Surface-decoration of Microtubules by Human Tau

Rachel A. Santarella¹, Georgios Skiniotis¹, Kenneth N. Goldie¹, Peter Tittmann², Heinz Gross², Eva-Maria Mandelkow³, E. Mandelkow³, and Andreas Hoenger¹*

¹European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117, Heidelberg, Germany
²Elektronenmikroskopiezentrum der ETH Zuerich, c/o Institut fuer Angewandte Physik, ETH Hoengerberg, 8093, Zuerich Switzerland
³Max Planck Unit for Structural Molecular Biology, c/o DESY-Hamburg, Notkestrasse 85, D-22607 Hamburg, Germany

Tau is a neuronal, microtubule-associated protein that stabilizes microtubules and promotes neurite outgrowth. Tau is largely unfolded in solution and presumably forms mostly random coil. Because of its hydrophilic nature and flexible structure, tau complexed to microtubules is largely invisible by standard electron microscopy methods. We applied a combination of high-resolution metal-shadowing and cryo-electron microscopy to study the interactions between tau and microtubules. We used recombinant tau variants with different domain compositions, (1) full length tau, (2) the repeat domain that mediates microtubule binding (K19), and (3) two GFP-tau fusion proteins that contain a globular marker (GFP) attached to full-length tau at either end. All of these constructs bind exclusively to the outside of microtubules. Most of the tau-related mass appears randomly distributed, creating a “halo” of low-density mass spread across the microtubule surface. Only a small fraction of tau creates a periodic signal at an 8 nm interval, centered on β-tubulin subunits. Our data suggest that tau retains most of its disordered structure even when bound to the microtubule surface. Hence, it binds along, as well as across protofilaments. Nevertheless, even minute concentrations of tau have a strong stabilizing effect and effectively scavenge unpolymerized tubulin.

Keywords: tau; microtubule-associated proteins; kinesin; surface metal-shadowing; cryo-electron microscopy

Introduction

Non-motor microtubule-associated proteins (MAPs) such as tau interact with microtubules (MTs) and tightly regulate their stability.¹ MTs consist of polymers of αβ-tubulin dimers that are arranged head-to-tail, forming polar protofilaments. The structures of tubulin and MTs have been solved by electron crystallography.² The subunits are mostly globular except for the highly acidic C-terminal tail, which is disordered. This tail, as well as the C-terminal helices (H11 and H12), dominate the outer surface of MTs. It is likely that they interact with a diverse range of MT-interacting proteins, including motor proteins and MAPs, but the details of these interactions are currently unknown at high resolution. This limits our understanding of why motors move along MTs, how MAPs stabilize them, and how different proteins compete or cooperate for binding sites on MTs.

Tau proteins, a family of six isoforms generated by alternative splicing in the human central nervous system, are prominent in neurons and promote the outgrowth of axons. Tau may also aggregate into pathologically relevant “paired helical filaments” (PHFs) that are hallmarks of Alzheimer’s disease and other dementias.³ This behavior is counterintuitive because tau is one of the most soluble proteins known, due to its hydrophilic amino acid composition.⁴ The malfunction of tau in neurons appears to be intimately related to its MT interactions. Affected neurons loose their MTs (resulting in an interruption of axonal transport), because tau protein in these cells no longer binds to them. Moreover, the repeat domain of tau is essential for MT binding and for PHF aggregation. Mutations of tau causing frontotemporal dementias...
dementias (FTDP-17) are concentrated in the repeat domain as well. Thus, it is important to understand the relationship between tau and MTs, both from a cell biological and a medical perspective.

