Functional Interactions between the Proline-rich and Repeat Regions of Tau Enhance Microtubule Binding and Assembly

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Tau is a neuronal microtubule-associated protein that promotes microtubule assembly, stability, and bundling in axons. Two distinct regions of tau are important for the tau–microtubule interaction, a relatively well-characterized "repeat region" in the carboxyl terminus (containing either three or four imperfect 18-amino acid repeats separated by 13- or 14-amino acid long inter-repeats) and a more centrally located, relatively poorly characterized proline-rich region. By using amino-terminal truncation analyses of tau, we have localized the microtubule binding activity of the proline-rich region to Lys^{215}–Asn^{246} and identified a small sequence within this region, $^{215}KKVAVVR^{221}$, that exerts a strong influence on microtubule binding and assembly in both three- and four-repeat tau isoforms. Site-directed mutagenesis experiments indicate that these capabilities are derived largely from Lys^{215}/Lys^{216} and Arg^{221}. In marked contrast to synthetic peptides corresponding to the repeat region, peptides corresponding to Lys^{215}–Asn^{246} and Lys^{215}–Thr^{222} alone possess little or no ability to promote microtubule assembly, and the peptide Lys^{215}–Thr^{222} does not effectively suppress in vitro microtubule dynamics. However, combining the proline-rich region sequences (Lys^{215}–Asn^{246}) with their adjacent repeat region sequences within a single peptide (Lys^{215}–Lys^{272}) enhances microtubule assembly by 10-fold, suggesting intramolecular interactions between the proline-rich and repeat regions. Structural complexity in this region of tau also is suggested by sequential amino-terminal deletions through the proline-rich and repeat regions, which reveal an unusual pattern of loss and gain of function. Thus, these data lead to a model in which efficient microtubule binding and assembly activities by tau require intramolecular interactions between its repeat and proline-rich regions. This model, invoking structural complexity for the microtubule-bound conformation of tau, is fundamentally different from previous models of tau structure and function, which viewed tau as a simple linear array of independently acting tubulin-binding sites.

INTRODUCTION

A number of different microtubule-associated proteins (MAPs) have been identified that have been shown to modulate microtubule assembly, dynamic behavior, and spatial organization (for reviews, Lee, 1993; Hirokawa, 1994; Kosik and McConlogue, 1994; Schoenfeld and Obar, 1994; Mandelkow and Mandelkow, 1995). Among the best characterized of the MAPs is tau (for MAP reviews, Wiche *et al.*, 1991; Mandelkow and Mandelkow, 1993; Goedert *et al.*, 1994; Trojanowski and Lee, 1994), a collection of MAPs (37–45 kDa) that colocalize with axonal microtubules in neurons.

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Although tau proteins are encoded by a single gene (Lee et al., 1988), alternative RNA splicing (Goedert et al., 1989a; Himmler, 1989; Himmler et al., 1989; Kosik et al., 1989; Goedert and Jakes, 1990) and differential phosphorylation (Butler and Shelanski, 1986) lead to the expression of more than 60 tau isoforms in the adult brain (Larcher et al., 1992). In addition, the number of different tau isoforms expressed increases markedly as brain development proceeds (Cleveland et al., 1977a,b; Mareck et al., 1980; Francon et al., 1982; Oblinger et al., 1991). Thus, these data suggest that tau might play important roles in neuronal development and maintenance. Investigations of tau action in neuronal cells have supported this view, demonstrating that tau can promote the net assembly and stabilization of microtubules, can drive neurite outgrowth (Drubin et al., 1985; Hanemaaijer and Ginzburg 1991; Shea et al., 1992; Esmaeli-Azad et al., 1994), and is critical for the establishment of neuronal cell polarity (Caceres et al., 1990–1992). Transfection studies in non-neuronal cells have shown further that tau can nucleate microtubule assembly from noncentrosomal sites, can increase microtubule stability, and can promote microtubule bundling (Drubin and Kirschner, 1986; Kanai et al., 1989, 1992; Lewis et al., 1989; Bass et al., 1991; Knops et al., 1991; Lee and Rook, 1992; Takemura et al., 1992).

