we suggest that these alterations have effects similar to that of taxol on mammalian MTs. We use these data to propose a structural model for the MT-to-HF transition in forams.

Materials and Methods

Genomic DNA

Species were selected from a wide range of environments and evolutionary lineages. *Reticulomyxa filosa* (Nauss 1947) and *Allogromia* sp. strain NF (Lee and Pierce 1963) were maintained in culture. All other species used in this study were harvested from marine sediments by serial sieving and isolation of individual cells. *Astrammina rara*, *Cornuspira antarctica*, *Pyrgo peruviana*, and *Rhabdammina cornuta* were collected at Explorers Cove, Antarctica (77°34′35″S, 163°31′39″E). *Ammonia tepida* and *Miliammina fusca* were obtained from tidal flats at Sapelo Island, Ga. (31°30′12″N, 81°14′09″W). After morphological identification, specimens were cleaned with a fine artist's brush and were allowed to purge ingested prey in sterile seawater for >24 h. After purging, 10–100 cells (depending on size) were used to obtain genomic DNA as described previously (Habura et al. 2004).

Cloning and Sequencing of α- and β-Tubulin Genes

Primer sets were designed with reference to previously published foraminiferal α- and β-tubulin genes. The primers used to identify α-tubulin genes (forward primers AlTuFN: TAYTGYTTRGARCAYGGHATHCA and AlTuFD: GTNGGHCARGCNGGHATHCA; reverse primer AlTuR: AAYTCDCCYTCYTCCATDCCYTC) are also complementary to most other eukaryotic α-tubulin gene sequences. Universal primers for β-tubulin genes (BTubA and BTubB; Edgcomb et al. 2001) were used to probe for the more conventional Type 1 β-tubulin, but these primers show some mismatches with the cDNA sequences of Type 2 foraminiferal β-tubulins reported previously (Linder, Schliwa, and Kube-Granderath 1997). We designed an additional primer set with reference to the published sequences of *R. filosa* and *A. laticollaris* Type 2 β-tubulin (GenBank X96478 and Y16168, respectively). Forward primer BTub1F (CAATGTGGTAACCAAATTGG) is complementary to both Type 2 sequences as well as to a number of β-tubulin gene sequences from nonforams. Forward primer BTub2F (AATTGGGCAAAAGGACATTA) was designed to recognize both the Type 1 *R. filosa* and Type 2 *A. laticollaris* genes, and it also matches some nonforam sequences. Reverse primer BTub1R (CATCTTGTTTGTCTTGATATTCAGT) is similarly designed to favor the Type 2 sequences. These primers were used together and in combination with the universal primers as appropriate.

Polymerase chain reactions (PCR) were performed on a Techne Genius thermocycler, using TaKaRa proofreading ExTaq premix. Cycle parameters were as follows: 40 cycles at 94°C for 30 s, 48°C for 30 s, and 70°C for 2 min. Products were analyzed by gel electrophoresis and cloned into pGEM-T Easy (Promega, Madison, Wisc.) and replicated in *Escherichia coli* strain JM109. Individual clones were purified with the SpinPrep mini kit (Qiagen, Valencia, Calif.), and multiple clones for each product were sequenced in both directions using primers M13 and M13 reverse, with a PE-Biosystems ABI PRISM 377XL automated DNA sequencer.

Sequences for the new genes, and a taxonomically representative sampling of other sequences retrieved from GenBank, were aligned using ClustalW (Thompson, Higgins, and Gibson 1994). This alignment was adjusted manually in SEAVIEW (Galtier, Gouy, and Gautier 1996) to accommodate introns and other regions of variable length in the tubulin consensus structure (for a review, see Burns and Surridge 1994). The alignment was used to infer amino acid sequences from the foraminiferal genes, and these were then aligned with 47 previously deposited amino acid sequences. All unambiguously aligned positions were retained for phylogenetic analysis. Alignments are available for viewing at www.bowserlab.org/supplemental.

Table 1
Positions of Introns in Foraminiferal Sequences

Species and Tubulin Gene	Position in Reference Sequence	Length (nt)
Miliammina fusca α	422	70
Reticulomyxa filosa α	191	76
	263	105
	422	76
Allogromia sp. β	436	114
Pyrgo peruviana β	789	54, 52
	1,147	61, 46
R. filosa β	393	71
Rhabdammina cornuta β	693	74, 86

FIG. 2.—Comparison of consensus sequences of 25 conventional β-tubulins (top line) and 8 foraminiferal Type 2 β-tubulins (bottom line). Positions for which there is more than 10% sequence heterogeneity are shown in lower case. Positions for which no consensus exists are represented as X's; gaps are represented by dashes. Residues that are nonconservatively substituted in Type 2 foram sequences (defined as BLOSUM62 score <0) are highlighted.