Tau is a “natively unfolded” protein that does not have a well-defined shape, as judged by structural, spectroscopic, and biochemical evidence. It can be subdivided into two major domains, the N-terminal “projection domain” and the C-terminal “assembly domain” which binds and stabilizes MTs (Figure 1). The assembly domain contains three or four pseudo-repeats of ~31 residues important for MT binding, flanked by proline-rich regions which enhance MT binding. The absence of compact folding has precluded a detailed structural analysis so far. Protein cross-linking studies suggested preferential regions of interaction with tubulin, but this could not be interpreted in terms of a specific pattern of folding on the MT surface. Several studies using different electron microscopy techniques revealed that tau is located on the outside of MTs. The visible parts were due to the projection domains, which are not involved in MT binding, and there was no obvious periodicity in the binding pattern consistent with the MT lattice. This is in strong contrast to the clear visibility of the globular head domains of kinesin motor proteins (review). Recent cryo-EM studies of unstained vitrified specimens, a method commonly considered to preserve structures most faithfully, revealed MT-bound tau only with very low contrast. This required extensive data averaging and image processing, with concomitant uncertainties in the interpretation. Thus, these studies arrived at different conclusions: Al-Bassam et al. proposed that tau runs parallel with the outer protofilament rim, whereas Kar et al. suggested that some portion of tau locates to the inside surface. The repeats of tau represent the core of the MT-binding domain, and in addition, they also constitute the core of Alzheimer paired helical filaments. Therefore, in order to relate tau’s structure in the physiological and pathological polymer and to clarify the existing discrepancies, we chose an electron microscopy approach, which allows us to observe the MT binding properties of MAPs in a very direct way and with high contrast. To accomplish this, we took advantage of the MIDILAB instrumentation installed at the ETH-Zuerich. We applied surface metal shadowing to freeze-dried MTs and tubulin sheets decorated with either full length human tau40 (htau40), a tau derivative (K19) which contains only three MT-binding domains (R1, R3 and R4), and a fusion construct between htau40 and green-fluorescence protein at its N (GFP_N-htau40) or C terminus (GFP_C-htau40) (Figure 1). In contrast to usual projection methods (negative staining, cryo-EM), metal shadowing has the advantage of revealing surface-related features of tau-MT complexes without interference attributed to internal protein densities. The signal/noise ratio is extremely high and allows the interpretation of molecular detail to approximately 2 nm resolution without the need for computational image averaging methods.

Furthermore, using cryo-electron microscopy (cryo-EM) and helical 3-D image reconstruction, we analyzed the major tau-binding site on tubulin and its interaction with kinesin motor heads. We confirmed that kinesin motor heads displace tau only partially from the MT surface, and the two proteins are centered on different binding sites on tubulin. Tau binds to MTs in an unstructured fashion, reminiscent of its disordered structure in solution. Higher amounts of tau or K19 obstruct the surface features on the MT outer surface, but not on the inner one. Even very low amounts of htau40 and K19, invisible by either microscopy techniques used here, show dramatic effects on MT stabilization. We found novel evidence that tau can bind not only along protofilaments, but cross-links them laterally, thereby forming large

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**Figure 1.** Domain structure of tau isoforms and constructs. A, htau40, the longest isoform in the human CNS (441 residues). The N-terminal ~120 residue domain has an acidic character (in red), the remainder is basic (in blue). The core of the MT-binding domain is formed by the four ~31 residue pseudo-repeats R1–R4 (Q244-N368); this domain also forms the core of Alzheimer paired helical filaments. Two 58 residue inserts near the N terminus (I1 and I2) and R2 may be absent due to alternative mRNA splicing. B and C, htau40 with GFP fused to the N terminus or C terminus as a globular marker. D, K19 comprises the repeat domain of fetal tau (R1, R3 and R4).
rafts of intact protofilaments. Using GFP-tau we were able to visualize tau on the MT surface at very low concentrations due to the added globular domain. We observed no evidence that the tau-GFP construct binds to the inner surface of MTs and tubulin sheets. The MT-binding affinity of tau is strongly modulated by the C-terminal ends of tubulin (here referred to as CTTs). Removal of CTTs by subtilisin treatment reveals 3-D maps approaching the state of a non-decorated MT.

Results

Tau stabilizes MTs and forms a fuzzy coat on the outer surface

Figure 1 shows an overview of the constructs used here, and scheme of the tau domain organization. Several tau and tubulin concentrations were initially tested to optimize the visibility of the structures. The decoration experiments shown here for metal-shadowing and cryo-electron microscopy.
microscopy were carried out at a constant MT concentration of 4.5 μM. For the metal-shadowing experiments, htau40 was added in concentrations of either 0.9 μM (Figure 2B) or at 10.0 μM (Figure 2C). Even at sub-stoichiometric concentrations the presence of tau had a strong stabilizing effect on MTs, as seen by the disappearance of MT breakdown products in the background (compare Figure 2A with B). This is consistent with the tight binding of htau40 (KD below 1 μM), which lowers the critical concentration of MT assembly to the μM range.

