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Microtubule Minus Ends can be Labelled with a Phage Display Antibody Specific to Alpha-Tubulin

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To investigate the orientation of α- and β-tubulin heterodimers within microtubules, we cloned a phage display antibody to α-tubulin. The N-terminal 100 residues of α-tubulin were bacterially expressed and used to select clones from a large repertoire of antibody-expressing phagemid particles. One clone reacted with the expressed α-tubulin N terminus and native tubulin dimer but not with the expressed β-tubulin N terminus. Electron microscopy showed 30 nm gold beads coated with the antibody binding to one end of brain microtubules. The beads bound to the minus ends of axonemes but not to the brain tubulin extensions from their plus ends. In sliding motility assays with a plus end directed motor, beads were pushed ahead of the microtubules. Our results indicate that an N-terminal epitope of α-tubulin is exposed only at the minus ends of microtubules.

Keywords: microtubules; tubulin; phage display antibody; polarity

Microtubules are built from heterodimers of α- and β-tubulin. A recent solution of the tubulin structure at 6.5 Å (Nogales *et al*., 1995) has confirmed that each tubulin protofilament is a linear arrangement of obviously similar 4 nm × 5 nm globular monomers, but the polar orientation of heterodimers within microtubules remains uncertain. This is important for understanding the mechanism of microtubule assembly and the sensing of microtubule polarity by interacting proteins, including motor molecules. α- and β-tubulin differ in that GTP bound to α-tubulin is non-exchangeable, whereas the GTP on β-tubulin is exchangeable. It has been shown (Mitchison, 1993) that beads coupled to GTP label the faster-growing (plus) ends of microtubules, but this result has not been taken as conclusive proof (e.g. Song & Mandelkow, 1995) that β-tubulin is the terminal species at the plus end. We have therefore confirmed the dimer orientation using a phage display antibody to alpha-tubulin which labels only the minus ends of microtubules.

Sequences for the first 102 N-terminal amino acid residues of α-tubulin (αNT) and residues 1 to 105 of β-tubulin (βNT) were each inserted into the pMal c2 vector (BioLabs) to make fusion proteins coupled to the maltose-binding protein (MBP) *via* thrombin-sensitive linkers. Expressed fusion proteins were purified by binding to amylose resin and the tubulin domains separated from the MBP by thrombin cleavage and further chromatography. Purified αNT/MBP was used to select clones from the ''Vogon'' library (Vaughan *et al*., 1996), a large repertoire of phagemid particles that display artificial antibodies, consisting of single-chain Fv format (scFv) antibody fragments fused to the pIII coat protein. The scFv sequences were built from antibody heavy and light chain variable region genes, amplified from unimmunised human donors, with sequences encoding both a hexahistidine (Janknecht *et al*., 1991) and *myc* tag (Munro & Pelham, 1986) appended to the end of the light chain to facilitate purification and immunological detection.

After two to three rounds of selection (Marks *et al*., 1991) on immunotubes coated with MBP/αNT or MBP/βNT fusion proteins, clones were sought that were positive for αNT/MBP but not for βNT/MBP. Individual clones in *E. coli* TG1 were rescued with VCS-M13 helper phage (Stratagene).

Abbreviations used: MBP, maltose binding protein; αNT and βNT, N-terminal 100 residues of α- and β-tubulin; scFv, single chain variable fragment artificial antibodies; ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PEG, polyethylene glycol; MalNEt, *N*-ethyl maleimide; DIC, differential interference contrast; GST, glutathione *S*-transferase; *ncd*, non claret disjunctional; FPLC, fast protein liquid chromatography.

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Binding of the phagemid particles to native tubulin, MBP/αNT- or MBP/βNT-coated microplates was detected with HRP/anti-M13 antibody conjugate (Pharmacia) by enzyme-linked immunosorbent assay (ELISA). Positive phagemids were transfected into HB2151 cells for expression after isopropyl-β-D-thiogalactopyranoside (IPTG) induction. The resulting soluble antibodies were purified on Ni-NTA Spin columns (Qiagen) and dialysed against phosphate-buffered saline (PBS) at pH 7.2.