Although these studies have begun to define the functions of tau in vivo, in vitro biochemical analyses have provided a more molecular view of tau action. Early investigations showed that tau promotes tubulin polymerization by lowering the critical concentration of microtubule assembly and suppressing microtubule depolymerization (Weingarten et al., 1975; Cleveland et al., 1977a). More recent studies with video-enhanced light microscopy have measured the effects of tau on individual parameters of microtubule dynamics (growing, shortening, catastrophe, and rescue). Studies performed under conditions of net microtubule assembly show that tau increases the apparent microtubule growing rate and suppresses catastrophe (Drechsel et al., 1992; Pryer et al., 1992; Trinczek et al., 1995). These observations are likely relevant to conditions in early neuronal development, when axonal processes are elongating and microtubule polymer mass is increasing. In contrast, under steady-state conditions (microtubule polymer mass equilibrium), tau has been shown to decrease both microtubule growing and shortening rates and to increase the percentage of time that microtubules spend in an attenuated (pause) state (Panda et al., 1995). These observations are consistent with recent microinjection studies on the effects of MAPs on microtubule dynamics in living cells (Dhamodharan and Wadsworth, 1995) and likely reflect conditions in adult neurons, where microtubule polymer mass levels do not change appreciably. Thus, tau may have different roles at different stages of development, initially driving new microtubule assembly and axonal extension and later performing a stabilizing function, suppressing both microtubule growing and shortening to add structural integrity to axons.

In contrast to the detail in which the effects of tau on microtubule dynamics and organization are understood, surprisingly little is known about the biochemical structure–function relationships underlying these effects. Essentially nothing is known about the three-dimensional structure of tau or tubulin (no crystal structures are yet available), the molecular nature of the tau–microtubule interaction is unclear and many fundamental questions remain. For instance, it is unclear whether tau binds to α- and/or β-tubulin in microtubules, whether individual tau molecules span multiple tubulin subunits in the microtubule lattice, and whether tau binds to microtubules as a monomer, dimer, or higher multimer. Biophysical studies have failed to detect significant secondary or tertiary structure for tau in solution (Cleveland et al., 1977b; Schweers et al., 1994), but it remains possible that tau might adopt a more complex and folded structure upon functional association with microtubules.

One series of investigations that has provided valuable clues regarding the tau–microtubule interaction is the identification and molecular dissection of tau microtubule-binding domains. Two general approaches have been taken in defining these sequences. First, wild-type and mutated tau cDNAs have been transfected into non-neuronal cells (lacking endogenous tau) and their relative abilities to bind to microtubules have been compared by double immunofluorescence microscopic localization of tau and tubulin (Lee et al., 1989; Kanai et al., 1989, 1992; Lewis et al., 1989; Lewis and Cowan, 1990; Lee and Rook, 1992). Alternatively, wild-type and mutant tau polypeptides have been expressed in bacteria or by in vitro transcription/translation and their microtubule binding affinities have been compared by using in vitro microtubule cosedimentation assays (for review, Butner and Kirschner, 1991; Goode and Feinstein, 1994; Gustke et al., 1994). Although transfection assays have the advantage that they are performed in living cells, in vitro assays are more quantitative, since the concentrations of tau and tubulin can be more easily varied. As a result, binding constants can be determined for wild-type and mutant tau polypeptides and relatively small changes in microtubule-binding affinities can be detected, allowing microtubule-binding domains to be mapped at high resolution.

From both the transfection and in vitro biochemical studies, it is well established that there are two general regions in tau that contribute strongly to microtubule-binding affinity, a repeat region and a proline-rich region. The repeat region, located near the carboxyl terminus of tau, is composed of either three or four

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imperfect 18-amino acid repeats, separated by 13 or 14 amino acid inter-repeat (IR) regions (Lee et al., 1988; Goedert et al., 1989a, b; Himmler, 1989; Himmler et al., 1989; Kosik et al., 1989). A single three-repeat tau isoform (3Rtau) is expressed in fetal neurons, while both three- and four-repeat tau isoforms (4Rtau) are expressed in adult neurons. Each repeat makes a significant but variable contribution to the microtubule-binding affinity in tau (Butner and Kirschner, 1991; Goode and Feinstein, 1994), and an especially strong microtubule-binding site specific to 4Rtau is located in the inter-repeat region between repeats 1 and 2 (the R1–R2 IR; Goode and Feinstein, 1994; Trinczek et al., 1995). Furthermore, synthetic peptides corresponding to repeat 1, repeat 3, and the R1–R2 IR alone are sufficient to promote microtubule assembly (Ennulat et al., 1989; Goode and Feinstein, 1994). Finally, peptides corresponding to repeat 1 and the R1–R2 IR alone are sufficient to suppress microtubule dynamics in a manner that is qualitatively similar to full-length tau (Panda et al., 1995).

