Hypothesis

Analysis of β-tubulin sequences reveals highly conserved, coordinated amino acid substitutions

Evidence that these 'hot spots' are directly involved in the conformational change required for dynamic instability

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Vertebrate β-tubulins have been classified into six classes on the basis of their C-terminal sequences [(1987) J. Cell Biol. 105, 1707–1720]. In particular, the sequences starting at residue 430 differ between isotypes of the same animal but are conserved between species. We extend this analysis and show that there are three 'hot spots', at residues 35, 55–57 and 124 which exhibit intra-species heterogeneity but inter-species conservation. There is a remarkable correlation between the identity of these residues and the C-terminal sequences, and suggests that the vertebrate β-tubulins fall into three broad types. This correlation extends to those non-vertebrate organisms which have the Type 1 C-terminal sequence. We propose that these three 'hot spots' and the C-terminal peptide interact in the tertiary structure. We have also noted that the C-terminal peptide almost always contains a single phenylalanine or tyrosine residue, and that there is a strong correlation between this residue and the amino acids at positions 217/218, in both the vertebrate and non-vertebrate sequences. We propose that the C-terminal aromatic amino acid interacts with residues 217/218 in the tertiary structure. Analysis of conditions which stabilise microtubules and/or lower the steady state critical concentration strongly suggests that these two sets of coordinated amino acid substitutions are directly involved in effecting the conformational change associated with GTP hydrolysis which results in dynamic instability. We propose that there is an interaction between the highly acidic sequence between residue 430 and the aromatic amino acid (termed peptide A) and conserved basic amino acids located close to the 'hot spots'. We suggest that this interaction is altered in response to the assembly-dependent GTP hydrolysis, with the consequential increase in the subunit dissociation rate constant.

β-Tubulin; Coordinated substitution; Dynamic instability

1. CLASSIFICATION OF VERTEBRATE β-TUBULINS

The α- and β-tubulins are both highly conserved, with the most extreme diversity being confined to the C-terminal sequences [1]. For example, only 15.6% of residues 1–430 are different between any of the seven chick β-tubulins, and almost 40% of these are unique to the β-6 isotype, while the C-terminal peptides (from residue 431) are both variable in length and amino acid sequence.

This heterogeneity is unusual since β-tubulin isoforms with virtually identical C-terminus sequences are found in different organisms. This led Cleveland and co-workers to divide all vertebrate β-tubulins into six classes based on their C-terminal sequences (successively developed in [2–5]). The limited evidence available indicated that the expression of these different isotypes in different tissues is also conserved. All cells of higher organisms yet examined contain a mixture of different isotypes [5–7].

We have applied this approach of identifying intra-species heterogeneity but inter-species homology to the highly conserved portion of the vertebrate β-tubulin sequences (1–430), and have also identified a conserved feature within the extremely heterogeneous C-terminal peptide (431 →).

2. IDENTIFICATION OF COORDINATED CHANGES

We have noticed three 'hot spots' which, like the C-terminal peptide, vary between different isotypes from the same vertebrate yet are conserved between species (see Table I). These 'hot spots' are located at residues 35, 55–57 and 124, positions previously recognised as