Phylogenetic Analysis

Analyses were performed using the maximum likelihood (ML) distance and ML quartet puzzling methods implemented in TREE-PUZZLE 5.0 (Schmidt et al. 2002). Bootstrap analyses were performed using SEQBOOT and CONSENSE (Felsenstein 1989) in conjunction with PUZZLEBOOT (Holder and Roger 1999). Partition homogeneity analysis (Farris et al. 1994) was performed using PAUP* (Swofford 2003).

Results

Characterization of Foraminiferal Tubulin Genes

We obtained PCR products of the appropriate size for nearly full-length α-tubulin (~400 codons) from four species of Foraminifera (*A. tepida*, *M. fusca*, *R. filosa*, and *R. cornuta*). Type 2–favoring primer sets that amplify a nearly complete gene (~415 codons) and a smaller region (~330 codons) generated product from templates derived from eight foraminiferal species (*Allogromia* sp., *A. rara*, *C. antarctica*, *Crithionina delacai*, *M. fusca*, *P. peruviana*, *R. filosa*, and *R. cornuta*). In each case, the identity of the product was confirmed as an α- or β-tubulin by sequencing. Foraminiferal DNA extracts were also challenged with the universal eukaryotic β-tubulin primers BTubA and BTubB (Edgcomb et al. 2001), but these did not succeed in amplifying foraminiferal β-tubulin genes. A primer pair highly specific for the *R. filosa* Type 1 gene (BTub2F and BTubB) was also used on several templates, including one from *R. filosa*, but no additional sequence types were recovered.

The obtained sequences were aligned with the corresponding α- and β-tubulin DNA sequences from 21 and 17 taxonomically diverse species, respectively. This alignment revealed the presence of insertions in some foraminiferal sequences, all of which contain canonical Type I intron splice sites. The positions of these introns relative to the published *R. filosa* sequences are detailed in table 1. These sequences have been deposited in GenBank as AY818714–AY818726 and AY818729–AY818732.

Previously reported sequences for foram α- and β-tubulins (derived from cDNA libraries) were compared to the new sequences. The previously reported *R. filosa* α1 and α2 sequences resemble one another closely, differing only by 10 conservative amino acid changes (Linder, Schliwa, and Kube-Granderath 1997), and the α-tubulin that we obtained from *R. filosa* aligned well with these. Our other foraminiferal α-tubulins are also comparable in sequence. The β-tubulin sequence from *Allogromia* sp. closely resembles that previously reported from *A. laticollaris*, and the β-tubulin sequence derived from *R. filosa* was consistent with the previously reported Type 2 sequence from that species (X96478). None of the β-tubulin sequences that we obtained from any species are Type 1; all resemble the more divergent Type 2.

The inferred amino acid sequences of these genes were compared with homologous sequences retrieved from GenBank. The α-tubulin sequences contained almost no insertions or ambiguously aligned positions. Although there were many nonconservative substitutions in the foraminiferal Type 2 β-tubulin sequences relative to all other reported sequences, these generally did not involve indels and were bracketed by blocks of strongly conserved sequence. Some modifications to the alignment were made in the region corresponding to the variable loop between the predicted H1 and B2 in the β-tubulin structure, which is expanded in Foraminifera relative to most other organisms. This region is known to accommodate insertions and deletions (Burns and Surridge 1994). Both alignments are available at www.bowserlab.org/supplemental.

Foraminiferal Substitutions in the Consensus β-tubulin Sequence

Our analyses show that the α-tubulin genes are essentially unsubstituted. While there are minor variations in the nucleic acid sequences, the deduced amino acid sequences for the gene products are very strongly homologous to those from a broad range of eukaryotic α-tubulins. However, the β-tubulin sequences are highly divergent. A comparison of the foram Type 2 consensus sequence to the standard eukaryotic consensus is shown in figure 2. This comparison reveals that some regions of the foram β-tubulin are almost perfectly conserved with respect to the eukaryotic consensus, whereas others are heavily modified in a manner that could have a profound effect on the biochemical properties of the molecule. Many substitutions are nonconservative

relative to the residues found in other organisms, yet strictly conserved within the Foraminifera.

The N-terminal region has two areas of strong sequence substitution. The area between positions 18 and 55 of the consensus contains a 4- to 10-aa expansion and an additional 11 sequence substitutions in forams; the zone between positions 72 and 90 contains another eight substitutions. A normally well-conserved region in the central domain, spanning positions 210–238, is only 57% homologous to the eukaryotic consensus, although the foram sequences are conserved well with respect to each other. Even more strikingly, several functionally important loops lying between positions 260 and 318 are very heavily substituted. Lastly, the C-terminus of the predicted sequence contains several substitutions, although many are conservative. An alignment comparing heavily modified regions of the foram β-tubulin with a representative set of nonforam sequences appears in figure 3.

