The Microtubule-Associated Protein Tau Cross-Links to Two Distinct Sites on Each α and β Tubulin Monomer via Separate Domains†

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ABSTRACT: The interaction between tubulin subunits and microtubule-associated proteins (MAPs) such as tau is fundamental for microtubule structure and function. Previous work has suggested that the "microtubule binding domain" of tau (composed of three or four imperfect 18-amino acid repeats, separated by 13- or 14-amino acid inter-repeat regions) can bind to the C-terminal ends of both α and β tubulin monomers. Here, using covalent cross-linking strategies, we demonstrate that there are two distinct tau cross-linking sites (designated as "C-terminal" and "internal") on each α and β tubulin monomer. The C-terminal tau cross-linking site is located within the 12 C-terminal amino acids of both α and β tubulin, while the internal tau cross-linking site is located within the C-terminal one-third of α and β tubulin but not within the last 12 amino acids. In addition, we show that tau cross-links to the C-terminal site via its repeat 1 and/or the R1−R2 inter-repeat. The cross-linking of tau to the internal site is mediated by some subset of its other repeat units. Integrating these and earlier data with the 3.7 Å resolution model of the αβ tubulin dimer recently presented by E. Nogales et al. [(1998), *Nature 391,* 199−203], we propose a new model for the tau−microtubule interaction.

Microtubules are critical elements in a wide variety of fundamentally important functions such as cell motility, cell division, and cell morphology. Their functions are regulated by a family of proteins known as microtubule-associated proteins, "MAPs", which includes tau, MAP2, and MAP4. All three of these MAPs colocalize with microtubules in vivo and can promote microtubule assembly, stability, and bundling as well as neurite outgrowth and the establishment of cell polarity (for reviews, see refs *1*−*4*). While MAP2 and tau are found mostly in neuronal cells (with MAP2 primarily in the dendrites and tau in the axons), MAP4 is present in both neuronal and non-neuronal cells (*5*−*7*), raising the possibility that each MAP may have some unique functions. In addition, abnormal tau that has lost its ability to associate with microtubules is known to be the major component of the neurofibrillary tangles associated with necrotic neurons in Alzheimer's disease (for reviews, see refs *8* and *9*).

The molecular basis of the MAP−microtubule interaction has been investigated extensively. The primary sequence of adult brain tau, deduced from cloned cDNAs, revealed that the C-terminal region contained four 18-amino acid imperfect repeats separated from one another by more variable 13- or 14-amino acid inter-repeats (*10*−*14*). Subsequently, very similar sequences were identified near the C-terminal ends of MAP2 and MAP4 (*15*−*17*). These observations led to the model in which each of the 18-amino acid repeats in these MAPs might serve as individual tubulin binding sites, suggesting that MAPs might stabilize microtubules by interacting with, and thereby cross-link, multiple tubulin subunits. Initial support for this model came from the observation that individual repeats are indeed capable of binding to microtubules, albeit with very weak affinities relative to that of the intact protein (*18*−*21*). On the basis of these observations, the entire repeat and/or inter-repeat region became known as the "microtubule binding domain".

More recent findings suggest that the biochemical mechanisms by which tau interacts with microtubules are much more complex than the initial view of a simple linear array of independent tubulin binding sites. First, different repeats contribute differently to the overall strength of microtubule binding, with repeat 1 ("R1") being the most important and repeat 4 ("R4") maybe being dispensable (*18*, *22*, *23*). Second, the R1−R2 inter-repeat ("R1−R2 IR") has inherent and relatively strong microtubule binding capability (*23*). Third, a number of studies revealed that regions flanking the microtubule binding domain play important regulatory roles in enhancing the binding affinity of the repeats (*24*−*27*; B. L. Goode et al., unpublished data). Fourth, tau function is regulated also by complex combinations of phosphorylation, much of which occurs in sequences flanking the

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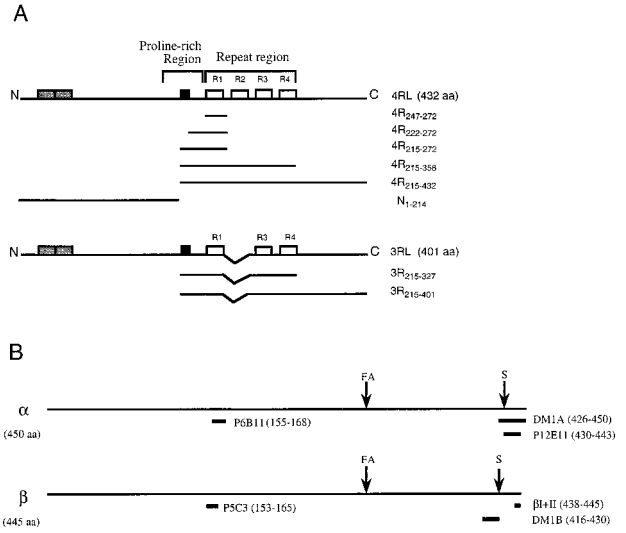

FIGURE 1: Schematic diagram of tau and tubulin polypeptides. (A) Schematic representations of three-repeat and four-repeat tau. The hatched boxes are the two 29-amino acid insertions at the N terminus. The solid box is the proline-rich domain (aa 215−221). Open boxes are repeat regions. Solid bars below each schematic correspond to either synthetic peptides or recombinant fragments used in this study. (B) Schematic representation of α and β tubulin. S denotes the subtilisin cleavage site. FA denotes the formic acid cleavage site. Solid bars represent regions recognized by monoclonal antibodies used in this study.

microtubule binding domain (*28*−*32*). Fifth, developmentally regulated alternative RNA splicing leads to the synthesis of multiple tau isoforms, including variation in the microtubule binding domain. More specifically, while fetal neurons express only one tau mRNA isoform (possessing only three repeat units in the microtubule binding domain), there are six different tau mRNAs expressed in adult CNS neurons, three of which possess an exon encoding an additional repeat and inter-repeat unit in the microtubule binding domain (repeat 2 and the R1−R2 inter-repeat; see Figure 1A for a schematic). Finally, it remains unclear whether each tau protein, harboring multiple regions capable of microtubule binding, interacts with a single tubulin monomer, an αβ tubulin dimer, or a polymeric tubulin structure.

Many questions also remain regarding the regions of α and β tubulin that interact with tau. While most studies suggest that the C-terminal ends of both α and β tubulins are important for the tau−tubulin interaction, there are a number of discrepancies regarding the exact location(s) of the tau binding sites on the tubulins. On the basis of studies using limited subtilisin digestion (which cleaves both α and β tubulins near their C terminal ends), two groups have concluded that tau binds very near the C-termini of both α and β tubulin, which is carboxy to the subtilisin site (*33*, *34*). Serrano et al. (*33*) further showed that tau can bind to the small C-terminal subtilisin fragment of α tubulin, often referred to as the "4 kDa" fragment in the literature. In contrast, other researchers have detected tau binding to subtilisin-digested microtubules (*35*−*38*), suggesting that tau binding sites could also be present on the amino side of the subtilisin sites of α or β tubulin. A possible explanation for these conflicting observations may be derived from the work of Redeker et al. (*39*), who showed that subtilisin can cleave at two closely positioned but different sites on each tubulin polypeptide: at amino acids (aa) 406 and 438 in α tubulin and at aa 396 and 433 in β tubulin, with the latter site in each tubulin subunit being more prominent. Since the digestion conditions and the source of subtilisin used by the above groups are not identical, it is possible that different subtilisin sites were cleaved in the various studies.

Studies of tau binding to synthetic tubulin peptides also have yielded conflicting results. Maccioni et al. (*40*) showed that peptides corresponding to aa 430−441 and aa 422−434 of α and β tubulin, respectively, can bind to tau. Additionally, these peptides can bind to synthetic peptides corresponding to either repeat 1 or repeat 3 of tau (*20*). Furthermore, idiotypic and anti-idiotypic antibodies raised against these same two tubulin peptides (α430−441 and β422−434) inhibited MAP-induced tubulin assembly (*41*, *42*); the anti-idiotypic antibodies recognized tau, and their inhibition of MAP-induced tubulin assembly could be overcome by the addition of excess MAPs (*42*). These antibody studies suggest that tubulin regions α430−441 and β422−434 are directly involved in the interaction of tubulin and MAPs. On the other hand, a blotting assay used by Littauer et al. (*43*) detected binding of tau to only three tubulin peptides: α1−75, β392−445, and β416−445; these investigators did not detect binding to the peptides corresponding to the C-terminal end of α tubulin (α426−450 and α424−450) even though they contained the region of aa 430−441 subsequently shown to be a tau binding site by Maccioni et al. (*40*).