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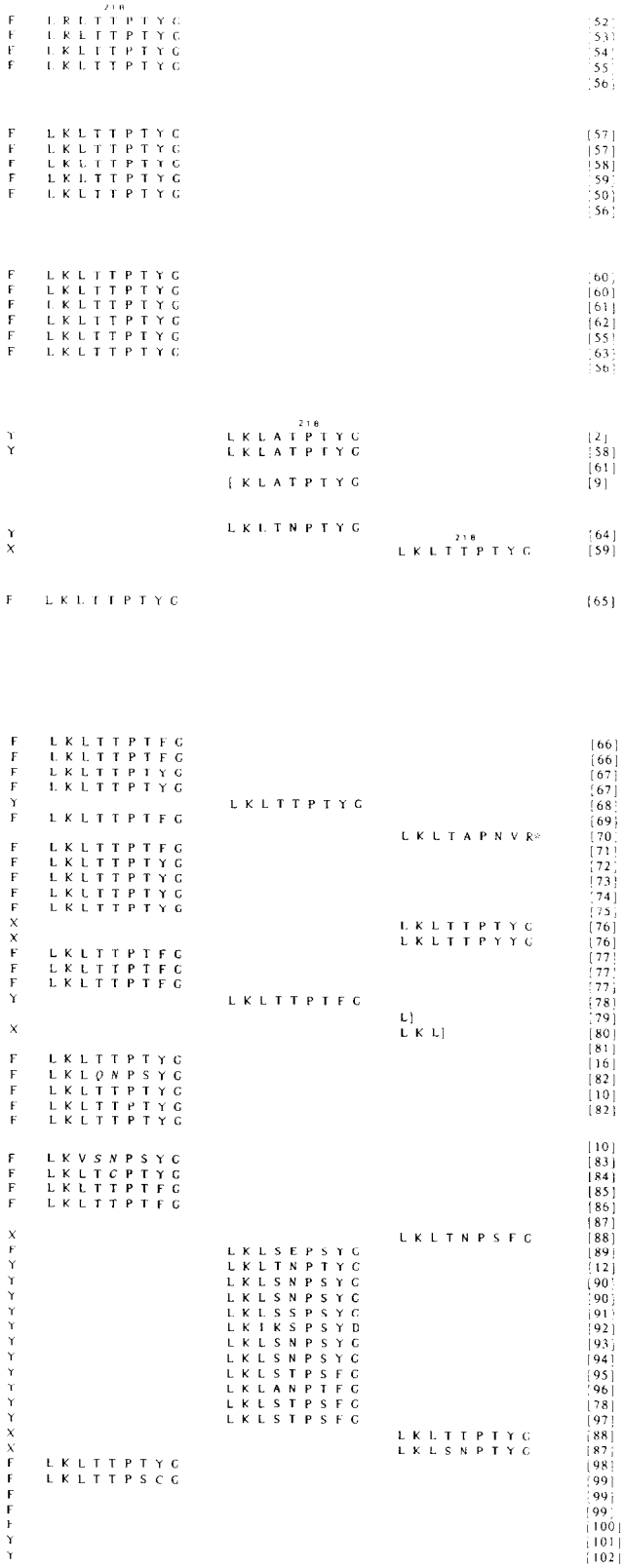
Table 1

Compilation of known vertebrate and non-vertebrate β-tubulin sequences around the 'hot spots' at residues 35, 55–57, and 124, the C-terminal peptide, and the sequences surrounding residues 217/218