In order to ensure that the alterations to the primary sequence had not disrupted the overall structure of the β-tubulin in forams, we subjected two alignments (one of 51 nonforam β-tubulins and one of five essentially full-length Type 2 foram β-tubulins) to secondary structure prediction using a hidden Markov model as implemented in HMMSTR (Bystroff, Thorsson, and Baker 2000). The consensus secondary structures predicted from the two alignments were nearly identical to one other (data not shown) and were very similar to the structure of β-tubulin derived by crystallographic methods (Nogales, Wolf, and Downing 1998). We conclude that, overall at the secondary and tertiary levels, the structure of the foram β-tubulin is not altered by the sequence substitutions that we have described.

Phylogenetic Analyses

Phylogenetic analysis of foram β-tubulin sequences (not shown) indicated that while multiple sequences were obtained from some species, these were very closely related and are likely to be simple isoforms of one another. (Multiple isoforms of tubulin are observed in many protists, e.g., Edgcomb et al. 2001.) The exception is the Type 1 and Type 2 forms of *R. filosa* β-tubulin; the Type 1 sequence appears as the basal member of the clade containing all the Type 2 isoforms.

To further investigate the evolutionary origin of the Type 1 isoform, we concatenated the β-tubulin sequences with the corresponding ribosomal small subunit (SSU) and α-tubulin sequences from the same species (species used were *R. cornuta*, *Allogromia* sp., *R. filosa*, and *M. fusca*, as well as a set of 19 other eukaryotic sequences). We then subjected these concatenated aligned sets to the partition homogeneity test (Farris et al. 1994). Partition homogeneity was not rejected for concatenated SSU and α-tubulin for the entire data set ($P = 0.27$), and data sets containing only Type 2 isoforms were also considered homologous by the model ($P = 0.29$ when concatenated with SSU and $P = 0.40$ when concatenated with α-tubulin). However, the Type 1 isoform violated the assumption of homogeneity in both cases ($P = 0.01$, 0.01), suggesting that its evolutionary history may be different from that of other foram β-tubulins.