In the evaluation of these various data and their inconsistencies, it is important to note that the number of possible tau binding sites per α and β tubulin monomer remains an open question. If more than one tau binding site exists per tubulin monomer, then one might be able to reconcile the above seemingly conflicting results as different groups using different assays detecting different subsets of the total number of binding sites.

Thus, despite intensive efforts, the molecular details underlying the tau−microtubule interaction remain poorly understood. Here, we have sought to investigate the sites of interaction between tau and both α and β tubulin with much higher resolution than previous studies have provided. To achieve this goal, we have conducted an extensive chemical cross-linking study employing full-length tau and tubulin proteins as well as a large number of different tau and tubulin fragments. The data reveal that tau can cross-link to two distinct sites on each α and β tubulin molecule. The "C-terminal" tau cross-linking site is located within the 12 C-terminal amino acids of each α and β tubulin polypeptide, while a second "internal" tau cross-linking site is located within the C-terminal one-third of each monomer but amino to the last 12 amino acids. In addition, we show that tau cross-links to the C-terminal sites of α and β tubulin via repeat 1 and/or the R1−R2 inter-repeat. The cross-linking of tau to the internal site is mediated by some subset of the other repeat units, with repeat 2 likely to be most influential in four-repeat tau and repeat 3 likely to be the main influence in three-repeat tau. Integrating our data with the recently presented 3.7 Å resolution map of the αβ tubulin dimer (*44*), we present novel models for the tau−microtubule interaction.

MATERIALS AND METHODS

Protein Purification. MAP-depleted tubulin was purified from bovine brain by two cycles of temperature-controlled

polymerization and depolymerization, followed by phosphocellulose chromatography (*45*) in PEM buffer [50 mM PIPES[1] (pH 6.9), 1 mM EGTA, and 1 mM $MgSO_4$] supplemented with 1 mM GTP (Sigma). Aliquots were then drop-frozen in liquid nitrogen and stored at −70 °C. On the basis of the literature, a subset of this tubulin is expected to possess a variety of post-translational modifications, including tyrosination, glutamylation, and acetylation. Protein concentrations were determined by the method of Bradford (*46*). SDS−polyacrylamide gel analysis of the MAP-depleted tubulin stock revealed no detectable MAP contamination, even when the lane was grossly overloaded (data not shown). Further, immunoblots using an anti-tau probe revealed no contamination of tau in the MAP-depleted tubulin stock (data not shown).

Recombinant rat tau polypeptides were expressed in BL21-(DE3) cells using the pET vector expression system of Novagen, Inc. (Madison, WI), and purified as described by Goode et al. (*27*).

Synthesis of Tau Peptides and β Tubulin Peptides Used in Cross-Linking Experiments. Synthetic rat tau peptides $4R_{247-272}$ (VRSKIGSTENLKHOPGGGKVQIINKK), $4R_{222-272}$ (TPPKSPSASKSRLQTAPVPMPDLKNVR-SKIGSTENLKHOPGGGKVQIINKK), and $4R_{215-272}$ (KKVAV-VRTPPKSPSASKSRLQTAPVPMPDLKNVR-SKIGSTENLKHOPGGGKVQIINKK) and human β tubulin peptides β2 (NDLVSEYQQYQDATADEQGEFEEEE-GEDEA) and β4 (NDLVSEYQQYQDATAEEGEFEEE-AEEEVA) were synthesized on a Millipore 9050 Plus peptide synthesizer by DIPCDI/HoBT chemistry. Following cleavage and deprotection (*47*), all tau peptides except tau $4R_{215-272}$ were purified to >95% purity by reverse phase HPLC, lyophilized, and resuspended in H_2O. Peptide integrity was verified by electrospray mass spectrometry. In the case of the 58-amino acid $4R_{215-272}$ peptide, the preparation was >80% pure, but had a mass ∼16 Da greater than the predicted mass of 6265 Da, suggesting that the methionine residue had become oxidized during synthesis or workup. Peptide concentrations were determined from dry peptide weights and confirmed after resuspension in H_2O by A_{205} absorption.

Cross-Linking with EDC. Paclitaxel (Taxol)-stabilized microtubules were prepared by incubating purified MAP-depleted tubulin with 1 mM GTP at 37 °C for 30 min, then adding paclitaxel to a final concentration of 20 μM, and incubating for an additional 5 min. Tau peptides, fragments, or full-length proteins were incubated with paclitaxel-stabilized microtubules, at a final concentration of 20 μM each, for 30 min at 37 °C. EDC was added to a final concentration of 1.5 mM [from a 24 mM stock in 100 mM PIPES (pH 5.9)], and the mixture was incubated for 30 min at room temperature. The reaction was stopped with the addition of 2× sample buffer and the mixture fractionated using an 8% polyacrylamide gel.

Formic Acid Digestion. Digestions were performed using the procedures described by Sonderegger et al. (*48*). Briefly, the cross-linked samples described above were drop dialyzed on VSWP membranes (Millipore) against 100 mM PIPES (pH 5.9) for 30 min at room temperature to remove any unreacted EDC. Formic acid was then added to a final concentration of 75% (v/v), and the reaction mixture was incubated at 37 °C for 14 h. The reaction was stopped with the addition of 3 volumes of water, and the mixture was lyophilized to remove the formic acid. The lyophilized sample was resuspended in 500 μL of water and lyophilized, and the procedure was repeated once more. The final sample was resuspended in 1× sample buffer and fractionated using a 15% polyacrylamide gel.

Subtilisin Digestion. Paclitaxel-stabilized microtubules ("αβ" microtubules) were incubated in PEM buffer at 37 °C and subjected to 1% subtilisin (w/w, Subtilisin Carlsberg, type VIII, Sigma) digestion for either 13 min (removing just the C-terminal fragment of β tubulin, thereby generating "$\alpha\beta_s$" microtubules) or 14 h (removing the C-terminal fragments of both α and β tubulin, thereby generating "$\alpha_s\beta_s$" microtubules). Digestions were stopped by the addition of PMSF at a final concentration of 2 mM. As shown in the Results, epitope mapping demonstrates that subtilisin cleavage under these conditions occurs ∼12 amino acids from the C termini in both α and β tubulin.

Subcloning of the α Tubulin C-Terminal Fragment. The human hkα1 cDNA clone was a kind gift from K. Sullivan (Scripps Research Institute, La Jolla, CA). An *Nco*I−*Bam*HI fragment of the α tubulin clone, encoding C-terminal amino acids 309−451, was subcloned into the pET3c vector in-frame with the T7 tag sequence of the vector (Novagen, Inc.). DNA sequencing confirmed that the α tubulin fragment was in-frame with the T7 tag sequences. The plasmid was transformed into BL21(DE3, lysS) cells for expression. The purified α tubulin fragment has noticeable secondary structure similar to full-length tubulin as assessed by circular dichroism (data not shown).

Gel Electrophoresis and Immunoblotting. Proteins were fractionated by SDS−PAGE using pure (99%) SDS (*49*). For better resolution of α and β tubulin monomers, 95% pure SDS (Sigma, catalog no. L-5750) was employed (*50*). The gels were either stained with Coomassie blue or electroblotted onto nitrocellulose filters at 40 V for 2 h, using a transfer buffer of 10% methanol, 10 mM sodium bicarbonate, and 3 mM sodium carbonate. Filters were blocked with 20 mM Tris (pH 7.4), 150 mM sodium chloride, 0.2% Tween 20, and 2% nonfat dry milk for 1 h at room temperature or overnight at 4 °C.