On the other hand, the mechanism(s) by which the proline-rich region affects microtubule binding and assembly remains poorly characterized. Although deletion of the proline-rich region from tau results in a marked drop in microtubule-binding affinity in vitro (Gustke et al., 1994; Figure 1) and in vivo (Lewis et al., 1989; Lee and Rook, 1992), the proline-rich region alone binds extremely weakly to microtubules (Butner and Kirschner, 1991; Goode and Feinstein, 1994; Gustke et al., 1994). Thus, the proline-rich region contributes to the microtubule-binding affinity of tau by poorly understood mechanisms, perhaps distinct from those utilized by the repeat region. In this study, we have used amino-terminal deletion analyses, site-directed mutagenesis, and synthetic peptides to examine the mechanisms by which the proline-rich region enhances the microtubule binding and assembly capabilities of tau.

MATERIALS AND METHODS

Amino-Terminal Truncations and Site-directed Mutagenesis

Amino-terminal deletions were required to map the microtubule-binding activities of the proline-rich region since carboxyl end truncation to the border of the proline-rich region abolishes almost all detectable microtubule-binding affinity (Figure 6 in Butner and Kirschner, 1991; Figure 4 in Goode and Feinstein, 1994). However, amino end deletions do not leave an intact start codon and 5' untranslated region for in vitro translation. Therefore, it was necessary to use oligonucleotide-mediated site-directed mutagenesis procedures to internally delete amino-terminal sequences in tau. All amino-terminal truncations/deletions (Δ21–151, Δ2–214, Δ2–221, Δ2–232, Δ2–246, Δ2–263, Δ2–277) were generated in parallel in 3R (Goode and Feinstein, 1994) and 4R rat tau (Kosik et al., 1989) cDNAs by using the Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA) based on the procedure of Deng et al. (1992). This oligonucleotide-mediated DNA "looping-out" proce-

dure allows the precise internal deletion of any desired coding sequences, leaving an intact 5' untranslated region and initiation codon for efficient in vitro translation. Oligonucleotides synthesized for this procedure were 30 nucleotides long, corresponding to 15 nucleotides on each side flanking the sequence targeted for deletion. Alanine substitutions were generated in full-length 3Rtau and 4Rtau at positions Lys^{215}/Lys^{216}, Arg^{221}, and $Lys^{215}/Lys^{216}/Arg^{221}$ using methods described previously (Goode and Feinstein, 1994) that also used the Transformer kit from Clontech. All deletions and point mutations were verified by direct DNA sequence analysis. Plasmid clones were then amplified and purified for use in in vitro transcription/translation reactions.

In Vitro Transcription and Translation of Tau Polypeptides

Coupled in vitro transcription/translation reactions were performed according to the specifications of the Promega TnT rabbit reticulocyte lysate kit (Madison, WI) by using [^{35}S]methionine (>1000 Ci/mmol, 10 μCi/μl, New England Nuclear, Boston, MA) to label the products. Prior to use in microtubule-binding assays, small aliquots (1 μl) of each translation product were fractionated on either 10% or 15% polyacrylamide gels, depending on the size of the products. Gels were fixed in 30% ethanol/10% glacial acetic acid, enhanced with APEX (55% glacial acetic acid, 15% xylenes, 30% ethanol, 0.5% 2,5-diphenyloxazole), rehydrated in H_2O for 5 min, dried, and fluorographed. Product yields were determined by the specifications in the Promega Protocols and Applications Guide as described previously (Goode and Feinstein, 1994). Routinely, specific activities of 10^6 cpm/ng of tau were obtained. Transcription/translation reactions yielded tau at 0.1 ng/μl. Translation products were stored at $-20°C$ and used in microtubule-binding assays within 1 wk of synthesis. All tau polypeptides used in a given set of microtubule-binding assays were synthesized at the same time.