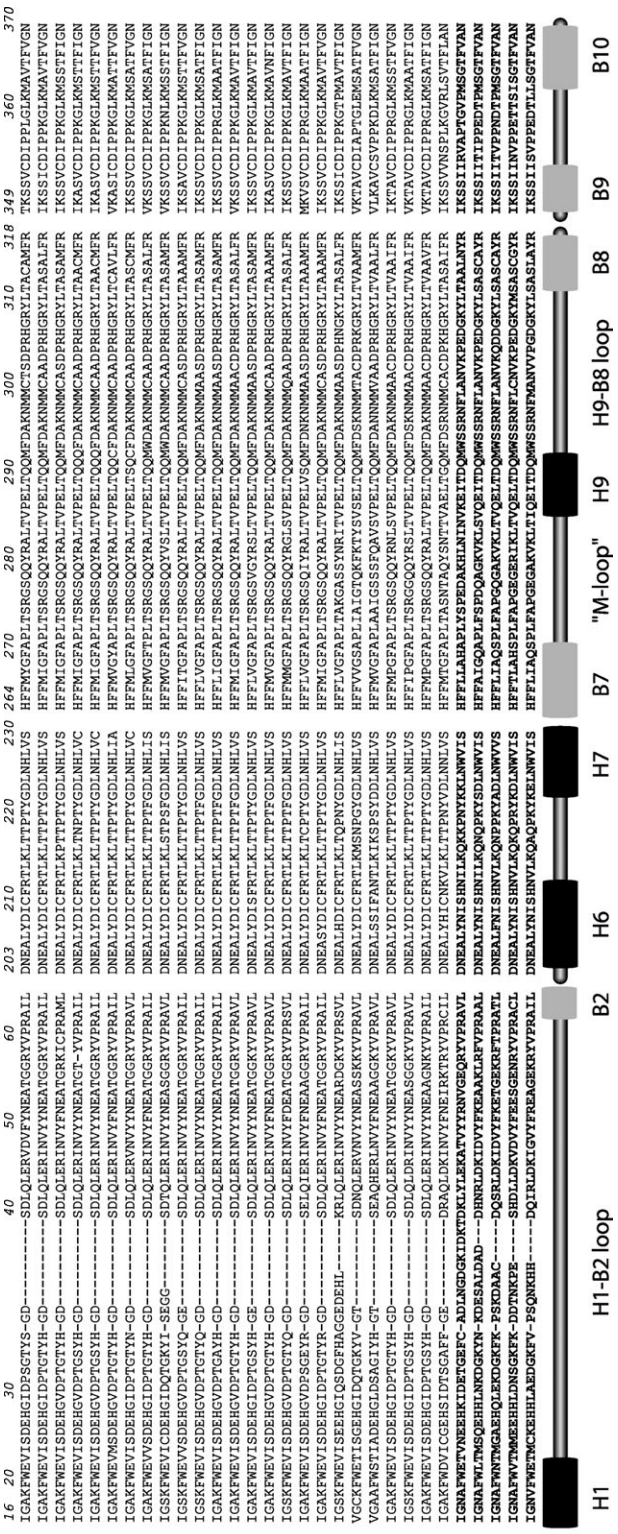
Discussion

The results reported here quadruple the size of the data set available for foraminiferal tubulin genes. Although previous analyses (Linder, Schliwa, and Kube-Granderath 1997) had noted the highly unusual β-tubulin isoform found in *R. filosa*, the present analysis has allowed us to better estimate which modifications are likely to be evolutionarily and functionally important. We find that the foraminiferal Type 2 β-tubulin isoform contains a number of dramatic and well-conserved substitutions with respect to the eukaryotic consensus sequence. In agreement with previously published data, we observe that foraminiferal α-tubulins are not noticeably different from those of most other protists.

The structure of the best-studied tubulin dimer, that of bovine brain tubulin, is shown in figure 4. The α- and β-tubulin monomers have very similar overall structure, consisting of an interior core of two beta sheets surrounded by helices (Downing and Nogales 1998). The two structures differ primarily by the presence of an insertion in the B9-B10 loop in α-tubulin relative to β-tubulin. The space that is occupied by this insertion forms the taxol-binding pocket in β-tubulin (Nogales et al. 1999). Despite the very strong structure conservation, the two monomers are only 40% homologous (Downing and Nogales 1998); the sequence differences confer the specialized functions of the two proteins.

As shown in figure 4, the great majority of substitutions to the consensus β-tubulin sequence are predicted to be located on the surface of the molecule, especially in two regions mediating lateral (inter-protofilament) contacts and taxol binding. Residues involved in other functions, such as nucleotide binding and longitudinal (intra-protofilament) contacts, are generally conserved in the foraminiferal sequences. For example, of the 48 residues designated as being involved in longitudinal contacts by Nogales et al. (1999), only 7 are substituted in forams; and most of these substitutions are conservative (Q94A, Q245K or E, A254G, K324Q or E) or occur in positions where other organisms show substantial sequence variability as well (T221R or K and G223A, S, or K). Two substitutions, Q94A and T178V, are nonconservative. (All residue numbers used in this report correspond to the positions in the *Sus scrofa* sequence.) By contrast, among the 50 residues implicated by the same study to be involved in lateral contacts, 18 are substituted, with 11 being substituted nonconservatively. Several other residues near these regions are also altered in ways that may affect lateral binding properties; we discuss these in more detail below. Finally, most of the residues involved in taxol binding in mammalian tubulins have been altered in foraminiferans.

Interestingly, a pair of cysteines near the nucleotide-binding pocket, C201 and C211, is substituted in forams. Either residue can be experimentally cross-linked with C12 in the absence of bound nucleotide (Nogales, Wolf, and Downing 1998). Other organisms may have cysteines in only one of these positions rather than both, but only in foraminiferans are both substituted. Because tubulin occurs intracellularly, the formation of disulfide bridges would not be expected, and neither C201 nor C211 participates directly in nucleotide contacts. We therefore do not



predict important differences in GTP binding in foram tubulins relative to other tubulins.

Predicted Changes to Lateral Contact Surfaces

Because foraminiferal HFs should require stronger lateral interactions and because pseudopodial motility apparently is not affected by taxol treatment (unpublished data), the locations of the substitutions described above raise suspicions that the changes in the foram sequences were driven by positive selection for lateral contact properties. The structures implicated in lateral contacts in bovine tubulin (Nogales et al. 1999) are parts of the H1-B2 loop, the H2-S3 loop, the H3 helix, H6 and its following loop, the B7-H9 loop (the "M-loop"), and H9 and its following loop. H3 is only slightly modified in foraminiferans, but the other five are altered in ways that may have implications for inter-protofilament stability (see fig. 4) as follows:

(1) H1-B2 loop. Studies based on intact MTs (Li et al. 2002) suggest that H1-B2 is the main binding partner for the crucial M-loop in inter-protofilament contacts. This loop is lengthened in forams, and many residues in the C-terminal portion of the loop are substituted (fig. 3). The region containing the insertion shows no sequence conservation among foram species. While this loop is expanded in some other protists such as *Dictyostelium*, the foraminiferal modifications are unprecedented. Finally, two negatively charged residues at the N-terminus of the loop (E22 and D26) are not conserved. Replacement of negatively charged residues in this region is lethal in yeast (Reijo et al. 1994), although the mutation in that study (*tub2-409*) replaced three residues, only one of which (E22) is found to be substituted here.