Antibodies. Monoclonal antibodies DM1A (Sigma), DM1B (Amersham), βI+II (Sigma), and T7 tag (Novagen) were used at 1:2000 dilutions. Monoclonal antibodies P12E11, P6B11, and P5C3 were generated as follows. Peptides corresponding to α tubulin (aa 155−168, ERLS-VDYGKKSKLEC; aa 430−443, KDYEEVGVDSVEGE) and β tubulin (aa 153−165, SKIREEYPDRIMNC) were synthesized by Merriefield solid phase methods, purified to homogeneity by HPLC, and coupled to KLH as described previously (*51*, *52*). Following immunization and boosts, spleen cells were fused with the Sp2/0-Ag14 myeloma line in the presence of PEG 1000 in DMEM as described by Nowinski et al. (*53*). Screening of positive hybridomas was performed by ELISA, immunoblotting (*51*), and indirect immunofluorescence (*54*), leading to the subsequent clonal

[1] Abbreviations: EDC, 1-ethyl-[3-(3-dimethylamino)propyl]carbodiimide hydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP, guanosine triphosphate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

isolation of class IgG_1 hybridomas P6B11 (α155−168), P12E11 (α430−443), and P5C3 (β153−165). Ascites fluids generated by these hybridomas were used at a 1:10000 dilution. None of the anti-tubulin antibodies cross-react with tau or tau fragments (for example, see Figures 7 and 9). Polyclonal antisera anti-$tau_{215-246}$ and anti-$tau_{247-272}$ were raised in mice against HPLC-purified synthetic peptides corresponding to amino acids 215−246 (KKVAVVRTPPK-SPSASKSRLQTAPVPMPDLKN) and 247−272 (VRSKIG-STENLKHOPGGGKVQIINKK), respectively. They were used at 1:3000 dilutions. Secondary antibody (anti-mouse IgG-HRP, Bio-Rad) was used at a 1:10000 dilution. The secondary antibody was then detected by ECL, using the Supersignal chemiluminescense kit (Pierce).

RESULTS

Specific Cross-Linking of Tau and Tau Fragments to Microtubules. Our strategy was to define sites on α and β tubulin that serve as tau binding sites by identifying regions of tubulin that cross-link with either intact tau or defined fragments of tau known to bind to microtubules (see schematic in Figure 1A). To identify sites of close contact, we used the zero-length covalent cross-linker EDC. Products of cross-linking reactions were analyzed by SDS−PAGE.

We began our analyses in the important R1 and the R1−R2 IR region of tau (tau peptide $4R_{247-272}$, Figure 2A). Unfortunately, the small size of tau peptide $4R_{247-272}$ makes it difficult to distinguish the bands of cross-linked tau peptide−tubulin from un-cross-linked tubulin by Coomassie blue staining (Figure 2A, left panel). However, the data are clarified by immunoblotting. DM1A, an anti-α tubulin antibody (see Figure 1B for antibody epitope), identified a single cross-linked product (Figure 2A, middle panel), while the βI+II antibody, an anti-β tubulin antibody, revealed two cross-linked products (Figure 2A, right panel). Thus, R1 and/or the R1−R2 IR sequences cross-link effectively to both α and β tubulin. The observation that there are two cross-linked tau peptide−β tubulin bands might result from either one or two tau peptides cross-linking to individual β tubulin subunits or from cross-linking of a single peptide at different sites, the products of which fractionate from one another on SDS−PAGE.

Similar analyses using $4R_{215-272}$, $4R_{215-358}$, $4R_{215-432}$, $3R_{215-327}$, and $3R_{215-401}$ (see Figure 1A) all yielded two higher-molecular mass cross-linked species when viewed by Coomassie blue staining (Figure 2B), both of which were recognized by tau antiserum (data not shown). Immunoblotting with antibodies DM1A and βI+II demonstrated that the upper band of each doublet is the cross-linked product of tau with α tubulin while the lower band is tau cross-linked with β tubulin (data not shown). The similar amounts of α and β cross-linked products in each case indicates that there is no significant preferential cross-linking of tau to either α or β tubulin. In contrast, a fragment corresponding to the amino half of tau (N_{1-214}), which lacks the entire microtubule binding domain, fails to cross-link to microtubules (Figure 2B). Next, Figure 2C demonstrates that both full-length 4R tau and full-length 3R tau also cross-link to microtubules. Immunoblotting with antibodies DM1A and βI+II revealed that both full-length tau isoforms cross-link to both α and β tubulin (data not shown). Taken together, the data demonstrate that the microtubule binding domain of tau, and

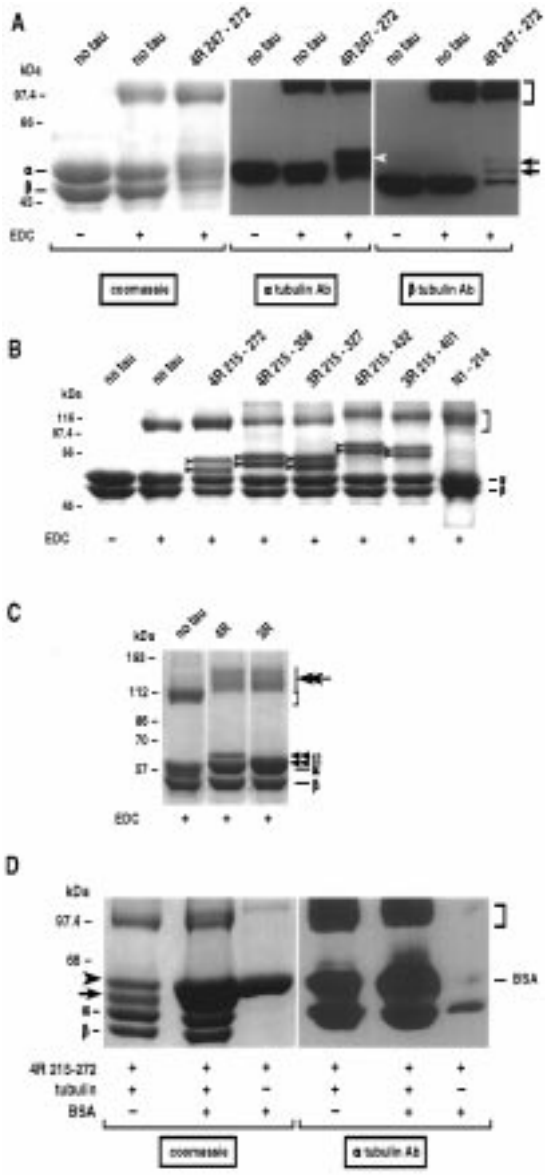

FIGURE 2: Cross-linking of tau peptides, fragments, and full-length proteins to paclitaxel-stabilized microtubules using 1.5 mM EDC. For all panels, tau peptides and proteins and paclitaxel-stabilized microtubules were each at 20 μM. Proteins were fractionated using an 8% polyacrylamide gel with 95% pure SDS (see Materials and Methods), which resolves the α and β tubulin monomers. The arrowhead denotes the cross-linked complex of tau peptide and the α tubulin monomer. The arrow denotes the cross-linked complex of the tau peptide and β tubulin monomer. The bracket denotes cross-linked tubulin dimers. (A) Cross-linking of tau peptide $4R_{247-272}$ to microtubules. Anti-α tubulin (DM1A) and anti-β tubulin (βI+II) antibodies were used in the immunoblots. (B) Coomassie-stained gel showing the cross-linked products of various tau peptides and fragments and microtubules. (C) Coomassie-stained gel showing the cross-linked products of full-length 3R and 4R tau and microtubules. The double-headed arrow denotes the cross-linked complex of full-length tau proteins and the α or β tubulin monomer. The double arrowhead denotes the non-cross-linked full-length tau protein. Full-length 3R tau has a mobility very similar to that of α tubulin, thus precluding complete resolution of the two bands. (D) Cross-linking of tau peptide $4R_{215-272}$ to microtubules in the presence or absence of a 10-fold molar excess of BSA. Anti-α tubulin antibody (DM1A) was used in the immunoblot. BSA has a mobility similar to that of the tau−tubulin complex, and this masks the signal in the Coomassie-stained gel; however, the data are clear with the immunoblot.

individual binding units within that domain, interact with both α and β tubulin subunits in microtubules.

Finally, to assess the specificity of the cross-linking reactions, we repeated all reactions in the presence of a 10-fold molar excess of BSA. No reduction in the extent of the cross-linking between α or β tubulin and any tau molecule is observed. As an example, Figure 2D shows that excess BSA has no effect on the extent of cross-linking between tau peptide $4R_{215-272}$ and α tubulin. In addition, controls containing the tau peptides, fragments, or full-length proteins alone revealed no significant tau−tau cross-linking under the same conditions used for tau−tubulin cross-linking reactions (data not shown). Thus, full-length tau and tau fragments bind to microtubules and can be cross-linked to tubulin specifically.