The characteristic surface features of MTs are clearly visible in Figure 2A and B. They are dominated by the longitudinal protofilaments spaced ~5 nm apart, and the 4 nm axial periodicity of tubulin subunits, better visible on sheets exposing the inner MT surface. In the present context, the important point is that at low density htau40 is practically invisible. There is no discernible structural change on either surface, even though tau’s influence is noticeable by the scavenging of the MT breakdown products. This means that sub-stoichiometric amounts of tau do not alter the apparent shape of tubulin but still render MTs much more stable. Surprisingly, even at a ~2-fold excess of tau (Figure 2C), the inner surfaces still remain “clean”, whereas the outer surfaces lose features (like a landscape after snowfall). These observations of tau are clearly distinct from kinesin. Even at low concentrations, kinesin stands out well on the outer surface, while the inner surface remains clean in both cases (see also Figure 7).

Similar observations were made with the repeat construct K19 (Figure 3). However, K19 has a lower affinity to MTs (>10 μM), and therefore,

Figure 3. MTs decorated with K19 after freeze-drying and unidirectional shadowing with Ta/W. MTs at 4.5 μM were decorated with the tau construct K19 (comprising the repeat domain of fetal human tau with the three repeats R1, R3 and R4) at 1.8 μM (A), 4.5 μM (B), and at 18.0 μM (C). While K19 decoration is virtually invisible at the lowest concentration (A), increasing amounts of K19 cause the MT surface features to disappear, similar to full length tau, but the efficiency is weaker, and the blurring of surface features requires higher concentrations because K19 binds less tightly (compare Figure 2C). As in Figure 1, decoration by K19 takes place exclusively on the outer surface of MTs. Stabilization of MTs by K19 is already noticeable at low density. Red arrows indicate the direction of shadowing.
we had to work at higher molar ratios to visualize surface effects (1.8 μM, 4.5 μM, and 18.0 μM in Figure 3A–C, respectively). Like htau40, K19 had a profound effect on MT dynamics. As seen with htau40, K19 at low concentrations could not be visualized, neither on the surface (Figure 3A) nor on helical 3-D reconstructions (see Figure 6B1–B3). When using higher concentrations of K19, again, the outer surface of MT loses its features, whereas inner surfaces remained unchanged (Figure 3).

The above data demonstrate that tau retains a featureless state upon attachment to the MT surface, but this still does not answer the question of whether the attachment is periodic or distributed in a random fashion. To address this point we attached a globular marker, GFP, to either the N terminus (GFP_N-htau40), or the C terminus of full-length tau (GFP_C-htau40) and decorated MTs (Figure 1). As before, this fusion protein attached only on the outside surface, and not the inside (Figure 4). The GFP domains became visible as globular dots. Significantly, no periodic pattern could be discerned, arguing that the distribution of tau, or at least of its N-terminal domain, does not strictly follow that of the underlying tubulin.

Figure 4. Shadow graphs of tubulin sheets decorated with a GFP-tau construct in the presence of taxol (A) and in the absence of taxol (B and C). In A and B, the GFP-tag is at the N-terminal end of htau40. In C the tag is at the C-terminal end. The GFP-tags produce directly visible, globular densities on the surface of the MT, indicating the presence of individual tau molecules. The decoration is clearly present on outer MT surfaces, but in either case (N- or C-terminal tag) inner surfaces stay free from decoration, both with and without taxol. Red arrows indicate the direction of shadowing.
molecules. The observed density of GFP-tau was significantly below a 1:1 stoichiometry with tubulin dimers, suggesting that one tau molecule may saturate more than one tubulin binding site (Figure 4). Figure 4 also demonstrates that the properties of tau binding do not depend on whether MTs are stabilized by taxol or not, since the distributions of tau are the same in both cases. This observation was confirmed for all tau variants tested.

K19 and tau stabilize the lateral contacts in MT sheets and widely spaced protofilament rafts

The presence of tau or K19 appears to stabilize lateral contacts in tubulin sheets (Figure 5A), which occur much more frequently than in the absence of tau. The lateral spacing of protofilaments in these sheets is 5 nm, typical of opened-up microtubule walls. In addition, we observe large protofilament rafts (Figure 5B), which display areas with regular lateral protofilament spacing of up to 8 nm (see inset and diffraction in Figure 5B). This lateral spacing is too large to account for direct lateral contacts between protofilaments. Hence, this suggests that tau molecules (or possibly dimers or oligomers) maintain these rafts by binding laterally across protofilaments.