(2) H6 and H6-H7 loop. In the MT-based structural prediction (Li et al. 2002), this region is a flexible structure lying on the lateral contact surface, partially enclosing the taxol-binding pocket. It contains seven nonconservative modifications in forams: C211S, F212H, R213N, T214I, L217Q, T219Q, and T221R or K. A C211F mutation in a mammalian cell line increases MT stability by conferring resistance to colchicine (Hari et al. 2003). Lateral contacts involve the N-terminal half of this region (approximately aa 212–216), whereas the C-terminus and parts of H7 participate in longitudinal contacts. The N-terminal half is both better conserved in other organisms and more thoroughly substituted in forams (see fig. 3), suggestive of a particularly dramatic change to the lateral contact surface in the latter

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FIG. 3.—Regions of the Type 2 β-tubulin are highly substituted. Sequences from a taxonomically diverse group of 24 nonforaminiferal species (including animals, fungi, green plants, and several protist lineages) and the *Reticulomyxa filosa* Type 1 sequence are compared with five Type 2 foraminiferal sequences. The partial alignment, corresponding to aa 16–92, 203–231, 264–318, and 349–370 in the pig (*Sus scrofa*) β-tubulin sequence, represents the main regions of sequence alteration in the foraminiferal genes. Structural assignments for these regions, predicted on the basis of the bovine brain tubulin structure (Nogales, Wolf, and Downing 1998), are given below the alignment. Type 2 sequences are shown in bold. Full alignment is available at www.bowserlab.org/supplemental.

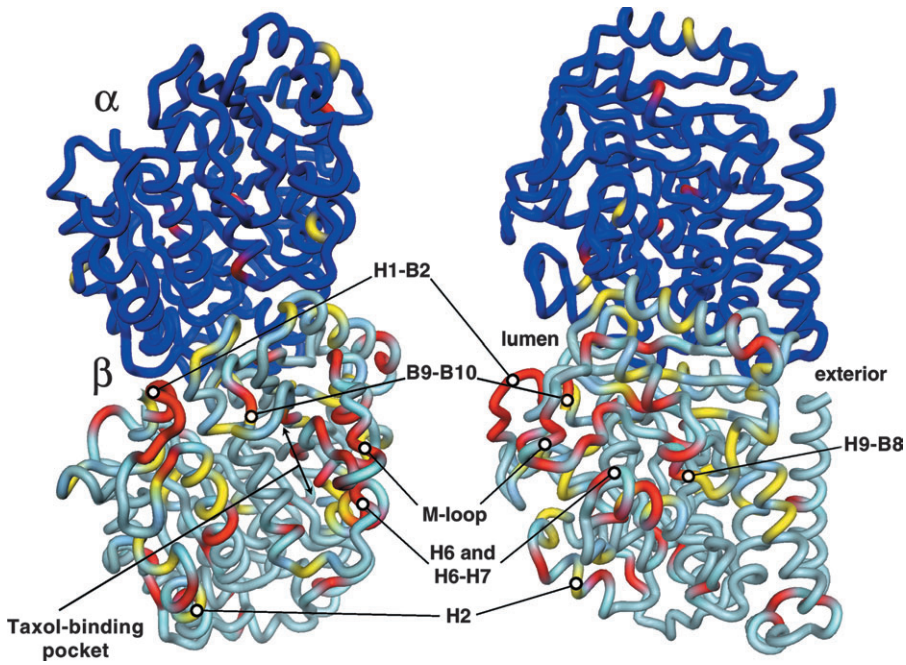

FIG. 4.—Predicted positions of substitutions in foraminiferal tubulins. Nonconservative (red) and conservative (yellow) substitutions found in foraminiferal α-tubulin and Type 2 β-tubulin relative to the consensus sequence are shown mapped onto the predicted structure of bovine brain tubulin (PDB 1JFF; Löwe et al. 2001). Left: view of the α (dark blue) and β (light blue) dimers from the lumen side of the MT. Lateral contact surfaces are to the left and right; longitudinal surfaces are at the top and bottom. Right: side view. Lumen surface is to the left; the taxol-binding pocket lies beneath the M-loop on the left side of the β-tubulin monomer. Surfaces associated with lateral contacts, such as the H1-B2 loop (which is not fully resolved in the crystal structure) and the M-loop, are strongly substituted in foraminiferal β-tubulins; other surfaces, such as the exterior helices, are essentially unchanged. Image is prepared using DINO (http://www.dino3d.org).

group. Intriguingly, the T219Q substitution has been observed in some other protists, in which it is correlated with taxol resistance (Mu, Bollon, and Sidhu 1999).

(3) M-loop. The structure and sequence of the M-loop differ between α- and β-tubulins. In α-tubulin, the M-loop is stabilized by nearby structures, especially the expanded H9-H10 loop; in β-tubulin, it is less well ordered except in the presence of taxol. This loop is also proposed to be a hinge that allows flexibility between adjacent protofilaments (Nogales et al. 1999). It is the main component in contacts between protofilaments, interacting with structures in the H1-S2 and H2-S3 loops of the adjacent monomer (Nogales et al. 1999; Li et al. 2002). Foraminifera exhibit many nonconservative substitutions in the M-loop compared to other taxa (see fig. 3). Of interest is the substitution at position 276, which is normally a positively charged residue but is a proline in forams. This position is near a predicted bend in the M-loop, and a proline residue here may serve to stiffen the M-loop. Such decrease in flexibility is also characteristic of taxol binding (see below).