Tubulin Purification

MAP-depleted tubulin was purified from bovine brains by two cycles of temperature-controlled polymerization and depolymerization, followed by phosphocellulose chromatography (Mitchison and Kirschner, 1984) in PME buffer [50 mM piperazine-N,N'-bis(ethanesulfonic acid) (pH 6.8), 1 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid] supplemented with 1 mM GTP (Sigma, St. Louis, MO). Aliquots were then drop frozen in liquid nitrogen and stored at $-70°C$. Protein concentrations were determined using the method of Bradford (1976). SDS-PAGE analysis of the MAP-depleted tubulin stock revealed no detectable MAP contamination, even when the lane was grossly overloaded (our unpublished results).

Microtubule-binding Assays

Microtubule-binding assays were performed as described (Goode and Feinstein, 1994) with the following modifications. Microtubules were assembled from 3.5 mg/ml MAP-depleted tubulin at 35°C for 30 min in the presence of 1 mM GTP and 30 μM Taxol (Calbiochem, LaJolla, CA). The integrity of the Taxol-stabilized microtubules was verified by electron microscopy, as described by Davis et al. (1993). Microtubule dilutions were made in BRB-80 buffer [80 mM piperazine-N,N'-bis(ethanesulfonic acid) (pH 6.8), 1 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid] supplemented with 1 mM GTP and 10 μM Taxol. Then, 9 μl of Taxol-stabilized microtubules, ranging from 0.01 μM to 40 μM, was mixed with 1 μl of in vitro-translated tau (~0.25 nM tau, final concentration) and incubated at room temperature for 15 min to reach binding equilibrium. At the minimum, all reactions contain at least a 40-fold molar excess of tubulin compared with tau. In most cases, the excess is much more.

The 10-μl binding reactions were layered over an 80-μl sucrose cushion (50% sucrose in BRB-80 buffer supplemented with 10 μM Taxol) in ultraclear centrifuge tubes, 5 $\times$ 20 mm (Beckman, Palo Alto, CA), and centrifuged for 12 min, 150,000 $\times$ g at room temperature in either a Beckman SW50.1 rotor or a Sorvall AH650 rotor, in each case with adaptors for tubes of 5 $\times$ 20 mm. Supernatants and pellets were collected as described (Goode and Feinstein, 1994) and fractionated on either 10% or 15% polyacrylamide gels. Gels were processed as described above and fluorographed. Relative levels of tau in the supernatants and pellets were determined by laser densitometry. The concentration of tubulin required to cosediment 50% of the tau is defined as the apparent K_d. Full-length tau was included as an internal control in each binding assay.

Peptide Synthesis and Purification

The following synthetic tau peptide amides were synthesized on a Millipore 9050 Plus peptide synthesizer by DIPCDI/HoBT chemistry (amino acid numbering is according to the sequence of the 4R rat tau cDNA; Kosik et al., 1989): [215]KKVAVVRT[222], a "scrambled" tau (residues 215–222) control peptide AVKVRTVK, [215]KKVAVVRTPPK-SPSASKSRLQTAPVPMPDLKN[246], [215]KKVAVVRTPPKSPSASKSR-LQTAPVPMPDLKNVRSKIGSTENLKHQPGGGKVQIINKK[272], [222]TP-PKSPSASKSRLQTAPVPMPDLKN[246], [233]RLQTAPVPMPDLKN[246], [247]VRSKIGSTENLKHQPGGGKVQIINKK[272], and [359]NKKIETH-KLT[368]. A subset of these peptides (peptides 215–246, 215–272, 222–246, and 233–246) are acetylated on their terminal amino group. After cleavage and deprotection (Solé and Barany, 1992), all peptides except 215–272 were purified to >95% purity by reverse-phase high performance liquid chromatography, lyophilized, and resuspended in H_2O. In the case of the 58-amino acid peptide 215–272, >80% of the material was within 32 daltons (by mass spectroscopy) of the predicted 6265 daltons, suggesting that relatively minor chemical modification(s) had occurred during synthesis. Peptide concentrations were determined from dry peptide weights and confirmed after resuspension in H_2O by A_{205} absorption. Peptide integrity was verified by electrospray mass spectrometry.