	35	55	124	430 1	
VERTEBRATE β-TUBULINS:					
TYPE 1:					
CLASS I					
Human β1/M40	TGTYHG	ATGGKY	VRKEAESC	AEEEFDFGEEAEEEA	
Human Dβ1	TGTYHG	ATGGKY	VRKEAESC	AEEEFDFGEEAEEEA	
Chick β-7	TGTYHG	ATGGKY	VRKEAESC	AEEEFDFGEEAEEEA	
Mouse β-5	TGTYHC	ATGGKY	VRKEAESC	AEEEFDFGEEAEEEA	
Rat RB13				EEEEDFGEEAEEEA	
CLASS IV					
Human β-2	TGTYHG	ATGGKY	VRKEAESC	AEEQEDFGEEAEEEV	
Human 5β	TGTYHG	ATGGKY	VRKEAESC	AEQGEEEEEAEEEVA	
Chick β-3	TGTYHC	ATGGKY	VRKEAESC	AEEEGEEEEEAEEEAL	
Mouse β-3	TGTYHC	ATGGKY	VRKEAESC	AEEEGEEEEEAEEEVA	
Mouse β-4	TGTYHC	ATGGNY	VRKEAESC	AEEGEEEEEAEEEVA	
Rat RBT2				EEGEEEEEAEEEVA	
TYPE 2:					
CLASS II					
Chick β-1	TGSYHG	AAGNKY	VRKESESC	ADEQGEEEEEGEEDEA	
Chick β-2	TGSYHG	ATGNKY	VRKESESC	ADEQGEEEEEGEEDEA	
Pig A	TGSYHC	AAGNKY	VRKESESC	ADEQGEEEEEGEEDEA	
Rat 15	TGSYHC	AAGNKY	VRKESESC	ADEQGEEEEEGEEDEA	
Mouse β-2			ESC	ADEQGEEEEEGEEDEA	
Xenopus laevis	TGSYHG	ATGNKF	VRKESESC	ADEQGEEEEEEDEA	
Rat RBT 1				DEQGEEEEEGEDEA	
TYPE 3:					
CLASS III					
Human 4			VRKECENC	AEEEGEMYEDDEEESESQGPK	
Chick β-4	SGNYVG	ASSHKY	VRKECENC	AEEEGEMYEDDEEESEQGAK	
Pig B	TGSYVG	ASSHKY			
Cow β2					
CLASS VI					
Chick β-6	AGNYCG	AYSHKY	VRNECESC	ADVEEYEEAEASPEKET	
Mouse β-1	ACSYCG	AYGKKY	VRRESESC	CLEDSEEDAEEAEVEAEDKDH	
CLASS V					
Chick β-5	AGGYVG	SSSQKY	VRKECEHC	ANDGEEAEEDDEEEINE	
NON-VERTEBRATE β-TUBULINS:					
Chlamydomonas β-1	TGTYHG	ATGGRY	VRKEAESC	AEEEGEEEGEEEEA	
Chlamydomonas β-2	TGTYHG	ATGGRY	VRKEAESC	AEEEGEEEGEEEEA	
Tetrahymena βTT1	TGTYHG	ATGGRY	VRKEAEGC	AEEEGEEEEEEEGEN	
Tetrahymena βTT2	TGTYHG	ATGGRY	VRKEAEGC	AEEEGEEEEEEEGEN	
Euplotes crassus	TGTYHG	ATGGRY	VRKEAEGC	AEEEGEYVFDEDEDMDGM	
Trypanosoma brucei	TGTYQG	ATGGRY	CCKEAESC	IEEEGEFDEEEQY	
Trypanosoma cruzi					
Volvox	TGTYHG	ATGGRY	VRKEAESC	AEEEGEEEGEEEEFN	
Paracentrotus	TGTYHG	ATGGKY	VRKEAESC	AEEEGEEFDEEEEGDEEAA	
Toxoplasma	TGTYCG	ATGGRF	VRKEAEGC	AEEEGEEFDEEEEGEMGAEEGA	
Plasmodium falciparum	SGTYCG	ATGGRY	VRKEAEGC	AEEEGEEEEEEEGDVEA	
Plasmodium falciparum	SGTYSG	ATGGRY	LRKEAEGC	AEEEGEEEEEEEGDVEA	
Stylonychia β1	TGTYHG	ATGGRY	VRKEAEGC	AEDEEEMDEFQME	
Stylonychia β2	TGTYHG	ATGGRY	VRKEAEGC	AEDEEEMDEEQME	