Tau Interacts with the C-Terminal One-Third of both α and β Tubulin. To begin mapping the location(s) of the tau cross-linking site(s) on α and β tubulin, our strategy was to cleave each α and β tubulin monomer into two major fragments (N- and C-terminal) and then identify the tubulin fragment(s) that cross-link with tau or tau fragments using specific tubulin antibodies (see Figure 1B for epitopes). In this first set of experiments, we used 75% formic acid digestion, which cleaves peptide bonds between aspartic acid and proline (*55*). Porcine α tubulin has only one Asp−Pro bond (between positions 306 and 307), and porcine β tubulin has two Asp−Pro bonds (between positions 31 and 32 and 304 and 305; see Figure 1B) (*56*, *57*). Although the bovine tubulin sequences have not been determined, the extraordinary degree of evolutionary conservation of the tubulins across species suggests a similar location of Asp−Pro bonds in bovine tubulins; indeed, the Asp−Pro sequence is present at the corresponding position even in yeast α and β tubulin (*58*, *59*). Consistent with this prediction, formic acid cleaves bovine tubulin into two populations of fragments with apparent molecular masses of ∼20 and ∼35 kDa (the C- and N-terminal fragments, respectively; Figure 3A); the heterogeneity of the formic acid digestion products is likely the result of slight sequence variations among the multiple isoforms of α and β tubulin present in brain. Monoclonal antibodies P12E11 (α430−443) and βI+II (β438−445) recognize the ∼20 kDa α and β tubulin fragments, respectively, as predicted on the basis of the Asp−Pro site in porcine tubulins (Figure 3B,C). Monoclonal antibodies P6B11 and P5C3 (see Figure 1B for epitopes) recognized the ∼35 kDa bands of α and β tubulin, respectively, again as predicted on the basis of the Asp−Pro site in porcine tubulins (data not shown).

Tau and various tau fragments were cross-linked to paclitaxel-stabilized microtubules as described above, followed by 75% formic acid digestion and gel fractionation. Since there are no Asp−Pro bonds in tau, no complications arise from formic acid digestion of the tau proteins. Tau synthetic peptides $4R_{215-272}$, $4R_{222-272}$, and $4R_{247-272}$ (each containing R1 and the R1−R2 IR, with or without additional sequences on their amino sides) and the recombinant fragments $4R_{215-358}$ and $3R_{215-327}$ (each of which contain the entire 4R or 3R microtubule binding domain, respectively) all cross-linked to the ∼20 kDa C-terminal fragments of α and β tubulin, as demonstrated by the generation of appropriately sized higher-molecular mass products that are recognized by the P12E11 (anti-α) and βI+II antibodies (panels B and C of Figure 3, respectively). Fragments with the same molecular mass are also recognized by tau antiserum, confirming that they are indeed cross-linked complexes of tau and tubulin (data not shown). Finally, the larger recombinant tau fragments $4R_{215-432}$ and $3R_{215-401}$, which extend from the start of the proline rich region to the

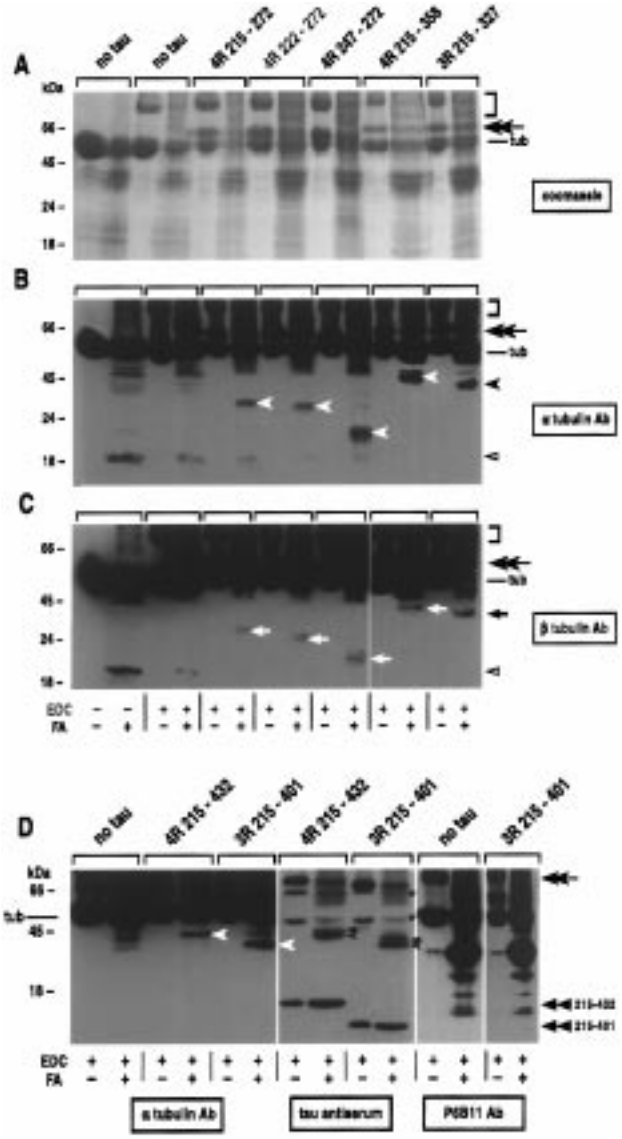

FIGURE 3: Formic acid digestion of microtubules cross-linked with tau peptides or fragments. For all panels, proteins were fractionated using 15% polyacrylamide gels with 99% pure SDS (see Materials and Methods). The arrowhead denotes the cross-linked complex of the tau peptide or fragment and the α tubulin C-terminal fragment. The arrow denotes the cross-linked complex of the tau peptide or protein and the β tubulin C-terminal fragment. The double-headed arrow denotes the cross-linked complex of the tau peptide or fragment and the α or β tubulin monomer. The bracket denotes cross-linked tubulin dimers. (A) Coomassie-stained gel. (B and C) Immunoblots probed with the anti-α tubulin (P12E11) and anti-β tubulin (βI+II) antibodies, respectively. All lanes are identical to those in panel A. The open arrowhead denotes the C-terminal fragment of α or β tubulin after formic acid digestion (see Figure 1B). (D) Immunoblots probed with anti-α tubulin (P6B11 and P12E11) antibodies and tau antiserum. The double arrowhead denotes non-cross-linked tau fragments. Two identical blots were each probed with the P6B11 and P12E11 anti-α tubulin antibodies. The immunoblot probed with the anti-α tubulin (P12E11) antibody was stripped and then reprobed with the tau antiserum. Some signal from the anti-α tubulin (P12E11) antibody was not totally removed even after stripping the blot (asterisk).

C termini of 4R and 3R tau, respectively, also cross-linked to the ~20 kDa C-terminal fragments of both α and β tubulin, again demonstrated by the reactivity of higher-molecular mass bands with the tau antiserum (Figure 3D), anti-α tubulin antibody (P12E11, Figure 3D), and anti-β tubulin antibody (βI+II, data not shown).

The fact that the tau antiserum only recognized two higher-molecular mass bands suggests that the tau fragments cross-linked only to the ~20 kDa C-terminal fragments of α and β tubulin. Western blots using monoclonal antibodies P6B11 (α155−168) or P5C3 (β153−165; see Figure 1B for epitope position), which recognize the N-terminal ~35 kDa fragments resulting from formic acid digestion of α and β tubulin, respectively, revealed no differences between the microtubule alone control samples and the tau−tubulin cross-linked samples (see Figure 3D for P6B11 anti-α tubulin data). Thus, these data confirm that the tau binding site(s) identified by EDC cross-linking are located exclusively within the C-terminal one-third of both α and β tubulin.

Tau Cross-Links to the 12 C-Terminal Amino Acids of both α and β Tubulin. Previous data have demonstrated that tau possesses multiple domains capable of binding to microtubules, raising the possibility that each microtubule binding domain might bind to more than one tubulin subunit (*22*, *23*). Alternatively, but not mutually exclusively, different microtubule binding domains of tau could interact with different sites on individual α or β tubulin subunits. From the data presented above, it is clear that all of the sites of EDC cross-linking are located exclusively in the C-terminal one-third of both α and β tubulin subunits. The next step was to map the location(s) of the tau cross-linking site(s) on α and β tubulin at higher resolution. To do so, we took advantage of the differential susceptibility of α and β tubulin subunits to subtilisin treatment (*60*, *61*). Previous studies have shown that brief subtilisin treatment (~10 min at 37 °C) efficiently removes the small C-terminal end (<4 kDa) of only β tubulin, while removal of the small α C-terminal end requires much more extensive digestion (>10 h at 37 °C; *35*). The ability of tau and tau fragments to cross-link to either α or β tubulin following removal of their C termini would indicate that at least one cross-linking site is internal to the C-terminal subtilisin site.