A clone (VαNT1) that was strongly positive both for expressed αNT and for native tubulin dimer on the ELISA plates (working dilution, 0.12 μg/ml), and for only the α-tubulin band on Western blots (Figure 1) was grown to express soluble scFv antibody for further experiments. On gel blots, VαNT1 labelled the ~11 kDa band corresponding to complete αNT but did not label a ~9 kDa breakdown product (Figure 1(B)). N-terminal sequencing of extracted bands showed that both αNT bands start with identical sequences, namely two residues of the linker sequence followed by the N-terminal amino acid residue sequence of α-tubulin. Thus, the ~9 kDa band contains residues 1 to ~80 of the α-tubulin sequence and the epitope for VαNT1 probably lies within residues ~80 to 102 of α-tubulin.

Soluble antibody (0.05 to 0.1 mg/ml in HPLC-grade water) was added to 30 nm diameter gold beads (Amersham), whose pH had been adjusted to 7.2, together with 1% (w/v) bovine serum albumin (BSA) plus 0.05% (w/v) polyethylene glycol (PEG) 20 K. The beads were centrifuged at 8000 ***g*** and 4°C for 15 minutes, and those in the uncompacted upper layer of the pellet were twice washed in storage buffer (20 mM Tris-HCl (pH 8.2), 0.15 M NaCl, 1% BSA, 0.05% PEG 20 K) and recentrifuged. They were kept in storage buffer at 4°C and used within two weeks.

The antibody-coated gold beads were added to various microtubule preparations and observed by electron and light microscopy. Because the antibody tended to precipitate at pH values below 7.0, the pH of microtubule preparations was raised as follows. Microtubules, assembled from 2 mg/ml tubulin in BRB80 buffer (80 mM Pipes (pH 6.8), 1 mM $MgCl_2$, 1 mM EGTA) plus 1 mM GTP and then stabilised with taxol, were pelleted and resuspended in HEMDT buffer (20 mM Hepes (pH 7.2), 1 mM EDTA, 3 mM $MgSO_4$, 1 mM DTT, 5 μM taxol). In electron microscope images, the microtubules appeared unchanged by the pH rise and they performed at least as well in motility assays (see below) as did the controls in BRB80. For labelling with beads, 0.1 mg/ml microtubules in HEMDT plus 0.1 to 0.5% BSA were combined with coated beads in storage buffer (~5:1). The mixture was incubated at room temperature for 20 to 30 minutes; then, after a further 5 to 10 times dilution in HEMDT, a 2 μl drop of the mixture was spread on each carbon-coated electron microscope grid and washed three to four times in HEMDT, before negative staining with 1% (w/v) UAc.