Polytomella β1	TGTALG	ATGSRY	VRKEAESC	AEEEGEEGEEEEEN	
Polytomella β2	TGTAIG	ATGSRY	VRKEAESC	AEEEGEEGEEEEEN	
Polytomella β3	TGTAIG	ATGSRY	VRKEAESC	AEEEGEEGEEEEEN	
Zea β10	TGRYGG	ASCGRY	VRKEAESC	ADEEGEYEDEEEGDLQD	
Physarum β1	TGSYHG	ATGGKY	VRKEAESC	IDDEEGGEEEEGGAEE	
Physarum betB	TGSYHG	ATGGKY	VRKEAESC	ADEDAAEAEDGE	
Caenorhabditis mec-7	SGQYVG	AGSNKY	VRKEAEST	VEDDEEVDENGDEGAPQNQDEPITENEE	
S. cerevisiae	NGTYHG	ASSGKT	IRREAEGC	ADEDAEEEEEQEAFVDEN	
Drosophila melano. β-1	TGTYHG	ASGGKY	VRKEAESC	ADEEGEEEEEEDEGCGDE	
Drosophila melano. β-2	TGTYYG	ATGAKY	VRKESEGC	ADEEGEEDEDEEGCGDE	
Drosophila hydei β-2	TGTYYC	ATCAKY	VRKESEGC	ADEEGEEDEDEEGCGDE	
		SAVTRS			
Drosophila melano. β-3	NGIYVG	ASGGKY	VRKECENC	ADDEEDPEVNQEEVEGDCI	
Giardia	SGEYRG	AAGGRY	VRKRSEAC	VDEGEEEEEEEEDFGDE	
Euglena	PDIPG	ATGGRY	VRKEAESC	VEEEGEEDEEEEAY	
Leishmania mexicana	TGSYQG	SAGGRY	CRKEAESC	VEEEGEEDEEEDVEQY	
Leishmania enriettii	TGSYQG				
Glycine Sβ-1	TGNYVG	ASCGRY	VRKEAENC	AVDDHEDEDEDEAMAA	
Caenorhabditis tub-1	DGTFKG	ANNGKY	VRKEAEGC	AEDDVDGYAEGEAGETYESSEQ	
Histoplasma capsulatum	AGYYNG	AAEKKY	VRRDAERC	ISEGEDEYEDYAWAM	
Neurospora	SGVYNG	ASGNKY	VRREAEGC	VDEEEEEEYEEEAPLEGEE	
Aspergillus Ben A	SGVYNG	ASGNKY	VRREAEGC	ASISEGEEEYATEEIMEGEE	
Aspergillus tub C	SGIYTG	AGGNKY	VRREAESC	ATVSDGEGAYDAEEGEAYEQEE	
Erysiphe graminis tub2	SGVYNG	ASGNKY	VRREAEGC	ISEGEEEYPEEVSNEEF	
Epichloe typhina tubB	NGVYNG	ASGNKY	VRREAEGC	IDEEEEEYEEAPVDEPLE	
Schizosaccharomyes pombe	AGIYHG	AAGGKY	VRREAEAC	ACIDEGDEDYEIEEEKFPLEY	
Candida tub-2	NGTYVG	ATSGKY	VRREAEGC	IDEEELEYADEIPLEDAMME	
Arabidopsis β-1	TGRYNG	ASCGRY	VRKEAENC	ADEEDEYEEEEEQVYES	
Physarum β2	AGTYKG	VAGSKY	VRKEAENC	VDDEAMEDDALAEGGAGQNAVEEE	
Caenorhabditis ben1	DGTYKG	ANGGKY	VRKEAEGC	AEEDGELDCTPGDAE	
Glycine Sβ-2	TGRYGG	ASCGRF	VRKEAENC	ADEDEYEEEEEEEEEFAQHDM	
Strongylocentrotus				AEEEGEEDEEEEGDEEAA	
Lytechinus pβ1				AEEEGEEDEEEGEDEEAA	
Lytechinus pβ2				AEEEGEEDEEEGEDEEAA	
Lytechinus pβ3				AEEEGEEDEEEGEDEEAA	
Lymnea				AEDEGEEDEEEAEGEGQEYA	
Brugia pahangi				DEEGDLQEGESEYLLQEE	
carrot				ADEEEYY.DEEEEEAQGM	