Given the uncertainty regarding the exact site of subtilisin cleavage (see the introductory section), we first mapped the sites of subtilisin cleavage using our reagents and experimental conditions. We prepared non-subtilisin-treated ($\alpha\beta$), lightly subtilisin-treated ($\alpha\beta_s$), and extensively subtilisin-treated ($\alpha_s\beta_s$) microtubules and used antibodies to map the cleavage site. As seen in Figure 4C (left-most panel), subtilisin-digested α tubulin remains reactive with DM1A, which has its epitope between amino acids 426 and 450 (*62*). Thus, under our conditions, subtilisin must digest α tubulin at the more C-terminal candidate site [position 438 rather than 406; see the introductory section and Redeker et al. (*40*)], cleaving off a small C-terminal fragment of ~12 amino acids. Parallel analyses with antibody DM1B (with epitope between aa 416 and 430; *63*) demonstrate that our conditions also lead to digestion of β tubulin at the more C-terminal candidate site, position 433 rather than 396, again generating a small C-terminal fragment of ~12 amino acids (data not shown). Usage of similar subtilisin sites has also been shown by de Pereda and Andreu (*64*).

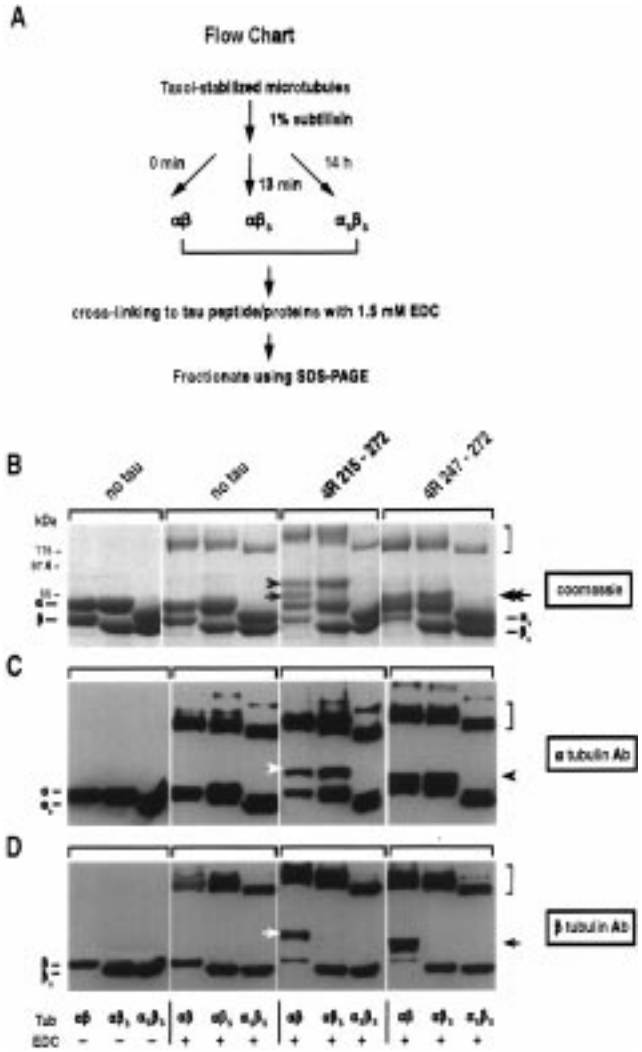

FIGURE 4: Cross-linking of tau peptides to $\alpha\beta$, $\alpha\beta_s$, and $\alpha_s\beta_s$ microtubules. Proteins were fractionated using 8% polyacrylamide gels with 95% pure SDS. Symbols are the same for all panels. The arrowhead denotes the cross-linked complex of the tau peptide and α tubulin monomer. The arrow denotes the cross-linked complex of the tau peptide and β tubulin monomer. The bracket denotes cross-linked tubulin dimers. (A) Flowchart showing the experimental protocol. (B) Coomassie-stained gel. (C and D) Immunoblots of the gel shown in panel B probed with the anti-α tubulin (DM1A) and anti-β tubulin (P5C3) antibodies.

We compared the ability of the various tau proteins or fragments described above to cross-link to non-subtilisin-treated ($\alpha\beta$), lightly subtilisin-treated ($\alpha\beta_s$), and extensively subtilisin-treated ($\alpha_s\beta_s$) microtubules using the experimental design depicted in Figure 4A. The analysis began with tau peptides containing the R1 and R1−R2 inter-repeat. Both $4R_{215-272}$ and $4R_{247-272}$ cross-linked to both α and β tubulin in $\alpha\beta$ microtubules, only to α tubulin in $\alpha\beta_s$ microtubules, and not at all to $\alpha_s\beta_s$ microtubules (Figure 4B−D). Therefore, both $4R_{215-272}$ and $4R_{247-272}$ cross-link within the 12 C-terminal amino acids of α and β tubulin. Since peptide $4R_{247-272}$ contains only R1 and part of R1−R2 IR, these data suggest that the binding of tau to the 12 C-terminal amino acids of α and β tubulin requires only R1 and/or the R1−R2 IR (aa 247−272) but not the proline rich domain located on the amino side of R1 (aa 215−246). This is consistent with previous findings suggesting that the proline rich domain, which does not bind to microtubules alone, plays a

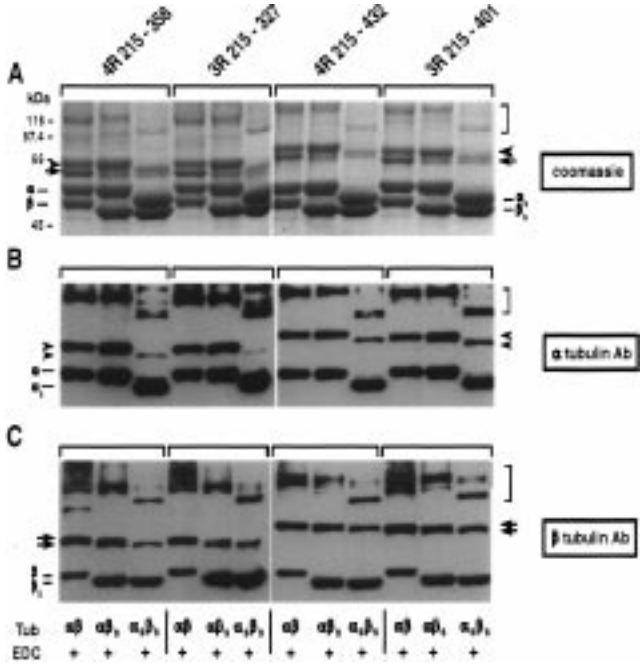

FIGURE 5: Cross-linking of tau fragments to $\alpha\beta$, $\alpha\beta_s$, and $\alpha_s\beta_s$ microtubules. Proteins were fractionated using 8% polyacrylamide gels with 95% pure SDS. Symbols are the same for all panels. The arrowhead denotes the cross-linked complex of the tau fragment and α tubulin monomer. The arrow denotes the cross-linked complex of the tau fragment and β tubulin monomer. The bracket denotes cross-linked tubulin dimers. (A) Coomassie-stained gel. (B and C) Immunoblots of the gel shown in panel A probed with the anti-α tubulin (DM1A) and anti-β tubulin (P5C3) antibodies.

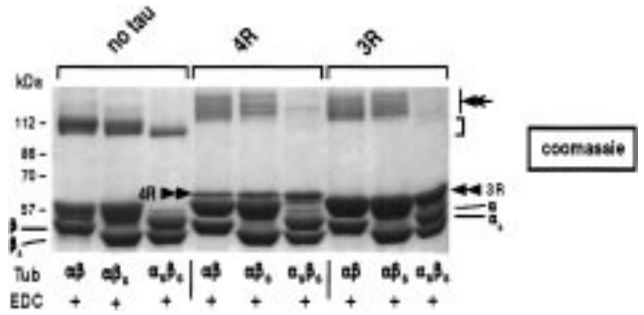

FIGURE 6: Cross-linking of full-length 3R and 4R tau to $\alpha\beta$, $\alpha\beta_s$, and $\alpha_s\beta_s$ microtubules. Coomassie-stained gel of proteins fractionated using 8% polyacrylamide gels using 95% pure SDS. The double-headed arrow denotes the cross-linked complex of full-length tau proteins and the α or β tubulin monomer. The double arrowhead denotes the non-cross-linked full-length tau protein. The bracket denotes cross-linked tubulin dimers.

regulatory role in enhancing the binding of R1 and/or R1−R2 IR (*27*).