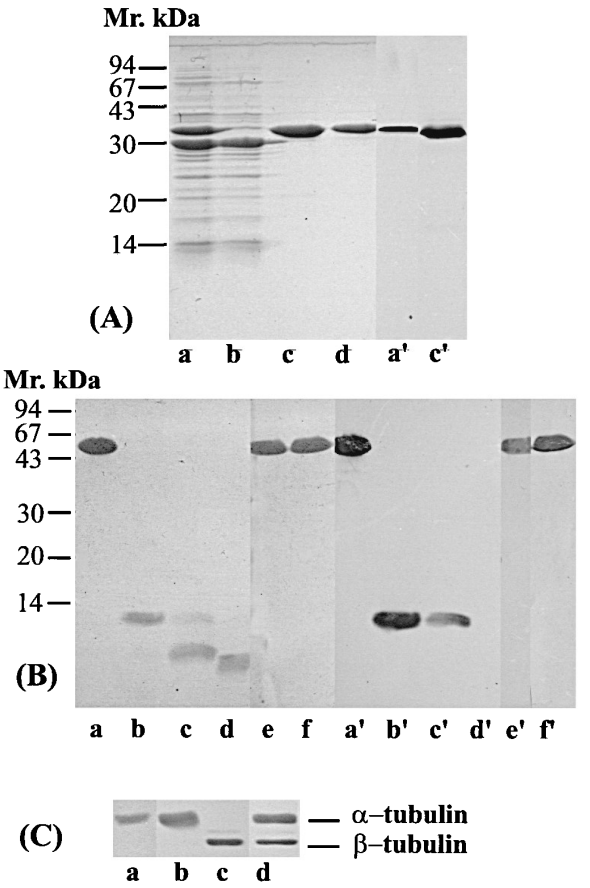

Figure 1. (A), Expression and purification of VαNTl, a soluble anti-αNT scFv antibody. a to d, Page Blue-stained 20% (w/v) polyacrylamide gel; a' and c', Western blot showing scFv fragments detected using mouse monoclonal 9E10 (specific for the *myc* tag) followed by alkaline phosphatase-labelled anti-mouse IgG (Sigma). a and a', IPTG-induced cell extract; b, flow-through fraction from Ni-NTA column; c and c', first eluted fraction; d, second eluted fraction. (B), Western blots of pig brain tubulin and bacterially expressed proteins labelled with VαNTl and the same secondary antibodies as in (A); after separation from the MBP, αNT and βNT each produce a lower molecular mass additional band. a to f, Ponceau Red-stained blot membrane carrying brain tubulin (a, e and f), two different fast protein liquid chromatography (FPLC) fractions of the ~11 kDa αNT and its ~9 kDa product (b and c) and one of ~11.5 kDa βNT and its ~7 kDa product (d). a' to f', Blots of brain tubulin (a', e' and f'), αNT (b' and c') and βNT (d') incubated with VαNTl antibody (a' to d') or with control anti-α- and β-tubulin (mouse monoclonal antibodies, clones DM1A (Sigma) and N357 (Amersham); e' and f'). (C), Western blots of native tubulin after carboxymethylation (Luduena *et al*., 1977), incubated with VαNTl (a), control anti-α (b and d) and control anti-β (c and d).

VαNT1-coated gold beads labelled many repolymerised brain microtubules at one end (Figure 2). A set of images in which there were 82 microtubules with visible ends showed 34 microtubules labelled in this way. When the grids were more widely scanned, to inspect an estimated number of 1000 microtubules, no examples with gold attached to

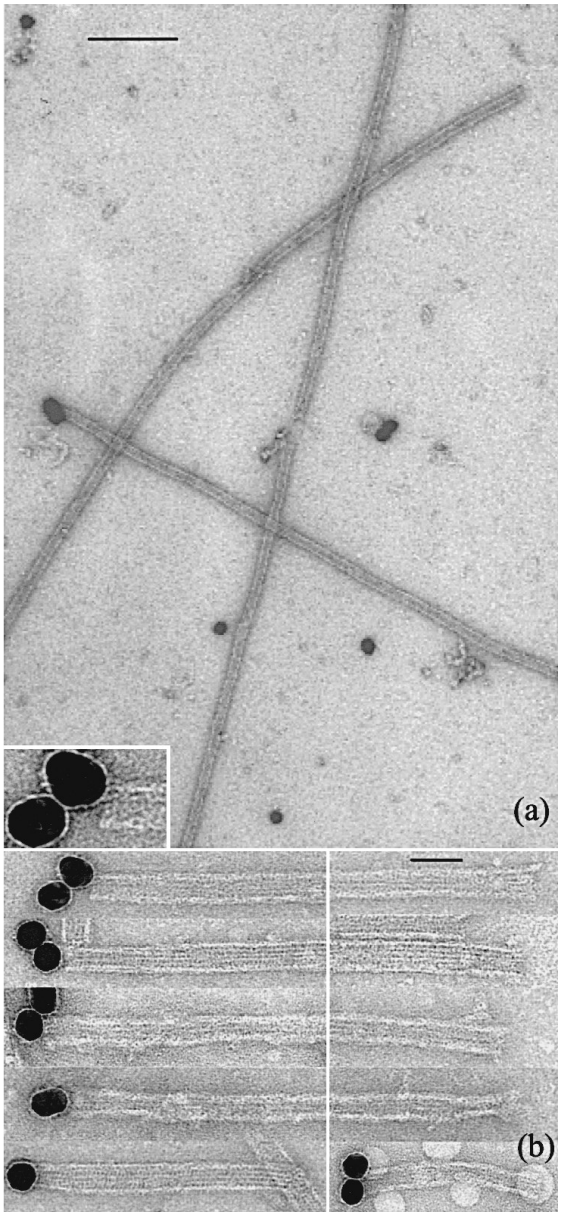

Figure 2. Electron micrographs of negatively stained, reassembled brain microtubules mixed with VαNT1-coated beads. (a), Typical area of carbon film, showing the low background concentration of beads. (b), Individual microtubules at higher magnification. Beads in contact with microtubules are mostly at their ends but never at both ends of the same microtubule. In the close-up view of a contact (inset), a bead appears to bind directly to the ends of three protofilaments. Scale bars represent 200 nm for (a) and 50 nm for (b) except for the inset, where the magnification is 2.25 times greater.

both ends were found and few gold particles appeared to be stuck to the sides of the microtubules. At high magnification, beads on the ends of microtubules often appeared to bind directly to the ends of several adjacent tubulin protofilaments (e.g. Figure 2(b), inset). Beads coated with a control scFv antibody, or with BSA only, showed no tendency to bind anywhere on brain microtubules.