Cryo-EM and helical image reconstructions reveal the major tau and kinesin binding sites on opposite sides of the protofilament ridge

We used cryo-EM and 3-D helical reconstruction methods (Figure 6) to define the binding site of tau to MTs with respect to the kinesin-binding site and the high-resolution structure of the αβ-tubulin dimer (Figure 9, below). Figure 6 shows helical reconstructions of 15 protofilament MTs (Figure 6A1–3), MTs decorated with K19 (Figure 6B1–3), htau40 (Figure 6C1–3) and co-decorated with htau40 and kinesin motor domains (Figure 6D1–3). Density differences compared with native MTs were investigated using Student’s t-test. The presence of 18 μM K19 was invisible in cryo-micrographs (Figure 6B1) and in 3-D reconstructions (Figure 6B2–3). The reconstructions with htau40 were made with a tau concentration of 10.0 μM. Cryo-micrographs reveal the presence of tau as a fuzzy density located around MTs and obstructing the image slightly (Figure 6C1). In 3-D reconstructions we found the same sharpening of the outer protofilament rim as described by Al-Bassam et al., indicating that a fraction of tau lies at the crest of protofilaments (Figure 6C2). This outer rim slewed with the opposite hand of kinesin motor domains (compare Figure 6C2 with D2 and C3 with D3). Difference mapping using statistical t-test analysis revealed that the major htau40-binding site repeats axially every 8 nm, commensurate with the spacing of αβ-tubulin dimers. The red volume shown in Figure 6C2 represents the t-test difference to native MTs at a significance level of 99.5% (note that this does not represent the shape and volume of tau in a literal sense, but rather the location of highest probability where tau interacts with tubulin protofilaments). Kinesin-decorated MTs pre-saturated with htau40 revealed the well-known features of kinesin–MT complexes, but showed no detectable traces of tau after averaging (Figure 7D2 and D3). Cross-correlation of the 3-D maps to each other and superposition of the kinesin-decorated map with the tau-decorated map and the t-test difference map from Figure 6C2 (red volumes) revealed the position of the major tau-binding site on α-tubulin and on the opposite side of the outer protofilament rim from where kinesin sits (Figure 6C2–3 and D2–3; Figure 9, below).

Kinesin dominates over tau when decorating the MT surface

Furthermore, we investigated the interference between tau and kinesin by co-decorating MTs with tau and kinesin motor domains. Figure 7A shows an example of MTs pre-saturated with htau40 (10.0 μM, generating images similar to Figure 2D) followed by addition of 10 μM monomeric Neurospora kinesin motor domains (nK355). Interestingly, tubulin sheets and MTs appear fully decorated with motor domains despite the pre-saturation of MTs with 10 μM htau40. The kinesin decoration pattern and the surface features of co-decorated sheets are essentially identical with that of tubulin sheets decorated exclusively with motor domains; in both cases a strong axial 8 nm repeat is observed, corresponding to a single motor complexed to one αβ-tubulin dimer. Similar to the assemblies found with K19, large extended sheets appeared, indicating an increased lateral stability between protofilaments due to the presence of tau. These features are observed both with surface-shadowed and ice-embedded samples (Figure 7B) and they are slightly different from MT–kinesin complexes, which often reveal long extended single protofilaments, or very small bundles of protofilaments, e.g. as shown recently. MT-pelleting assays analyzed by SDS-PAGE of htau40–MT, kinesin–MT, and htau40–kinesin–MT complexes (Figure 7C, +subt. tau–MT, +kine–MT, tau–kin–MT) revealed that htau40 and kinesin are both present on the MT surface despite the full saturation with kinesin motor heads (Figure 7A and B). In the presence of kinesin htau40 seems somewhat reduced on native MTs, and does not bind at all on subtilisin-treated MTs, which removes the CTTs (+subt. tau–kin–MT). In contrast, kinesin binds equally strong under all conditions (Figure 7C).

Removal of CTTs affects the affinity of tau to its major MT-binding site on α-tubulin