All sequences, apart from that of *Caenorhabditis* tub 1, *Epichloe* and *Limnea*, have been taken from published reports. The analysis assumes that there have been no sequencing errors. The vertebrate sequences are assigned to the proposed Types and to the Classes as defined by Cleveland. The insertion in the *Drosophila* β-3 sequence at residue 54 is shown. Each C-terminal peptide starts at residue 430, the last of the conserved sequence, and are aligned relative, when present, to the aromatic residue. The identity of this aromatic residue (F or Y) is shown: X indicates those C-terminal sequences lacking this feature. The residues surrounding positions 217/218 are sub-divided according to the identity of this aromatic residue. Residues at 217/218 which differ from the conserved Thr^{217}/Thr^{218} in the first (phenylalanine) block are shown in italics. The *Trypanosoma cruzi* sequence is labelled * as the C-terminal sequence is unknown. The ends of known sequences are marked by square brackets

Table I continued



being highly variable [2]. There is a remarkable correlation between the identity of these residues and the C-terminal sequence (Table I). The substitutions at these 'hot spots' represent a significant fraction of all those within the first 430 residues: for example, they include a quarter of the residue differences between chick β-7 and chick β-1/2.

These 'hot spots' have three striking features. First, a change at one site is always accompanied by the appropriate changes at the other two sites, apart from certain minor exceptions which will be considered later. Secondly, the same substitutions are found in all β-tubulins of the same class as defined by their C-terminal peptides. Thirdly, the converse does not apply: Class I and IV β-tubulins have distinct but related C-terminal peptides, but have identical residues at the 'hot spots'. Vertebrate β-tubulins can therefore be divided into three types; Type 1 includes Classes I and IV, Type 2 is equivalent to Class II, while Type 3 is more heterogeneous and includes Classes III, V and VI.

This heterogeneity of Type 3 is illustrative of the minor exceptions. Inspection of the 'hot spot' residues clearly shows that Classes III, V and VI are closely related to each other, with minor variants, e.g. Cys^{124} is replaced by a serine in the mouse β1 isotype. The second group of exceptions are point substitutions in the flanking sequence, such as the replacement of Ala^{55} by threonine in the Class II chick β2 isotype, and Lys^{58} by asparagine in mouse β4. Finally, the pig sequences, determined by peptide analysis, have been allocated to Classes II and III on the basis of their homologies, their neuronal origin, and the evidence that the tubulin contains a mixture of isotypes [8,9].

Several of the non-vertebrate sequences share a very high sequence homology with the vertebrate Type 1 at all three 'hot spots' and their flanking regions (Table I), and most of these (*Chlamydomonas* β1 and 2, *Tetrahymena* βTT1 and 2, *Trypanosoma brucei*, and *Volvox*) have a C-terminal sequence which is either identical or closely resembles that of the Type 1 Class IV (Table I). A similar homology with respect to the 'hot spots' and residues 431–436 applies to *Paracentrotus, Euplotes, Toxoplasma* β-tubulins and to one *Plasmodium* isotype. This does not apply to any of the other C-terminal sequences, with the exception of *Lytechinus* and *Strongylocentrotus* for which the 'hot spot' sequences are unknown. *Stylonychia* β-1 and β-2 have the vertebrate Type 1 'hot spots' but a significantly different C-terminal peptide, while *Polytomella* β-tubulins have the vertebrate Type 1 C-terminal sequences but different 'hot spots'.

Almost all the non-vertebrate sequences closely resemble the vertebrate Type 1 around residue 124, while greater heterogeneity is apparent around the other two 'hot spots'. This may relate to a common functional property of most β-tubulins.

The flanking regions of the three vertebrate and non-vertebrate β-tubulin 'hot spots' contain three conserved basic residues: $Arg^{58}/Lys^{58}/Asn^{58}$, Arg^{121} (except for *Trypanosoma brucei*), and $Arg^{122}/Lys^{122}/Asn^{122}$. His^{37} is also conserved in the vertebrate Types 1 and 2 (but not Type 3), while many non-vertebrate sequences have a basic residue at this position (Table I).

Almost all of the C-terminal sequences contain a single tyrosine or phenylalanine residue. This partially conserved residue splits the C-terminal sequence into what we shall define as peptide A, between the conserved Ala^{430} and the aromatic site, and peptide B which extends from this site to the C-terminus. The sequence of peptide A is virtually identical for all vertebrate Type 1 and non-vertebrate β-tubulins having the Type 1 'hot spots', while the sequence of peptide B is more variable (Table I).