A 1:1 mixture of unmodified and *N*-ethyl maleimide (MalNEt) modified brain tubulin was added to sea urchin flagellar axonemes in order to form hybrid microtubules with an identifiable polarity (Huitorel & Kirschner, 1988; Hyman *et al*., 1991). Brain tubulin extensions were stabilised with 10 μM taxol in BRB80 as described (Hirose *et al*., 1995a). The grid was washed, first in BRB80 plus 5 μM taxol, then in HEMDT, then twice in HEMDT plus 1% BSA, before being incubated for ten to 20 minutes at room temperature, with 1.5 μl of coated beads in HEMDT with 1% BSA. Finally it was washed four times with HEMDT and negatively stained. The extensions that were seen growing from the plus ends of flagellar doublet microtubules were always unlabelled (at least 100 axonemes were inspected, with more than 500 brain microtubules growing from their plus ends) but VαNT1-coated gold particles were bound to the minus ends and, less densely, to the sides of axonemes (Figure 3 shows typical examples). Possibly the labelling of the axoneme sides is due to excess tubulin dimers stuck on to the surface. Alternatively, there may be some cross-reactivity with a flagellar antigen; in this case, the images at least show that brain microtubules grown from the plus ends of axonemes are completely unlabelled; thus the reassembled brain microtubules in Figure 2 must be labelled on their minus ends.

Axonemes can be extended at both ends using unmodified brain tubulin but the two ends can only be distinguished for certain by differential growth if the incubation is carried out *in situ* on the electron microscope grid (Hirose *et al*., 1995a). Under these conditions, labelling of microtubule ends with beads was observed very infrequently, presumably because the microtubule ends were too close to the carbon support film; however, all those beads which did bind, did so to the end with shorter brain tubulin extensions. Beads coated with control scFv, or just BSA, attached occasionally to the sides of axonemes (data not shown) but did not show any preference for the ends of microtubules.

Antibody-coated beads were clearly visible by differential interference contrast (DIC)-light microscopy and were distinguishable as swellings on the ends of microtubules (Figure 4). Taxol-stabilised brain microtubules mixed with antibody-coated beads in HEMDT plus 0.5% BSA plus 1 mM ATP were added to a microscope slide chamber that had been incubated with bacterially expressed motor protein and blocked with 1% BSA plus 0.005% cytochrome *c* in HEMDT. The microtubule motor we used was a dimer consisting of residues 12 to 437 of the Eg5K2 gene (Sawin & Mitchison, 1995), with glutathione *S*-transferase (GST) fused to the C terminus (Lockhart & Cross, 1996). More than 95% of unlabelled microtubules (e.g. A in Figure 4) were seen to slide over the motor-coated coverslips and continued in motion for at least 40 minutes in each experiment. In contrast, microtubules with beads on their ends tended to remain stationary, apparently because the beads had stuck to the glass. In