Subtilisin cleaves ~10 amino acid residues from
the α- and ~18 amino acid residues from β-tubulin C termini. By choosing appropriate conditions of limited proteolysis one can cleave either the CTT only of β-tubulin, or the CTTs of both β and α-tubulin. CTTs are involved in the binding of tau and other MAPs, and regulate the binding states of kinesin. Here we calculated 3-D maps of tau–MT complexes using MTs at different stages of polymerization.
during subtilisin treatment (Figure 8). Figure 8A–C shows SDS-PAGE under highly separable conditions (revealing separate bands for α and β-tubulin) of htau40 complexed to native MTs (Figure 8A), to partially digested MTs (leaving most of α-tubulin intact; Figure 8B), and to fully digested MTs (Figure 8C). When only β-tubulin CTTs are cleaved off (Figure 8B) there is a notable decrease in binding affinity of tau, but some tau remains on the microtubule surface (Figure 8B). Complete digestion, eliminating the CTTs of both, α and β-tubulin, however, reduced the amounts of
tau below a detectable level (Figure 8C). Figure 8D–G shows cross-sections through the reconstructed 3-D maps of htau40–MT complexes at the position of the strongest difference signal for the complexed tau molecule (red contours in Figure 8D and E). Figure 8E shows an htau40–MT complex on native MTs corresponding to the condition shown in Figure 8A. Figure 8F shows a 3-D map of tau complexed to partially digested MTs (corresponding to Figure 8B). The fuzzy coat of tau is already visibly reduced (Figure 8B, first blue contour line above background). Figure 8G shows a cross-section obtained on fully digested MTs (corresponding to Figure 8C). The loss of tau is clearly visible in both, SDS-PAGE and 3-D maps.

Discussion

Tau and related MAPs are enigmatic molecules from several perspectives. They are highly soluble proteins and have a largely hydrophilic composition. As a result, they have a “natively unfolded” structure in solution; none of the domains of tau investigated so far show the compact folding of typical protein domains under physiological conditions. This class of proteins is receiving increasing attention because of their versatile functions. One of the questions addressed here was whether tau would retain its unstructured character even when binding to MTs, and indeed this is the case, at least for most of its part. The sequence of tau has a mostly basic character, except the ~100 residue N-terminal domain, which is acidic (see Figure 1). Since the C-terminal domain of tubulin has a pronounced acidic character, this means that the basic part of tau has a general tendency to bind to tubulin, whereas the acidic part does not. This leads to the distinction between the C-terminal assembly domain of tau, which binds...
and stabilizes MTs, and the N-terminal projection domain which does not bind. However, the explanation of tau–MT binding based merely on charge is oversimplified. This is because some of the basic regions, such as the repeat domain, bind to MTs productively (i.e. they bind and stabilize MTs), while others, such as the domains flanking the repeats, bind unproductively (binding without stabilization). Tight binding combined with efficient stabilization requires both, the repeat domain and the flanking regions. This indicates that there must be specific interactions as well, and indeed, cross-linking and other studies reveal proximity of the repeat-domain of tau and the C-terminal domain of α- and β-tubulin comprising mostly helix H12 and the disordered acidic C-terminal tail. This duality of specific and generalized binding is also revealed by binding studies. The reported apparent dissociation constants vary by an order or magnitude between ~0.1 μM and 1 μM, but a detailed analysis reveals biphasic binding of tau to MTs with a tight-binding initial phase and loose binding in the second phase. Significantly, the second phase is not saturable so that tau can pile up on the MT surface. In this regard tau differs from kinesin motor domains, which show single-phase and saturable binding behavior because the binding interface is well defined. How can this biochemical information be translated into structure? So far, tau has resisted all attempts of a high-resolution structure analysis, due to its disordered nature, and it has been difficult to visualize by electron microscopy. Tau in solution can be imaged only by very low angle shadowing after glycerol spraying, where it appears as a highly elongated molecule. MT-bound tau has been imaged by conventional metal shadowing techniques, thin sectioning, and metal-shadowing after quick-freezing and deep etching, where it reveals protein stubs on the surface which correspond roughly to the N-terminal projection domain. However, the technique of cryo-EM, which is best suited for preserving structures, does not reveal individual tau molecules to the unaided eye (Figure 6C1; in contrast to the binding of motor domains which is easily detectable; Figure 7B). The interpretation requires image processing and averaging over (seemingly) identical image elements, which suffers from potential pitfalls (e.g. assumptions about symmetry, about degree of occupancy, and others).
Because of these difficulties, we decided to also address the issue of tau–MT binding by a method that provides high contrast and resolution and is directly interpretable. This is achieved by a unique instrument, which allows high-resolution, unidirectional metal shadowing with low background (MIDILAB, ETH-Zuerich). We wanted to answer the following questions. (a) Where is the binding interface between tau and MTs? (b) Does tau follow a periodic binding pattern? (c) Does tau retain its largely unfolded, disordered structure upon binding to MTs? The last question is of particular interest because the tau–tubulin binding is a paradigm of the interaction between an unfolded protein versus a globular folded protein. Moreover, the tau–tubulin interaction bears analogies with the tau–tau interactions in Alzheimer PHFs so that the tau–tubulin paradigm should shed light on pathological aggregation as well. Our results can be interpreted as follows.