The conserved aromatic residue is tyrosine in chick β-4, β-6 and human β-4, but is phenylalanine in all other vertebrate isotypes. Examination reveals that these three exceptions contain a single unique change: residues 217/218. The amino acids at residues 217/218 correlate with the identity of the conserved aromatic residue for almost every known β-tubulin (Table I): with Thr^{217}-Thr^{218} when tyrosine is conserved but other residues for the phenylalanine C-terminal peptides. There are just five exceptions: *Drosophila* β-3, which is unusual in that it contains the sole known insertion [10], *Saccharomyces cerevisiae* with its highly divergent C-terminal peptide (Table I), and *Giardia*, while both *Zea* and *Euplotes* have a phenylalanine in the C-terminal peptide but the Thr^{217}-Thr^{218} characteristic of the tyrosine C-terminal peptide. Residues 217/218 are not noticeably dissimilar in the eight C-terminal sequences lacking a conserved aromatic residue.

We have therefore identified two sets of coordinated yet independent changes: a remarkable correlation between the 'hot spots' at residues 35, 55–57 and 124 and peptide A of the C-terminal sequence of all vertebrate and many non-vertebrate β-tubulins, and a strong correlation between the identity of aromatic residue of the C-terminal sequence and the identity of residues 217/218.

3. POSSIBLE ORIGINS OF THE COORDINATED CHANGES

One possibility is that the tubulin sequence is so highly constrained by the required tertiary structure that a substitution at one position requires the other changes for the protein to preserve its conformation. There is considerable evidence against this possibility for the correlation between the C-terminal sequence and the three 'hot spots'. First, the different β-tubulins contain many other amino acid substitutions, although there are no other equivalent 'hot spots' in either the β- or the α-subunit, while there are an increasing number of functional β-tubulin mutants (for examples, see

[11–13]). Secondly, the products of yeast-chick chimeric genes are assembled in vivo following transfection [14,15], even though *S. cerevisiae* tubulin and chick β-1 sequences only share ≈70% identity [16]. Furthermore, the *Drosophila* β-3 sequence contains an insertion of six residues at position 54, i.e., almost immediately adjacent to one of the 'hot spots', which does not appear to adversely affect its function [10]. Finally, many non-vertebrate β-tubulins show considerable heterogeneity at these three positions, while the vertebrate β-tubulins can accommodate amino acid changes at adjacent positions (Table I).

The alternative possibility is that the coordinated substitutions are functionally significant. This would imply that residues at the three 'hot spots' and the C-terminal peptide interact with each other in the tertiary structure, i.e. these four parts of the primary sequence are brought into close proximity by the folding of the peptide chain to form a 'complex'. The term 'complex', rather than 'pocket', has been used in order to avoid implying any specific geometry. The 'hot spots' and the C-terminal peptide would represent highly conserved sequences rather than permitted heterogeneity [17]. Similarly, the correlation between the C-terminal aromatic residue and positions 217/218 implies that they interact in the tertiary structure.

Consideration of residues 35, 55–57, 124 and the C-terminal peptide strongly suggests that the 'complex' is on or close to the exposed surface of the assembled microtubule. First, monoclonal antibodies against epitopes at residues 416–430 and 422–434 label assembled microtubules [18–20]. The N-terminal residues of peptide A therefore lie on the outer subunit surface of the assembled microtubule. Similarly, peptide B is also exposed as isotype-specific polyclonal antibodies raised against C-terminal peptides label assembled microtubules [4,5], while a second MAP-binding consensus sequence (EGEE, [21]), characteristic of α-tubulin, is located at residues 444–447 of certain of the Type 2 Class II isotypes (Table I).