Since the synthetic peptides were generated with amide groups at their carboxyl termini, we considered the possibility that an amide group on Arg[221] might affect the potential microtubule binding activity of [215]KKVAVVR[221]. For this reason, we included one additional residue (Thr[222]) in the peptide corresponding to this region, [215]KKVAVVRT[222]. The control peptide for [215]KKVAVVRT[222] has an identical amino acid content arranged in a scrambled order (AVKVRTVK).

Synthesis and Purification of Recombinant Tau Polypeptides

Recombinant tau polypeptides were expressed in BL21 (DE3) using the pET vector expression system of Novagen (Madison, WI). The coding sequences from cDNAs encoding the full-length 4R rat tau (Kosik et al., 1989) and a KK/AA (215/216) mutant 4Rtau were subcloned into the NdeI site of the pET-3c vector and introduced into BL21 (DE3) cells. Tau expression was induced by the addition of 0.4 mM isopropyl β-D-thiogalactoside to cells in logarithmic phase growth. Two hours after induction, cells were pelleted and resuspended in BRB-80 buffer supplemented with 0.1% 2-mercaptoethanol and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Cells were lysed by sonication and cleared by centrifugation at 18,000 $\times$ g for 15 min at 4°C, and the supernatants were transferred to new tubes and placed in a boiling water bath for 10 min. Boiled supernatants were centrifuged as above and then further purified by phosphocellulose chromatography. A 30- to 50-ml column of P11 phosphocellulose (Whatman, Hillsboro, OR) was used to purify ~10 mg of tau (from ~1 l of BL21 (DE3) cells). After addition of the boiled and cleared cell lysate, the column was washed with 10 vol of BRB-80/0.1% 2-mercaptoethanol buffer, and the tau was eluted with a linear

gradient of NaCl (0.1–0.6 M) in the same buffer. Tau-containing fractions were identified by PAGE, pooled, then desalted, and concentrated simultaneously in Centriprep 10 columns (Amicon, Beverly, MA). The tau stocks were drop frozen in liquid nitrogen in 50-μl aliquots. Final stock concentrations of wild-type and mutant 4Rtau preparations were determined by optical density at 278 nm with an extinction coefficient for tau of 0.29 (Cleveland et al., 1977b). The concentration of 4Rtau (Lys[215]–Gly[358]) was determined by comparison to 4Rtau by using the Bradford assay and Coomassie blue staining of SDS-PAGE-fractionated tau.

Microtubule Assembly Assays and Electron Microscopy

To initiate microtubule assembly, 8 μl of MAP-depleted tubulin (2.5 mg/ml) in BRB-80 buffer supplemented with 1 mM GTP were added to 2 μl of H_2O, recombinant tau protein, or synthetic tau peptide at different concentrations. Reactions were mixed by pipetting, incubated at 35°C for 30 min, then gently layered over a prewarmed 80-μl 50% sucrose cushion in BRB-80 buffer, and centrifuged as described above for microtubule-binding assays. After centrifugation, pellets and supernatants were collected, fractionated on 10% polyacrylamide gels, Coomassie blue-stained, and quantitated by using laser densitometry to determine the percentage of tubulin in polymer (pellet). To verify that the pelleted material was composed of microtubules, not tubulin aggregates, samples of reactions were fixed by a 1:20 dilution in PME buffer containing 0.5% glutaraldehyde and 30% glycerol, then negatively stained with uranyl acetate, and visualized by electron microscopy as described in Davis et al. (1993).

Video-enhanced Light Microscopy

The effects of the tau peptide [215]KKVAVVRT[222] and the control scrambled peptide AVKVRTVK on microtubule dynamics were measured by video-enhanced light microscopy as described for the repeat 1 and the R1–R2 IR tau peptides (Panda et al., 1995). Briefly, tubulin was mixed with axoneme seeds and polymerized in PME buffer containing 1 mM GTP in the presence or absence of 1 mM peptide. After steady-state equilibrium was achieved (~30 min), the polymerization dynamics of individual microtubules were recorded at 37°C for a maximum of 45 min. A computer-based analysis system (a kind gift from Dr. E. D. Salmon, University of North Carolina, Chapel Hill, NC) was used to determine microtubule length changes at plus ends over time. For a more detailed method of analyses, see Panda et al. (1995).