1. Tau or its derivatives bind exclusively to the outside surface of MTs, the inside remains clear, even when it is readily accessible (Figures 2–4). Tau–GFP constructs with GFP at either end revealed the globular GFP domains only on the surface, no matter whether MTs were preformed prior to decoration, or polymerized with GFP–tau present. This conclusion is possible because the surface layer of tau can be easily visualized, and because the two surfaces are clearly distinguishable by their characteristic features. This is consistent with most previous studies, but contradicts the recent study by Kar et al. In our hands, the localization of tau does not depend on whether MTs are stabilized by taxol or not, so that taxol cannot explain the divergent published results (see Figure 4).

2. At lower occupancies, tau is essentially invisible on the surface, even when its presence is already obvious by highly effective stabilization of MTs. This means that the initial layer of bound tau either adapts smoothly to the MT surface, without creating bumps detectable by shadowing. This induces a cooperative structural change to adjacent tubulin dimers, or acts predominantly on MT ends. This argument strongly that tau remains mostly unfolded and retains its very low-contrast shape even when bound to MTs. It is reminiscent of the shape of single tau molecule in images by the observed density (Figure 6D1–3). In contrast, the periodic part of tau that follows the protofilament symmetry appears as a slight extension of the protofilament ridges. It accounts for approximately 5–10% of the tubulin volume (Figure 6C2 and 8, volumes in red). Accordingly only a small fraction of tau follows the protofilament ridges (in agreement with Al-Bassam et al.).

3. At higher densities the presence of tau creates a fuzzy coat, obliterating or "snowing in" the surface features of MTs. This fuzzy tau density is also faintly visible on cryo-micrographs (Figure 6C1). Remarkably this occurs in a largely random fashion. In other words, the major part of tau does not adopt fixed and repetitive positions relative to the tubulin lattice. This is in pronounced contrast to the decoration by kinesin (Figure 7), which faithfully highlights the underlying lattice of tubulin heterodimers. This is seemingly in contradiction to the biochemical evidence suggesting specific interaction sites. However, it is simply explained by assuming that only a small fraction is involved in specific interactions with MTs, the rest being mostly free to adopt a variety of orientations and possibly cross-linking protofilaments laterally (see Figure 5).

4. Tau appears to bind not only along protofilaments, but also across. This is suggested by the large loosely coupled rafts of protofilaments (Figure 5B), and the increased lateral stability observed on tubulin sheets (Figures 5A and 7). The rafts show areas with a regular lateral spacing of protofilaments in the order of ~8 nm. This distance would be too large for regular lateral tubulin contacts (5 nm). Hence, the bridging interactions are presumably mediated by the repeat domains of tau.

5. Cryo-EM and helical averaging revealed 3-D reconstructions of the tau–MT complex, but at the expense of losing non-periodic parts of the structure. The additional features emerging after image processing concern the periodic components. As a reference, kinesin binds at a ratio of one head per tubulin heterodimer, which is fully accounted for by the observed density (Figure 6D1–3). In contrast, the periodic part of tau that follows the protofilament symmetry appears as a slight extension of the protofilament ridges. It accounts for approximately 5–10% of the tubulin volume (Figure 6C2 and 8, volumes in red). Accordingly only a small fraction of tau follows the protofilament ridges (in agreement with Al-Bassam et al.), while the majority of tau remains highly flexible and could fill up the gaps between protofilaments and bind across protofilaments (Figures 5 and 6). The disorder becomes even more apparent when considering axial periodicities: the "major binding site" of tau is revealed by t-test analysis and represented here in a volume including a 99.5% probability for the presence of tau-related mass (Figures 6C2, 8D, E and 9). This argues that specific binding to tubulin is restricted to only a small fraction of tau. The finding that tau binds more visibly to α-tubulin than to β-tubulin is also supported by our results from limited subtilisin proteolysis. In conditions where the CTTs are mostly removed from β-tubulin but retained on α-tubulin, the affinity of tau is reduced, but not eliminated (Figure 8B and F). However, in conditions of more extended proteolysis where both CTTs are removed completely, the binding of tau falls below a detectable limit (Figure 8C and G). Hence, the CTT of α-tubulin is clearly involved in tau binding.