Interestingly, the sequence of the M-loop in forams is still more closely related to that of other β-tubulins than it is to the sequence of α-tubulin. No substitution found in the foram M-loop matches the residue found in that position in the α-tubulin sequence. This may be important for MT assembly, perhaps functioning to preserve the distinction between the two lateral contact sites.

(4) H9 and H9-B8 loop. This loop lies nearer the outer surface of the MT, on the border of the taxol-binding pocket. This region exhibits a remarkable degree of sequence substitution in forams. In addition to two changes involving gain or loss of charge (Q291D and D295S), three residues in this loop (D304K, R306D, and H307D) have experienced complete charge reversals. Because lateral contacts are thought to be mediated at least in part by ionic interactions, the alteration of the surface charge in this region should have a strong effect on the interdimer contact here.

(5) B9-B10 loop. In α-tubulin, this loop is expanded by 8 aa relative to β-tubulin, and it appears to act to stabilize the M-loop and thereby strengthen lateral contacts. The insertion is proposed to fill the same site in α-tubulin as is filled by taxol in β-tubulin (Nogales et al. 1999). The foraminiferal B9-B10 loop is of normal length but is altered in sequence. The most notable substitution is the loss of a cross-linkable cysteine (C354I), one which is also implicated in colchicine binding (Keskin et al. 2002), but several other positions are either not conserved (D355, G360, K362) or are substituted (K359E, L361T).

Implications for Taxol Binding and MT Stabilization

As described above, many of the modifications to the foram β-tubulin sequence surround the taxol-binding pocket. Two of the foram modifications (the loss of C211 and C354) may also confer resistance to the MT-destabilizing drug colchicine, but most residues involved in the binding of this drug are unaltered in forams (Downing 2000; Hari et al. 2003).

Not all tubulins bind taxol. Many protist and fungal tubulins are only slightly affected by the drug (e.g., Gull 2001), despite the fact that they are structurally normal

and can even coassemble with mammalian tubulins (McKean, Vaughan, and Gull 2001). It has been shown that sensitivity to this molecule is controlled by relatively few residues; one study (Gupta et al. 2003) generated a taxol-binding domain in yeast β-tubulin, which is normally taxol resistant, by alteration of five residues to their mammalian counterparts (A19K, T23V, G26D, N227H, and Y270F). In support of these results, the mutation F270V has been shown (Giannakakou et al. 1997) to cause partial taxol resistance in a cultured cell line, suggesting that the presence of a phenylalanine in that position is crucial for taxol binding. Forams have A19, T23, W227, and Q or H270 and do not conserve position 26. Other residues that have been implicated in taxol binding (E22, L217, T219, T274, Q279, and R282) are also altered in forams. As mentioned above, the presence of an N or Q at position 219 seems to be correlated with taxol resistance in protists; forams generally have a Q in this position. Even more intriguing, T274, which is implicated in three separate contacts with the taxol molecule (Snyder et al. 2000), has been replaced with a much bulkier residue (F or Y) in forams. A T274I mutation was shown to cause resistance to both taxol and epothilone, a smaller analog (Giannakakou et al. 1997). It seems very likely that the modifications to these residues make forams insensitive to taxol, as earlier evidence had suggested (unpublished data).

A current hypothesis for the mode of action of taxol (Nogales et al. 1999) is that the drug stabilizes the helices and loops immediately surrounding the binding pocket. This stiffens the M-loop (making it more "alpha-like") and strengthens the lateral contact between the residues surrounding the pocket and residues from the neighboring monomer. This view explains the decrease in flexibility in four important lateral contact surfaces (the H1-H2 loop, the H6-H7 loop, the M-loop, and the B9-B10 loop) in the presence of taxol (Keskin et al. 2002). The action may also be influenced by the volume occupied by the taxol molecule itself as its presence serves to increase the packing density in the region.

The specific residues involved in lateral contacts are not well understood, but the consensus is that many are involved in ionic interactions (see, for example, fig. 10 of Li et al. [2002]). In the case of forams, some of the changes we observe—especially those involving charge, such as those in the H9-B8 loop—may affect the lateral contacts. The mutations are also predicted to decrease the hydrophobicity of the lateral contact surface (J. Bell, personal communication), as taxol does when it binds (Snyder et al. 2000). In addition, we propose that the effect of many of the foraminiferal modifications to this region is to change the conformation of the binding site in a manner similar to that induced by taxol in mammalian MTs. The insertion of a proline in the M-loop may stiffen it, much as taxol does. Although the insertion in the H1-B2 loop is not likely to be in direct contact with residues in the adjacent filament, the lengthening of this loop may increase the packing density of the contact surface, by pressing in from the lumen side. This may also explain why the sequence of the foraminiferal insert is nonconserved: if the function of the extra residues is simply to occupy space, their presence is important but their identities are not.