Tau Cross-Links to an Additional Site Located within the C-Terminal One-Third but Internal to the Last 12 Amino Acids on both α and β Tubulin. Larger tau fragments ($4R_{215-358}$, $4R_{215-432}$, $3R_{215-327}$, and $3R_{215-401}$) and full-length tau proteins (4R and 3R), each possessing the entire microtubule binding domain, yielded different cross-linking results compared to the smaller peptides ($4R_{247-272}$ and $4R_{215-272}$) in the subtilisin experiment described above. Each of these tau proteins cross-links to $\alpha\beta$, $\alpha\beta_s$, and $\alpha_s\beta_s$ microtubules (Figures 5 and 6). The cross-linked products of tau with α_s tubulin and β_s tubulin are slightly smaller than their respective cross-linked products with undigested α and β tubulin, as expected, since the last 12 amino acids are

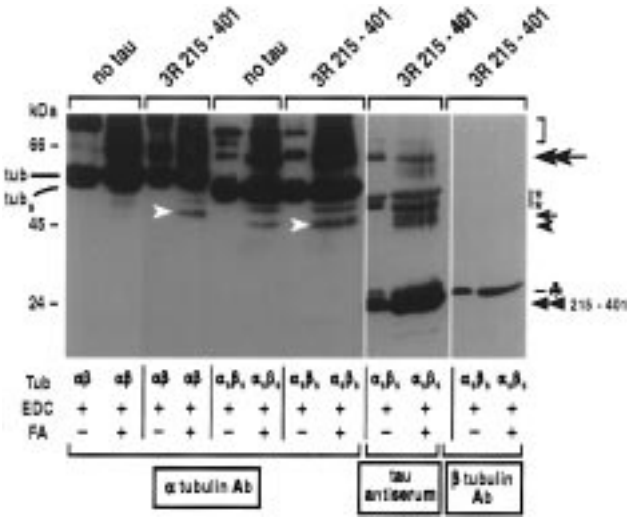

FIGURE 7: Formic acid digestion of cross-linked products of tau fragment $3R_{215-401}$ to $\alpha\beta$ and $\alpha_s\beta_s$ microtubules. Proteins were fractionated using 15% acrylamide gels with 95% pure SDS, blotted, and then probed with the anti-α tubulin (P12E11) and anti-β tubulin (βI+II) antibodies and tau antiserum. The arrowhead denotes the cross-linked complex of tau fragment $3R_{215-401}$ and the α tubulin monomer. The arrow denotes the cross-linked complex of tau fragment $3R_{215-401}$ and the β tubulin monomer. The double-headed arrow denotes the cross-linked complex of tau fragment $3R_{215-401}$ and the α or β tubulin monomer. The double arrowhead denotes non-cross-linked tau fragment $3R_{215-401}$. The club sign denotes the cross-linked complex of tau fragment $3R_{215-401}$ and the small C-terminal subtilisin fragment of β tubulin. The immunoblot was first probed with the anti-α tubulin (P12E11) antibody, stripped, and then reprobed with the tau antiserum. Some signal from the anti-α tubulin (P12E11) antibody was not totally removed even after stripping the blot (asterisk).

removed in the digested samples (Figure 5A−C). On the basis of these observations, we conclude that tau fragments containing the entire microtubule binding domains (with either three or four repeat regions) can bind to α_s and β_s tubulin, in contrast to peptides possessing only repeat 1 and the R1−R2 inter-repeat. Therefore, there must be a second tau cross-linking site on both α and β tubulin located internal to the subtilisin site, which is located ~12 amino acids from their C termini. Taken together, it is reasonable to propose that these additional tau cross-linking sites on the tubulins interact with tau sequences located C-terminal to repeat 1 and the R1−R2 inter-repeat (i.e., repeats 2, 3, and/or 4). Although we cannot yet determine which repeats are involved, previous findings suggest that repeat 2 of 4R tau and repeat 3 of 3R tau are especially important for tau binding to microtubules (*65*).

Next, we mapped the internal site directly by cross-linking tau fragments $3R_{215-401}$ and $4R_{215-432}$ to $\alpha_s\beta_s$ microtubules (as well as the control $\alpha\beta$ microtubules) and then performing formic acid digestions on the cross-linked products. Tau fragment $3R_{215-401}$ cross-links to the C-terminal formic acid fragment of α_s tubulin, as shown with the antibody P12E11 (α430−443) and the tau antiserum (Figure 7). As expected, the cross-linked product with α_s tubulin is smaller in size than the cross-linked product with the undigested α tubulin control since the 12 C-terminal amino acids are removed by subtilisin in the former. We infer from tau antiserum data that the band immediately above the α_s cross-linked species is the cross-linked product with β_s tubulin (Figure 7, arrow). Hence, we conclude that the second tau cross-linking site is

located within the C-terminal one-third of α and β tubulin but amino to the last 12 amino acids, i.e., a region of ~130 amino acids. Similar results were obtained with tau fragment $4R_{215-432}$ (data not shown).

Finally, both tau fragments $3R_{215-401}$ and $4R_{215-432}$ also appear to have cross-linked to the small subtilisin fragment containing the last 12 amino acids of β tubulin, on the basis of the presence of an additional band above the tau fragment visualized by both tau antiserum and the βI+II antibody (Figure 7). The ability of the tau fragments to cross-link to the small subtilisin fragment demonstrates that the binding of tau to the 12 C-terminal amino acids does not require the presence of full-length tubulin, in agreement with previous studies where it was found that tau can bind to small subtilisin fragments (*33*).

Tau Can Bind to Free α and β Tubulin C-Terminal Fragments Independent of One Another. The above data indicate that all tau cross-linking capability resides in the C-terminal one-third of each tubulin subunit. Therefore, we next sought to test whether the C-terminal one-third of each α and β tubulin subunit is capable of interacting independently with tau. In other words, is sufficient structural information present in these smaller tubulin fragments to allow binding to tau? In addition, by using recombinant tubulin fragments and synthetic tubulin peptides, we can also determine if post-translational modification within the C-terminal fragments of tubulin (e.g., glutamylation or tyrosination) is necessary for tau binding.

We subcloned the C-terminal region (amino acids 309−451) of a human α tubulin cDNA (hkα1) in-frame with the T7 tag sequence of the pET3c expression vector (see Materials and Methods). This fragment corresponds to the smaller C-terminal domain of tubulin (aa 301-end) described by Kirchner and Mandelkow (*66*). On the basis of the formic acid digestion data described above, it should contain all the tau cross-linking sites. The recombinant α tubulin fragment has an apparent molecular mass of ~18 kDa and is expressed in bacteria at high levels but with low solubility (Figure 8A). Fortunately, the small quantity of soluble α tubulin in cell extracts can be detected with either a T7 tag antibody or the anti-α tubulin antibody DM1A (Figure 8A). Cross-linking experiments were performed using various tau polypeptides and total *Escherichia coli* soluble lysates, which contain a complex mixture of bacterial proteins and a small amount of soluble recombinant α tubulin fragment. The results revealed clear cross-linking of tau peptide $4R_{215-272}$ and the two tau fragments ($4R_{215-358}$ and $3R_{215-327}$) to the α tubulin C-terminal fragment as detected by the DM1A antibody (Figure 8B). In addition, $4R_{215-432}$ and $3R_{215-401}$ also cross-link to the α tubulin fragment (data not shown). That these tau peptides and proteins cross-link to the α tubulin C-terminal fragment even though it is present only at low levels in the complex lysate suggests that the cross-linking is specific.

Detectable levels of β tubulin expression in bacteria were not achieved either with pET3c or with other expression systems. Therefore, we generated two β tubulin synthetic peptides corresponding to amino acids 410−445 of the human β2 and β4 isotypes (see Materials and Methods). These peptides contain the C-terminal tau cross-linking site within the last 12 amino acids and, possibly, also the internal tau cross-linking site. Both peptides cross-linked to the tau