If we combine this with biochemical data on tau–MT binding and structural data on tubulin we can conclude that the major tau binding site (presumably comprising the repeat domain of tau, notably R1/R2) is located next to the C-terminal helix H12 (residues 415–435) and the acidic tail on α-tubulin (beyond residue 435). This does not exclude further regions of interaction along protofilaments, but these are not well enough defined
in terms of the tau or tubulin sequence. However, both shadowing and cryo-EM data argue that tau does not undergo a notable condensation upon binding to MTs, as seen from the distributed fuzzy density in cryo-EM images (Figure 6C1). Extended compact domains would become visible by shadowing (compare the dot-like visibility of GFP domains, equivalent to about half of the tau mass, in Figure 4). The absence of compaction upon binding is typical of other unstructured proteins when they bind to their targets (review). This allows them to reach across and connect different adjacent domains or protein subunits. In the case of MTs, this leads to the stabilization of the polymeric state. The structural methods used here reveal only “still” pictures of static disorder, but they are complemented well by NMR observations on MT-bound tau, which reveal a high degree of dynamic disorder. Thus, we may depict tau as a highly mobile molecule on the MT surface, with an immobilized portion centered on α-tubulin.

Given that tau and motor proteins bind to the outside of MTs, an interesting question concerns their interference or competition. The kinesin binding site (mostly on β-tubulin) and the major tau site (here identified to be on α-tubulin) are separated and point to different directions from the protofilament ridge (Figure 6). As demonstrated here, both by the shadowing and the cryo-EM data, kinesin completely decorates the MT surface despite the presence of tau. SDS-PAGE revealed that both molecules bind MTs simultaneously, but we could no longer find a statistically significant tau signal on co-decorated MTs, probably due to increased disorder or partial absence. Therefore, kinesin seems to partially displace tau and may impose more flexibility but it does not displace tau from the MT surface entirely. In view of this, it is remarkable that tau is able to profoundly interfere with kinesin-based transport in vitro and in cells, to the extent that elevation of tau in neurons causes the inhibition of organelle transport into axons, imposing a retrograde bias on axonal transport. Given the stronger equilibrium binding of kinesin, this is likely due to either a kinetic effect, and/or an alteration of the kinesin binding properties during an ATP-hydrolysis cycle. Skiniotis et al. have shown recently that kinesin motor domains complexed to subtilisin-treated MTs assume a rigor binding state (nucleotide-free state), under all nucleotide conditions. Accordingly, one could speculate that tau may saturate the CTTs with its basic units, rendering them ineffective for kinesin motor binding and stalling kinesin in a rigor conformation. However, such a model could not explain the full mechanism as it contrasts the findings of Seitz et al. who concluded that tau reduces primarily the attachment rate of kinesin, but not directly its capability to walk. Hence, the “fuzzy coat” of tau seen on the MT surface (Figures 2C and 7C1) would provide a simple explanation of the cellular effects.

Figure 9. High-resolution model of the kinesin–tubulin complex in ribbon representation and the proposed major interaction site of htau40 (red wire frame). A, View from outside the MT cylinder. B, Tangential view from a point between two protofilaments. According to cross-correlation studies with reconstructions obtained from MTs decorated with kinesin, the major tau-binding site locates to α-tubulin, in close proximity to helices 11 and 12. However, the volume indicated in red accounts for less than 5% of the mass of htau40, the remainder is distributed non-periodically. The exposed position of this site, near the outer protofilament ridge and almost between two protofilaments, suggests that tau may also bind across protofilaments (see also Figures 5 and 6B).
Materials and Methods

Proteins

**Tau**

Recombinant human tau isoforms and constructs were expressed in *Escherichia coli* as described. The numbering of the amino acids follows that of isoform htau40, the largest in the human CNS, containing 441 residues. Construct K19 is equivalent to the repeat domain of fetal tau (htau23), containing the amino acid repeats R1, R3, R4 (but lacking R2 = exon 10 due to alternative splicing). The proteins were expressed and purified as described by making use of the heat stability and FPLC Mono S chromatography with subsequent gel-filtration (Superdex 200 for full-length tau and Superdex 75 for the construct K19; Amersham Pharmacia Biotech, Freiburg, Germany). The purity of the proteins was analyzed by SDS-PAGE. Protein concentrations were determined by Bradford assay or by UV absorption at 214 nm. The fusion protein GFP-htau40 (where EGFP was coupled to the N terminus of htau40) was generated as described.

**Tubulin**

Bovine brain tubulin was purchased from Cytoskeleton (Denver, CO). MTs were polymerized for 30 minutes at 37 °C in BRB80 buffer (80 mM Pipes (pH 6.8), 4 mM MgCl₂) at a concentration of 5 mg/ml, in the presence of 10% (v/v) dimethyl sulfoxide (DMSO), 2 mM GTP and 20 μM taxol and in the absence of phosphate.