In conventional MTs, the α-α lateral contact is thought to be substantially stronger than the β-β contact (Downing and Nogales 1998). Taxol binding is predicted to increase the strength of the β-β contact to be at rough parity with the α-α contact. If the foram modifications to the β-tubulin structure do, in fact, mimic the effects of taxol, this may explain why there are no analogous modifications to the α-tubulin sequence.

Models of Foraminiferal MT Assembly States

Based on the previous studies of HF formation in foraminiferans, we a priori expected to find sequence modifications in regions that would weaken longitudinal interdimer bonds, which are widely accepted as being the strongest and most stable bonds in a conventional MT subunit lattice. Our results, however, do not show extensive modification of either the α- or β-tubulin genes in regions expected to affect longitudinal contacts. The nucleotide-binding regions of the molecules also appear unaffected, implying that foraminiferal β-tubulins bind and hydrolyze GTP in an essentially conventional way. We believe, therefore, that the main contribution of the tubulin modifications to the formation of HFs is in the stabilization of lateral contacts.

As noted above, HFs are too wide to be composed of a single protofilament; therefore, the mechanics of MT disassembly in forams must differ in at least some respects from that observed in other organisms. At least two alternate possibilities can reconcile HF formation with the established behavior of vertebrate tubulin polymers (fig. 5), and both would be affected by altered properties in the β-β interaction. The first (fig. 5*B*) is that foram HFs are composed of laterally associated heterodimers as originally proposed by Hauser and Schwab (1974). In this model, strengthening of the β-β interaction causes the lateral interaction to be hyperstable relative to the intra-protofilament longitudinal bond, allowing the MT to unwind into a coil. Figure 5(*C*) illustrates an alternative model, in which the lateral bonds in foram MTs are stronger and more flexible than those in conventional MTs, allowing the protofilaments to splay out from the MT in pairs during disassembly. The modifications occurring in the H1-B2 and M-loops of foram Type 2 β-tubulins may stabilize the lateral interdimer interaction just enough to allow protofilament pairs to remain associated as they bend from the MT. Alternatively, the stronger interactions might allow individual protofilaments to reassociate after disassembly.

We do not favor the model shown in figure 5(*C*). This model predicts that the 13-protofilament MT would initially splay into five or six paired strands (or coils), with the remainder being either a single- or triplet-protofilament strand. However, neither we nor other investigators have observed more than a single HF extending from the end of a foram MT.

The model in figure 5(*B*) also requires selective disruption of the longitudinal contacts, but the sequence modifications identified in our study are unlikely to affect longitudinal contact properties. The simplest explanation is that the apparent lability of the longitudinal bonds reflects a decrease in their relative strength compared to the lateral

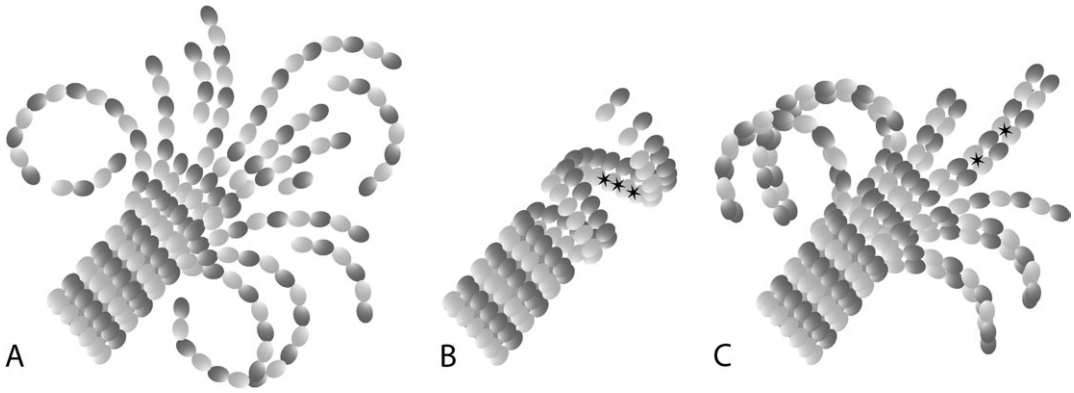

FIG. 5.—Models of MT disassembly. In most organisms (*A*), MT disassembly begins by breaking of lateral contacts, resulting in long strands of individual protofilaments. HFs in foraminiferans are too wide (~10–11 nm) to be composed of single protofilaments. The Hauser and Schwab model (*B*) proposes that the interdimer longitudinal bonds break first, producing a coil one dimer in width. The coil would be stabilized by strengthened β-β contacts (black stars). An alternative model (*C*) is that the lateral bonds in foraminiferal MTs are stronger and more flexible than those in conventional MTs, allowing the protofilaments to disassemble in pairs. As foraminiferal MTs contain 13 protofilaments, 1 of the protofilaments must be unpaired (or participate in a triplet; not shown) in this interpretation.