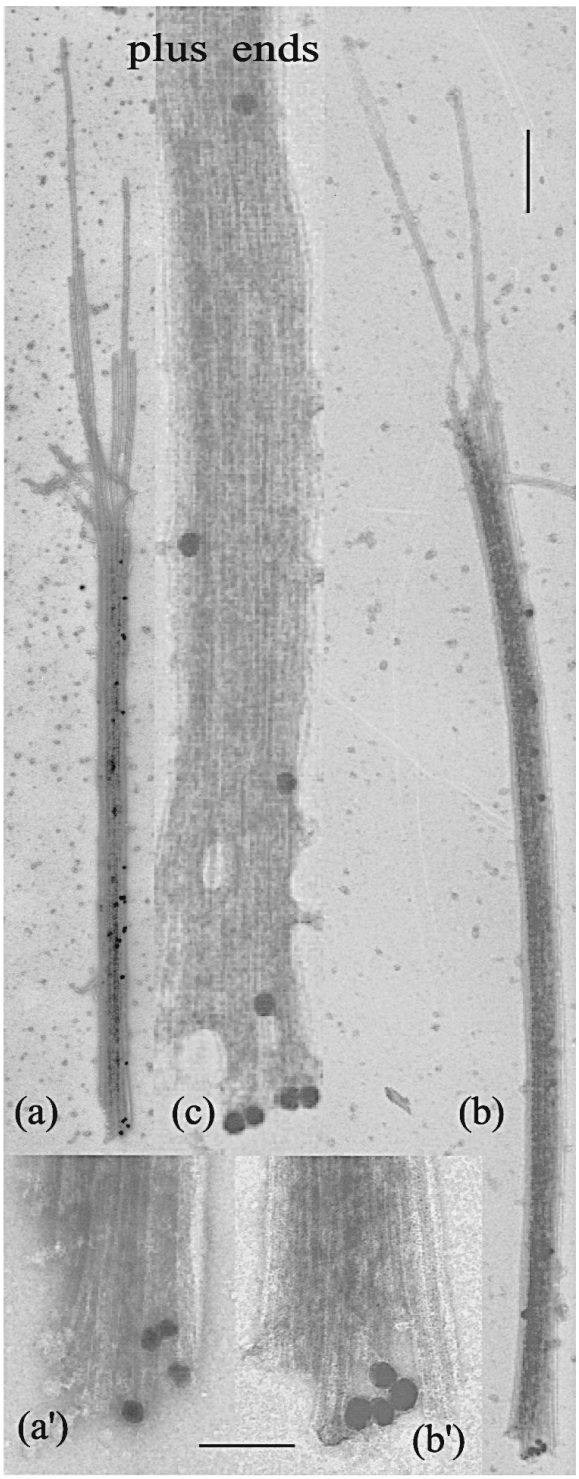

Figure 3. Electron micrographs of sea urchin axonemes extended at their plus ends with a 1:1 mixture of native tubulin and MalNEt-treated tubulin (Huitorel & Kirschner, 1988; Hyman *et al*., 1991; Hirose *et al*., 1995a). VαNT1-coated beads bind to the minus ends of axonemes and, to a lesser extent, to their sides. But no beads were seen binding to the plus ends of brain tubulin extensions ((a) and (b)). (a') and (b') are higher magnification views of the minus ends of (a) and (b). Scale bars represent; top 500 nm for (a) and 350 nm for (b), bottom, 100 nm for (c) and (a') and 70 nm for (b').

experiments where the concentration of blocking agents was too low, no beads were seen moving, but with the concentrations quoted, a few microtubules were seen coiling and writhing behind beads and a few microtubules (11 examples in three experiments) slid over the surface with beads attached to their leading ends. In three of these cases the beads were followed for long distances (including the example in Figure 4), before either becoming stuck or disappearing into the medium as the microtubule detached from the surface. Only one bead seen moving appeared to be attached to the side of a microtubule. In two additional cases, beads moved bidirectionally, apparently because more than one microtubule was attached to the same bead. BSA-coated beads were also observed by light microscopy but only two were ever seen moving, both apparently attached to microtubule sides.

Thus, both light and electron microscopy show that the antibody VαNT1 specifically recognises only the minus ends of microtubules and not the plus ends. Since electron microscopy experiments also indicate that the antibody fails to bind to the inside surface or to either edge of opened-out protofilament sheets, we conclude that the epitope of VαNT1 is exposed at the minus end of each protofilament and that α-tubulin is the ultimate subunit at the minus ends of microtubules. Our evidence complements that of Mitchison (1993), who showed that the exchangeable GTP-binding site of β-tubulin is localised to the plus ends of microtubules. Together, the two results provide a robust assignment of microtubule polarity.

A combination of the available data allows a tentative identification of the tubulin dimer within the recent 6.5 Å map of tubulin protofilaments. The localisation in this map of the site at which taxol binds to β-tubulin (Nogales *et al*., 1995) means that β-tubulin can be identified in other maps showing tubulin dimers, such as those published by Hoenger *et al*. (1995). The alignment of their images of undecorated tubulin sheets with those of sheets decorated with the motor domain of non claret disjunctional (*ncd*) protein suggests that the main mass of *ncd* is associated with β-tubulin, although the whole molecule straddles both an α- and a β-tubulin monomer. Finally, images of the very ends of decorated sheets (Song & Mandelkow, 1995; Hirose *et al*., 1995a,b) indicate that the pairs of tubulin monomers straddled by motor molecules correspond to individual tubulin dimer subunits. The maps of Hoenger *et al*. (1995) thus need reorienting by 180° to put β-tubulin at the plus end of the tubulin dimer. This would bring them into agreement with our determination of the polarity of kinesin- and *ncd*-decorated microtubules and protofilament sheets (Hirose *et al*., 1995a,b). The 6.5 Å map of Nogales *et al*. (1995) is probably already oriented plus end upwards, but the pair of monomers labelled may not correspond to a tubulin dimer subunit.