**Kinesin**

Recombinant motor domains of rat brain conventional kinesin (rK354) were expressed in *E. coli* and purified as described. Neospora crassa conventional kinesin heads (NcK355) were prepared as described by Song *et al.* These proteins contain the core motor domain but lack most of the stalk so that dimerization does not occur.

Sample preparation for electron microscopy

During cryo-EM and shadowing experiments, MT concentrations were initially varied to find optimal conditions for imaging, but then kept constant at 4.5 μM for the experiments shown here. In cryo-EM experiments, MTs were decorated with htau40 at concentrations of 10.0 μM and K19 at 18.0 μM. Decoration by rat brain kinesin heads (rK354) was done at 10.0 μM. The MAPs were incubated with MTs at room temperature for 20 minutes and subsequently adsorbed to holey carbon film for one minute. In separate experiments, htau40 was added to MTs, held at room temperature for five minutes, followed by addition of rK354, and adsorbed to holey carbon film as mentioned above. Grids were quick-frozen into liquid ethane using a plunger. For the metal shadowing experiments, htau40 was added to MTs at a concentration of 0.9 μM (low density) and 10.0 μM (high density). Tau construct K19 was added to MTs at concentrations of 1.0 μM (low density), 4.5 μM (medium density) and 18.0 μM (high density). Decoration by the GFP-htau40 fusion protein was done at a concentration of 7.0 μM. Finally, experiments were carried out using *N. crassa* kinesin (NcK355) at a concentration of 10.0 μM and co-decoration experiments were carried out using htau40 and NcK355 at 10.0 μM.

**Subtilisin treatment**

MTs at a tubulin concentration of 4.5 μM were incubated with subtilisin for one hour at 30 °C at a subtilisin/tubulin ratio of 0.04% (w/w) for partial digestion (removing only the CTT from β-tubulin), and 0.4% (w/w) for complete digestion (removing the CTTs from β and α-tubulin). Tau decoration was performed as described above. The reaction was terminated with 5 mM phenyl-methyl-sulfonyl fluoride (PMSF).

**Freeze-drying and unidirectional surface shadowing**

Each of the above samples were applied to carbon-coated grids and absorbed for two to three minutes. The grids were washed twice with BRB80 buffer (80 mM Pipes (pH 6.8)) and once in distilled water, followed by blotting to remove excess liquid, and finally quick-frozen in liquid nitrogen. Frozen grids were then transferred to the Midilab instrument, a unique design in the laboratory of H. G. It consists essentially of a freeze-drying/metal shadowing unit, mounted onto the column of a Philips-CM-12 electron microscope at the height of the specimen stage, and allows for a direct vacuum cryo-transfer of specimens into the microscope onto a modified GATAN cryo-holder. In the Midilab, samples were freeze-dried for two hours at a temperature of 180 K and a pressure of <5 × 10⁻⁷ mbar. Completely dried specimens were unidirectionally shadowed with a 0.5 nm thick layer of tantalum/tungsten (Ta/W) at an elevation angle of 45° (180 K). Unlike regular preparations that have to be transferred through air, this instrument does not require a carbon backing, which results in a significant increase of resolution and a higher signal/noise ratio. The contrast created by the metal coat is so high that the contracting component resulting from unstained material below the surface remains negligible. Micrographs of shadowed specimens were recorded with a GATAN-794 Multiscan CCD camera (GATAN, Pleasanton, CA, USA), using an electron dose of 500–1000 electrons/nm².

**Cryo-EM, image processing and image analysis**

Cryo-electron microscopy experiments were carried out using a Philips CM200 FEG microscope, and a GATAN-626 cryo-holder. Images were recorded on Kodak SO-163 electron microscopy film at a defocus around -2.5 μm. Micrographs were screened for 15 protofilament MTs with a two-start helix. Suitable images were scanned using a Zeiss-SCAI scanner at a step size of 21 Å and MTs were reconstructed according to their helical symmetry using the software packages PHOELIX and SUPRIM. Three-dimensional maps were visualized using VOLVIS (Research Foundation of SUNY) and Bobscript 2.3. The atomic coordinates for the αβ-tubulin dimer are from Löwe *et al.* (PDB 1jff), and that for kinesin from Sack *et al.* (PDB 2kin).
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References


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