bonds formed by the novel foram β-tubulins. Alternatively, longitudinal bonds may be partially mediated by uncharacterized microtubule-associated proteins (MAPs) known to be present in forams (Travis and Bowser 1986; Jensen et al. 1990). In other organisms, MAPs may stabilize intact MTs by binding along the outer surface of individual protofilaments, possibly by bridging the longitudinal interdimer interface (Al-Bassam et al. 2002). These proteins associate primarily with structures along the outer ridge of the protofilament, including helices 11 and 12. As shown in figure 4, these regions are unaltered in foraminiferal tubulins. However, if the MT-to-HF transition is regulated by MAPs, these must remain bound in both conformations because HFs induced in vitro by divalent cation treatment transform back into intact MTs following cation washout (Welnhofer and Travis 1998).

Implications for Cold Stability

At low temperatures, tubulins from non–cold-adapted organisms may fail to assemble. Changes to certain residues in β-tubulin, primarily in regions associated with lateral contacts, have been suggested to confer cold stability on the MTs of Antarctic icefishes (Detrich et al. 2000). One sequence from that study (from *Notothenia coriiceps*) is included in figure 3. Four of our sequences are from Antarctic forams (*A. rara*, *C. antarctica*, *R. cornuta*, *P. peruviana*), while the remaining four species are native to temperate waters; each group is taxonomically diverse. We do not find the "cold-specific" mutations described in the previous fish study in any of our foram sequences, nor is there any consistent difference between the tubulins of temperate and polar forams.

These species do, however, exhibit differences in the cold stability of their MTs. The MTs of temperate-water forams such as *Allogromia* form HFs upon cold shock (Travis and Bowser 1986), suggesting that the lateral contacts are cold stable in all forams. Therefore, the modifications reported by Detrich and co-authors may be superfluous in forams. Stabilization of the longitudinal contacts in cold temperatures, which is apparently much more effective in polar foram species than in temperate ones, may be accomplished by a different mechanism in these protists.

Absence of β-tubulin Type 1 Isoform

A previous study (Linder, Schliwa, and Kube-Granderath 1997) reported two β-tubulin isoforms in *R. filosa*. In our hands, amplification with β-tubulin universal primers failed to recover product from all but one of the foraminiferal templates tested for this study. The last amplification product, on sequencing, proved to be strongly homologous to ciliate β-tubulin genes, especially those from several members of the genus *Euplotes*, and probably represents a contaminating organism within that template. (As discussed below, such contamination can result from the nonaxenic growth conditions required by forams.) A phylogenetically very similar ciliate α-tubulin sequence was obtained from the same template, reinforcing the idea that this particular DNA extract was polygenomic. Further, a primer pair (BTub2F and BTubB) that was highly specific for the reported Type 1 β-tubulin gene of *R. filosa* failed to generate product when used on an *R. filosa* template. We have no evidence that a sequence corresponding to the Type 1 β-tubulin isoform is found in any foram species.

Reticulomyxa filosa cells are large (often >1 cm across), delicate syncytial networks and cannot be grown axenically. Therefore, it is possible that the Type 1 sequence is from an organism living in coculture with *Reticulomyxa*, as addressed by Linder, Schliwa, and Kube-Granderath (1997). Their report described several methods (immunofluorescence microscopy using antibodies against the MTs of *R. filosa* pseudopodia, western blotting etc.) used to determine whether both Type 1 and Type 2 sequences were expressed in *Reticulomyxa*. Western blots failed to detect the presence of the Type 1 β-tubulin, although immunofluorescence suggested that both were present. Other immunofluorescence studies (e.g., Centonze and Travis 1983) found that foram MTs were stained with a wider range of conventional anti–α-tubulin antibodies

than anti–β-tubulin antibodies. Indeed, only Type 2 β-tubulin sequence data have been deposited for *A. laticollaris*. We therefore feel confident in stating that the "typical" foram β-tubulin is the Type 2 form.

Conclusion

The evolution of this highly unusual β-tubulin may have been a key factor in the origin of foraminiferan protists—it apparently enables the rapid and efficient transport of "prepackaged" tubulin, as paracrystals of HFs, to/from distal sites in the extended reticulopodial network (reviewed in Travis and Bowser 1991). Indeed, the formation of reticulopodia may not be possible without such a tubulin transport system. Foram tubulin innovation also represents a valuable natural experiment in examining the role that lateral contacts play in MT assembly in other organisms. We are currently engaged in immunocytochemical and ultrastructural studies of foram MTs in order to clarify the appropriate model of HF formation, determine whether MT lattice is altered in these organisms, and discern whether associated proteins are implicated in assembly dynamics.

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