Secondly, the predicted hydrophilicity, as reported by De la Vina et al. [19], and independently confirmed, shows that residue 124 lies in one of the most hydrophilic regions. It is therefore unlikely to be buried in either the hydrophobic core or at a subunits/subunit interface, and is probably on the exposed surface of the assembled microtubule. Residues 55–57, which are also moderately hydrophilic, lie immediately adjacent to the inserted six additional amino acids of the *Drosophila* β-3 sequence [10]. This insertion probably forms a loop which extends from the assembled microtubule, thus placing residues 55–57 at or close to the exposed subunit surface. Finally, the sequence at residues 33–38 of *Euglena* β-tubulin is totally different from those of all other β-tubulins (see Table I), despite a high homology elsewhere, and involves the deletion of a single residue. It is extremely unlikely that this highly altered sequence is buried within the tertiary structure since other changes would be predicted, and therefore it may lie at or close to the subunit surface.

There is no equivalent corroborative evidence showing that the conserved aromatic amino acid and residues 217/218 are spatially related. Significantly, no antibodies or other proteins have been shown to bind at or close to the aromatic residue, while the predicted hydrophilicity of residues 217/218 is not significantly high.

4. FUNCTIONAL ROLE OF THE COORDINATED SUBSTITUTIONS

The C-terminal peptide has been implicated in the binding of Microtubule Associated Proteins such as the neuronal MAP2 and tau [22]. The function of the proposed 'complex' is unlikely to relate specifically to either of these proteins since both are neuron-specific, while the three types are each found in both neuronal and non-neuronal tissues. The 'complex' could be the binding site for more ubiquitous proteins, e.g. microtubule motors such as kinesin, dynein and dynamin; other MAPs, such as MAP1A and the 205–210 kDa family; bundling proteins, such as glyceraldehyde-3-phosphate dehydrogenase [23]; or proteins which specifically interact with the β-subunit such as the protein kinase which phosphorylates the Type 3 Class III β-tubulin [24] and tubulinyl tyrosine kinase [25]. The most persuasive argument against this possibility is that CV1 cells only contain Type 1, Classes I and IV [5], yet such cells are likely to have typical requirements for the various associated proteins. Furthermore, the function of this 'complex' is unlikely to be simply an on/off switch, since this could be effected by just two classes. Indeed, the vertebrate Class I and Class II β-tubulins have identical 'hot spot' residues but have different C-terminal peptides, which suggests that their exact sequences are functionally important in specifying the properties of the 'complex'.

An alternative possibility is that the function of the coordinated changes relates to an intrinsic property of the tubulin subunit. Microtubules exhibit dynamic instability [26]. This behaviour reflects the existence, at steady state, of two slowly interconverting sub-populations of microtubules, one which is elongating while the other is rapidly shortening [27]. The two sub-populations have different critical concentrations (C_o or the ratio of the dissociation to association rate constant), such that one sub-population, with the lower C_o, continues to elongate at the expense of the other.

The transition between the elongating and shortening phases is generally believed to be a direct consequence of the assembly-dependent hydrolysis of GTP bound to the β-subunit [28], such that only the former terminate in a 'GTP-cap'. GTP hydrolysis greatly enhances the

dissociation rate constant (i.e. $k_{-1}^{GTP} < k_{-1}^{GDP}$), and this underlies the difference in the C_o of capped and uncapped microtubules. The change in the dissociation rate constant indicates that the tubulin dimer alters its conformation. As GTP hydrolysis occurs at the exchangeable site on the β-subunit, the primary conformational change is likely to occur in this subunit.

Tubulin can therefore exist in a minimum of two conformational states, with the transition from one state to the other influencing the lability of the assembled microtubule. One intriguing possibility is that the various isotypes respond differently to this assembly-dependent GTP hydrolysis, and that microtubules assembled from specific isotypes exhibit dynamic instability to different extents, while those consisting of a mixture of isotypes have intermediate properties.

Unfortunately, it has not yet been possible to prepare significant amounts of tubulin containing single α- and β-isotypes. However, the successive use of isotype-specific antibody affinity chromatography has permitted the purification of pig α- and β_2- (Type 3, Class III) tubulin [29]. Microtubules assembled from this protein are reportedly more stable than those from the unfractionated tubulin [30]. The enhanced stability may be a direct consequence of the β_2-isotype or microtubules assembled from a mixture of isotypes may be intrinsically more labile.