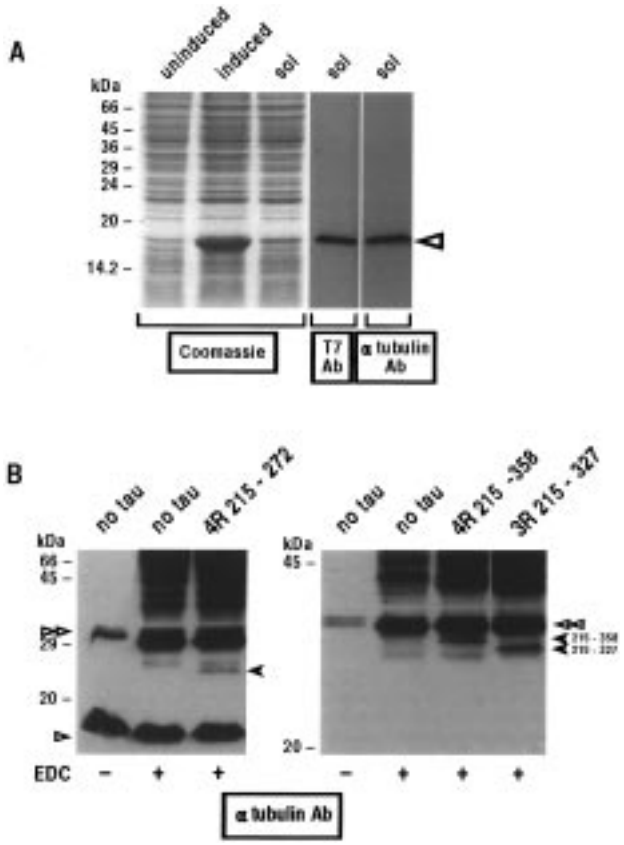

FIGURE 8: Expression of the α tubulin C-terminal domain and its cross-linking with the tau peptide or fragment. All proteins were fractionated using 15% acrylamide gels with 99% pure SDS. The arrowhead denotes the cross-linked complex of the tau peptide or fragment and the α tubulin C-terminal domain. The open arrowhead denotes the recombinant α tubulin C-terminal domain. The double open arrowheads denote cross-linked dimers of the α tubulin C-terminal domain. (A) Analysis of *E. coli* lysate containing the recombinant α tubulin C-terminal domain. For immunoblots, the T7 tag and anti-α tubulin (DM1A) antibodies were used. Sol denotes the soluble fraction of total lysate of the induced recombinant bacterial cells in 0.1 M PIPES (pH 6.8). (B) Immunoblots of products of tau peptide $4R_{215-272}$ and tau fragments $4R_{215-358}$ and $3R_{215-327}$ cross-linked to *E. coli* lysate containing the α tubulin C-terminal domain using the anti-α tubulin (DM1A) antibody. To fractionate the larger complexes present in the right-hand gel, the gel on the right was run longer than the gel on the left. As a consequence, the un-cross-linked α tubulin seen near the bottom of the gel on the left has run off the bottom of the gel on the right.

fragment $4R_{215-358}$, and the cross-linked species have the expected size of ~20 kDa (Figure 9). The extent of cross-linking of the β2 peptide and $4R_{215-358}$ was not reduced in the presence of a 10-fold molar excess of BSA (a nonspecific competitor) but was totally eliminated by a 10-fold molar excess of microtubules (a specific competitor; Figure 9), suggesting that the cross-linking of the β C-terminal peptides to $4R_{215-358}$ is specific. Both β peptides also cross-link to tau peptide $4R_{215-272}$ (data not shown). These results are consistent with previous studies in which tau and tubulin peptides were used (*20*).

The ability of tau to bind to the recombinant and synthetic tubulin C-terminal fragments demonstrates (i) that sufficient structural information is present within these relatively small fragments to allow binding to occur and (ii) that post-translational modifications are not required for the binding of tau to tubulin, although they may play a regulatory role.

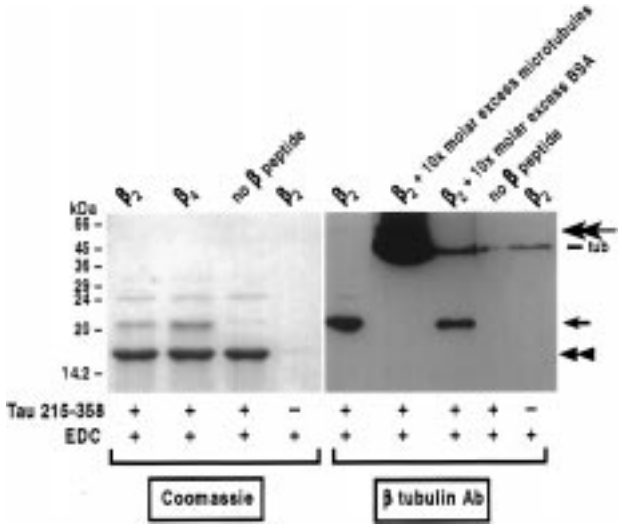

FIGURE 9: Cross-linking of tau fragment $4R_{215-358}$ with the β2 and β4 tubulin peptides. All proteins were fractionated using 15% acrylamide gels with 99% pure SDS. The immunoblot was probed with the anti-β tubulin (βI+II) antibody. The high-molecular mass band (~50 kDa) in the last two lanes is overflow of tubulin from the adjacent lane. The arrow denotes the cross-linked complex of tau fragment $4R_{215-358}$ and the β2 or β4 tubulin peptide. The double arrowheads denote non-cross-linked tau fragment $4R_{215-358}$. The double-headed arrow denotes the cross-linked complex of tau fragment $4R_{215-358}$ and the α or β tubulin monomer.

DISCUSSION

α and β Tubulin Each Possess Two Tau Cross-Linking Sites. Using different combinations of tau and tubulin fragments and a covalent cross-linking strategy, we have demonstrated that tau cross-links to two distinct sites on both α and β tubulin. These sites are located near the C termini of both α and β tubulin, one on either side of their respective C-terminal subtilisin cleavage sites, which we have mapped to ~12 amino acids from the C terminus in both α and β tubulin. Use of a zero-length cross-linker (EDC) allows identification of regions associating extremely closely with one another, thereby implicating these cross-linked regions as being in the vicinity of binding sites. On the basis of these observations and considerations, our data indicate the presence of either two distinct tau binding sites on each tubulin molecule or a single binding domain on each tubulin molecule that is bifurcated by the subtilisin digestion site. In either case, tau is interacting with tubulin sequences located on both sides of the subtilisin sites.

Our data demonstrate that 4R tau associates with the C termini of α and β tubulin via the R1 and/or the R1−R2 IR. It is also known that the R1−R2 IR has both microtubule binding and microtubule assembly activities (*23*). However, since the R1−R2 IR is absent in 3R tau, it is possible that only repeat 1 is involved in the binding of 3R tau to the tubulin C termini. Alternatively, the function of the R1−R2 IR could be replaced by the R1−R3 IR in 3R tau, since recent work demonstrates that this inter-repeat appears to be important for microtubule interaction in 3R tau (B. L. Goode et al., unpublished data). The data also suggest that tau associates with the tubulin sequences between amino acids ~305 and 430 via repeats 2, 3, and/or 4 in 4R tau and repeats 3 and/or 4 in 3R tau. In 4R tau, repeat 2 makes an especially strong contribution to microtubule binding, as does repeat 3 in 3R tau (*65*). On the basis of these observations, we suggest that repeat 2 and repeat 3 of 4R and 3R tau, respectively, are most likely to bear primary responsibility for tau binding to tubulin amino acids ~305−430.

The interaction of tau with tubulin sequences on both sides of the subtilisin sites may help explain some of the earlier discrepancies in the literature regarding the location of tau binding site(s) on microtubules (see the introductory section). The observations of Maccioni et al. (*40*), who showed that β422−434 is important for tau binding, and Littauer et al. (*43*), who suggest β434−440 is important for tau binding, are not necessarily conflicting but could result merely by detecting the interaction of tau with tubulin sequences on one or the other side of the subtilisin site. Our results also corroborate the finding of Maccioni et al. (*40*) that α430−441 is a tau binding site. However, we do not detect any tau cross-linking to the amino end of α tubulin, as suggested by Littauer et al. (*43*). In addition, our data do not explain the data of Marya et al. (*34*), who did not detect binding of tau to subtilisin-digested microtubules even though subtilisin cleaved at a site beyond amino acid 430 on both α and β tubulin in that study.

Finally, Cross et al. (*67*, *68*) have shown that MAP2 binds to two regions of β tubulin: aa 422−434 and the variable C-terminal domain (within the last 10−12 aa). Taken together with our data showing tau cross-linking to the two corresponding regions on β tubulin, it appears that MAP2 and tau may bind to β tubulin in a similar manner. The MAP2-binding site(s) on α tubulin is inferred to be on the C-terminal end since MAP2 failed to bind to subtilisin-digested microtubules (*69, 70*). However, it is unclear if there is one or more MAP2 binding sites on α tubulin.

Tau Can Bind to α and β Tubulin Independently. Considerable debate has focused upon the mechanism of tau binding to tubulin. Since tau promotes the assembly of tubulin dimers into microtubules at concentrations below that required for stable self-assembly, it has been suggested that tau acts by binding to tubulin dimers in solution (*71*). Alternatively, but not mutually exclusively, tau could stabilize otherwise unstable tubulin polymers to promote net assembly.