The conclusion that the minus ends of microtubules end in α-tubulin also fits well with the

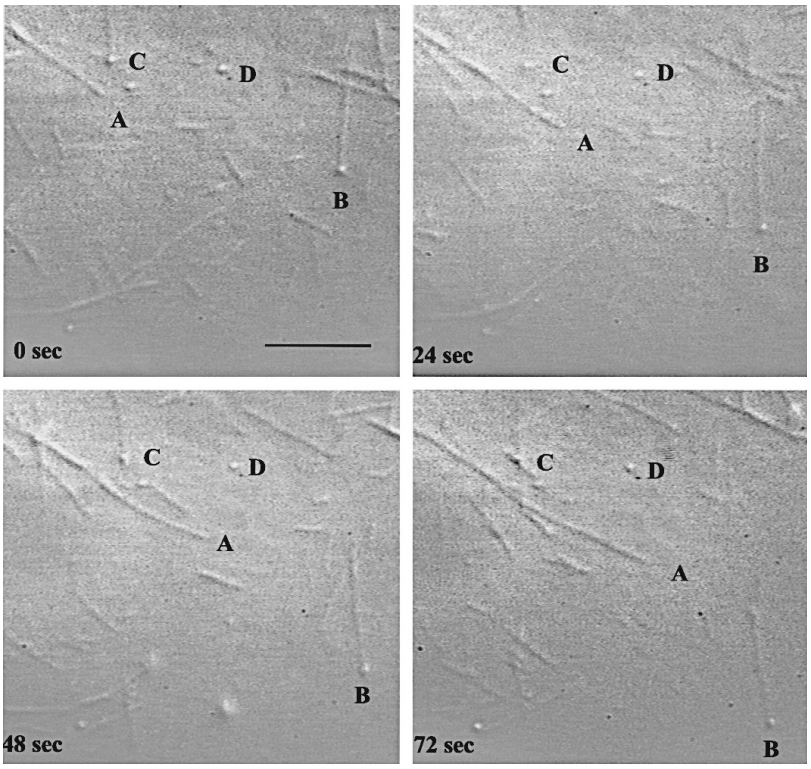

Figure 4. Time-lapse series showing microtubules and VαNT1-coated gold beads by video-enhanced DIC optics. Microtubules were sliding over a coverslip surface which was coated with expressed Eg5-GST kinesin-like motor molecules (Lockhart & Cross, 1996). A indicates the leading end (i.e. the minus end) of an unlabelled microtubule. B, C and D are gold beads; B moved at the leading end of a microtubule; C seems to be on the end of a microtubule that has become stuck to the glass surface, in spite of the presence of 1% BSA as a blocking agent; D is not associated with a microtubule. Both A and B moved at similar speeds ($\sim 0.08\ \mu m/s$). Sliding speeds were measured using the RETRAC tracking software, obtained from the Institute of Applied Biology, University of York (e-mail: NJCl @york.ac.uk). Scale bar represents 5 μm.

prediction (Mandelkow & Mandelkow, 1989) that the lower rate of dynamic instability observed at the minus end *in vitro* might be due to the stabilising influence of terminal subunits whose bound GTP is never hydrolysed. Gamma tubulin is known to be bound to the minus ends of microtubules assembled *in vivo*. Since a known mutation in β-tubulin has been rescued by a further mutation in γ-tubulin (Oakley & Oakley, 1989), it is likely that there are direct bonds between γ- and β-tubulin but these could be lateral interactions which would occur in either of the two possible microtubule subunit lattices (Amos, 1995).

Our results also confirm that high-affinity monospecific antibody fragments can be obtained from large and diverse phage display libraries (Griffiths *et al*., 1994; Vaughan *et al*., 1996). The strategy of expressing specific protein domains in bacteria and rapidly selecting single clones coding for recombinant antibodies should be useful for investigating a large range of questions about macromolecular structures.

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