The erythrocyte tubulin provides the clearest evidence that the dynamic properties relate to the isotype composition. Erythrocyte tubulin consists of a mixture of Types 1 and 3 [4], while brain tubulin contains all three types [29]. Assembly studies have shown that the erythrocyte tubulin differs from the brain protein in having a lower steady state C_o [31,32]. This C_o reflects the number average of the relative dissociation/association rate constants of the elongating and shortening microtubule sub-populations, and therefore the lower value may be due, in part, to an altered response to the assembly-dependent GTP hydrolysis.

We also note that four of the experimental methods for either lowering the steady state C_o or enhancing the extent of assembly relate to either the C-terminal peptide or to a probable interaction between this highly anionic peptide and a cationic site. These are: (i) the addition of MAPs such as MAP2 and tau, which bind to consensus sites at either end of the C-terminal peptide. Such MAPs inhibit dynamic instability [33,34] and lower the steady state C_o (for example, [35]) to experimental values which are similar to those determined for the elongating sub-population of pure tubulin microtubules (for example, see [26,27]). Significantly, tau lowers the steady state C_o but does not affect the initial elongation rate [36]. (ii) The truncation of both α- and β-tubulin by 7–15 residues by subtilisin digestion [37–39]. (iii) The addition of polycations, with the formation of double-walled microtubules and other polymorphs, which probably bind to the highly acidic C-terminal peptide [40,41]. (iv) The use of assembly buffers containing very high ($\approx$1 M) sodium glutamate which may saturate sites normally occupied by the C-terminal glutamic acid residues [42].

Peptide A, rather than peptide B, is involved in lowering of the steady state C_o. Subtilisin digestion results in the complete removal of peptide B and an unknown amount of peptide A, yet truncation of peptide B to $\approx$ residue 438 by carboxypeptidase Y has no effect on the steady state C_o [43]. Peptide A is also directly involved in the binding to the repeated consensus sequences of MAP2 and tau [44–46], both of which lower the steady state C_o, since these proteins bind to a synthetic peptide of residues 422–434 [47]. Other studies, using [^{125}I]MAP2 and [^{125}I]tau, have detected strong binding to synthetic peptides of residues 426–440, 426–445 and 434–445 but not to the peptides 416–425 or 416–431 [48]. At least one MAP-binding site therefore appears to be specified by residues close to position 434, i.e. towards the N-terminus of peptide A, and directly implicates peptide A in effecting the conformational change associated with dynamic instability.

This conformational change presumably involves relative movement between the three domains apparent from low angle X-ray diffraction [49]. One interesting, but speculative, possibility is that this change involves an association of peptide A with two of these domains. A synthesis of the available sequence information suggests that the N-terminus of peptide A interacts with the 'hot spots' at residues 35, 55–57 and 124, while the C-terminal aromatic amino acid interacts with residues 217/218. Consequently, peptide A may link two of the structural domains, and be directly involved in effecting the conformational change induced by the assembly-dependent GTP hydrolysis. In particular, the adoption of a lower energy state as a result of this hydrolysis may involve a change in the interaction between the multiple acidic residues of peptide A and the identified, and highly conserved, basic residues flanking the three 'hot spots' and at residue 215. This proposal specifically relates to the β-subunit: additional conformational changes presumably occur in the α-subunit induced by the β-tubulin GTP hydrolysis.

An important feature of this possibility is that it makes the precise predictions which are a pre-requisite for any informed site-directed mutagenesis. Although genetic insertions and deletions have been examined (for example, [15]), these have generally been made on the basis of available restriction sites rather than in respons to any specific structural model. The current difficulty in preparing significant amounts of monotypic tubulins, the various post-translational modifications, the mingling of isotypes in single microtubules (for example, [50,51]), and the cytoplasmic MAPs will all complicate any analysis of the functional role of the coordinated substitutions.

Possible methods, short of determining the crystallographic structure, include combining site-directed mutagenesis with transplacement and transfection.

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