Taken together with earlier work, our data reveal several important features of the tau−tubulin interaction. First, tau can bind to unpolymerized tubulin polypeptides. This is supported by the fact that tau can bind to a recombinant α tubulin fragment and β tubulin synthetic peptides (*40*; Figures 8 and 9 in this study). Consistent with this conclusion, we have observed that tau and tau fragments can bind to tubulin maintained in an unpolymerized state by podophyllotoxin (data not shown). Thus, tau does not require a microtubule surface (i.e., a polymer-dependent conformation) to bind to tubulin. Rather, sufficient structural information for interaction is inherent in fragments of both tau and the tubulins. Second, tau can bind to α and β tubulin independently. This conclusion is supported by the observation that tau binds to the recombinant α tubulin fragment and the purified β tubulin peptides (Figures 8 and 9). Third, the ability of tau to bind to recombinant or synthetic tubulin fragments, which lack the normal post-translational modifications found in brain tubulin, demonstrates that post-translational modification is not required for the binding of tau to tubulin. Post-translational modification can however affect the affinity of the tau−tubulin interaction, as shown by Boucher et al. (*36*).

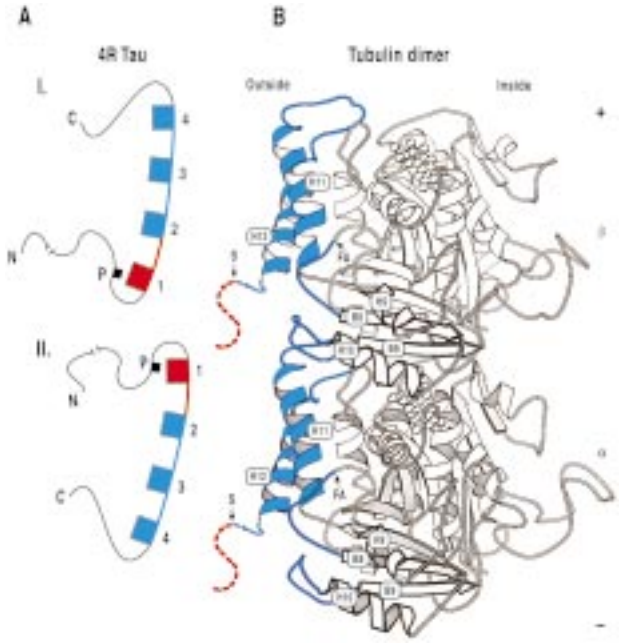

FIGURE 10: Two possible models by which tau might interact with microtubules. Tau is proposed to bind either to a single tubulin monomer or, alternatively, to two adjacent tubulin monomers. In the first case (A, part I), sequences in the R1 and/or the R1−R2 inter-repeat of a given tau molecule (shown in red) interact with the extreme C-terminal end of a tubulin monomer (the small C-terminal tail released by subtilisin cleavage; these sequences are also shown in red) while at the same time other tau sequences from the same tau molecule (some subset of the region shown in blue) interact with regions of tubulin located between the formic acid and subtilisin sites of the same tubulin subunit (shown in blue). In the second case (A, part II), the interactions are similar except that the "red region" of a given tau molecule interacts with the red region of one tubulin monomer while the "blue region" of the same tau molecule binds to the blue region of an adjacent tubulin monomer. The tubulin dimer drawing, constructed with the program Molscript (*80*), is based upon the model of Nogales et al. (*44*) and was kindly provided by K. Downing. Since the structures of the extreme C termini of each tubulin subunit could not be determined in that work because of disorder, we have drawn it in as a simple dotted line. S denotes the subtilisin sites; FA denotes the formic acid sites. For other sites of interest, please refer to Nogales et al. (*44*). Also note that the tau protein is not drawn to scale relative to the tubulin monomers.

Molecular Mechanism of the Binding of Tau to Tubulin. The data presented here demonstrate that tau binds to the C-terminal one-third of both α and β tubulin. This conclusion is consistent with the recent 3.7 Å density map of the tubulin dimer obtained by electron crystallography of zinc-induced tubulin sheets (*44*), which shows that these regions of the tubulins correspond to the entire outer surface of the microtubule. Using the nomenclature presented by Nogales et al. (*44*), the outer surface of the microtubule is composed of (i) the loops connecting H9 and B8, H10 and B9, and H11 and H12 (Figure 10B, marked in blue), (ii) the entire lengths of H11 and H12 (Figure 10B, marked in blue), and (iii) the highly acidic and variable C-terminal tail of ∼20 amino acids (Figure 10B). The loop connecting helices H11 and H12 is involved in the interaction with the adjacent monomer along the protofilament. Unfortunately, the positions of the 10 and 18 C-terminal amino acids of α and β tubulin could not be determined because of disorder.

Any model of the tau−tubulin interaction must account for the fact that tau can promote stable microtubule assembly and suppress microtubule dynamics (*72*−*75*). One activity very likely to contribute to the ability of tau to assemble and stabilize microtubules is promoting polymer-stabilizing allosteric effects upon binding to tubulin subunits. In support of this possibility, it is important to recall that tau can suppress microtubule dynamics when present at a very low molar stoichiometry relative to tubulin (*74*, *75*). This situation is quite similar to microtubule stabilization by paclitaxel (also effective substoichiometrically), which is believed to stabilize microtubule dynamics by increasing the number of tubulin subunit−subunit contact sites through conformational changes in the tubulin subunits upon drug binding (*76*, *77*). Unfortunately, in the absence of atomic level structural information on the tau−tubulin complex, we cannot yet envision how such an allosteric effect might occur at the molecular level. In addition to allosteric effects, it is also possible that tau could act by noncovalently cross-linking adjacent tubulin subunits by binding to microtubules at junctions between adjacent subunits as well as by neutralizing electrostatic repulsion between potentially adjacent subunits.

Via integration of our data with the Nogales et al. (*44*) structure and previous studies, it is possible to develop testable general models of the tau−tubulin interaction and, therefore, the mechanism(s) by which tau promotes microtubule stability. First, our data demonstrate that R1 and/or the adjacent inter-repeat of tau interacts with the short C-terminal tails of α and β tubulin (Figure 10, marked in red). Although the overall sequence of the C-terminal tail is somewhat divergent among the different tubulin isoforms, they are all highly acidic. We have shown previously that two particular lysine residues in the R1−R2 inter-repeat are critical for the tau−tubulin interaction (*23*). Further, these lysines require proper relative position to one another to function. There are also two similarly spaced lysines in R1. Thus, it seems highly likely that the interaction between repeat 1 and/or the first inter-repeat of tau and the 12 C-terminal amino acids of α and β tubulin is, at least in large part, driven by ionic interactions. In addition, it has long been known that removal of the C-terminal tails of the tubulins by subtilisin increases the inherent self-assembly ability of tubulin (*55*, *78*). The tubulin C-terminal tails somehow inhibit self-assembly, perhaps by simple electrostatic repulsion and/or by imposing a conformational state incompatible with efficient self-assembly. Maccioni et al. (*79*) have presented spectroscopic evidence consistent with the latter. Taken together, the simplest conclusion is that at least part of the interaction between tau and the 12 C-terminal amino acids serves to reverse the inhibitory effects of the tubulin C-terminal tails.

Integrating our data with the structure of Nogales et al. (*44*), we can propose two general models for the tau−microtubule interaction (Figure 10). Binding of repeat 1 and/or the first inter-repeat of tau to the C terminus and tau repeats 2, 3, and/or 4 to the loops and/or helices H11 or H12 of the same tubulin subunit could induce a polymer-stabilizing conformational effect (Figure 10A, part I, and Figure 10B). Alternatively, binding of repeat 1 and/or the first inter-repeat of tau to the C terminus of one subunit (marked in red) and tau repeats 2, 3, and/or 4 to the loops and/or helices H11 or H12 (marked in blue) of an adjacent tubulin subunit would noncovalently cross-link the two subunits, and at the same time, it could impose a polymer-

stabilizing conformational effect (Figure 10A, part II, and Figure 10B). The work of Maccioni et al. (*40*), demonstrating that a peptide corresponding to β tubulin amino acids 422−434 (which are present in helix H12) binds to tau, suggests that helix H12 is likely to be involved directly in the tau−tubulin interaction.

In summary, we have demonstrated that two distinct tau cross-linking sites exist on both α and β tubulin. One of these sites has been localized to a fragment of only 12 amino acids. The internal site is less well defined, and future investigations with additional recombinant fragments and synthetic peptides will make it possible to define this interaction at much higher resolution.

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