RESULTS

Microtubule-binding Activity in the Proline-Rich Region of Tau Is Localized Primarily between Lys[215] and Asn[246]

Our initial strategy was to use amino end deletion analyses to localize sequences within the proline-rich region of tau that influence the ability of tau to bind to microtubules. As shown in Figure 1, deletion of a large segment from the amino end of tau (amino acids 21–151) had no significant effect on microtubule binding in 3Rtau and perhaps a slight effect on microtubule binding in 4Rtau. These results suggest that the amino third of tau does not strongly influence microtubule binding, a view consistent with previous studies (Lewis et al., 1989; Lee and Rook, 1992; Gustke et al., 1994). On the other hand, further truncation analyses revealed that deletion of the relatively small region
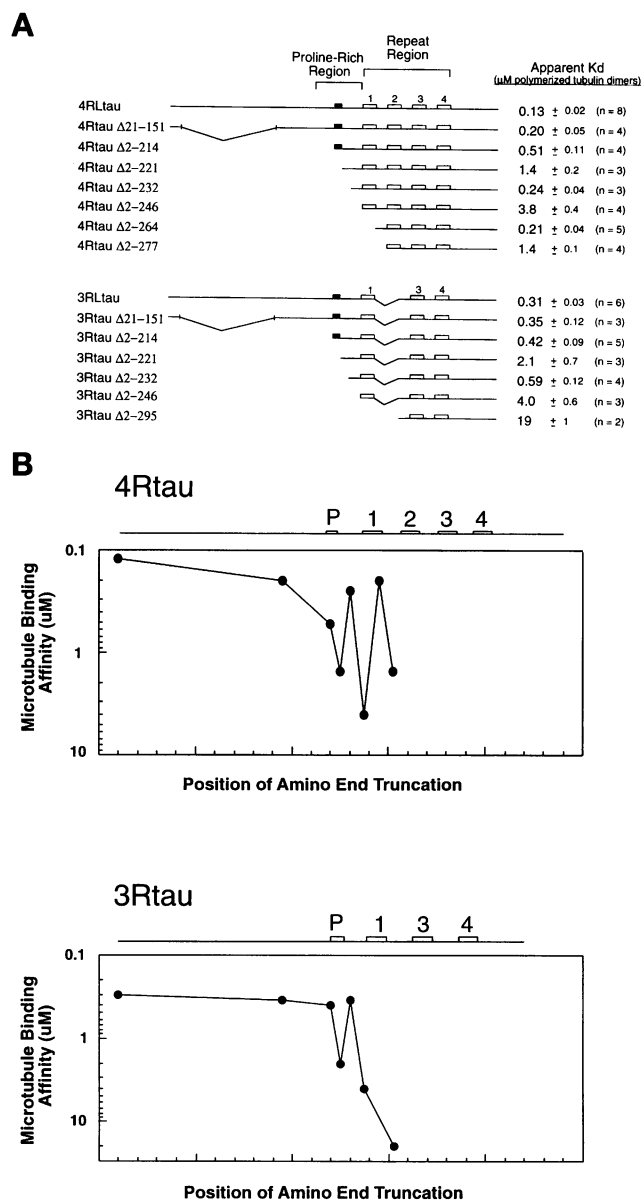
A

		Apparent Kd (uM polymerized tubulin dimers)
4RLtau		0.13 ± 0.02 (n = 8)
4Rtau Δ21–151		0.20 ± 0.05 (n = 4)
4Rtau Δ2–214		0.51 ± 0.11 (n = 4)
4Rtau Δ2–221		1.4 ± 0.2 (n = 3)
4Rtau Δ2–232		0.24 ± 0.04 (n = 3)
4Rtau Δ2–246		3.8 ± 0.4 (n = 4)
4Rtau Δ2–264		0.21 ± 0.04 (n = 5)
4Rtau Δ2–277		1.4 ± 0.1 (n = 4)
3RLtau		0.31 ± 0.03 (n = 6)
3Rtau Δ21–151		0.35 ± 0.12 (n = 3)
3Rtau Δ2–214		0.42 ± 0.09 (n = 5)
3Rtau Δ2–221		2.1 ± 0.7 (n = 3)
3Rtau Δ2–232		0.59 ± 0.12 (n = 4)
3Rtau Δ2–246		4.0 ± 0.6 (n = 3)
3Rtau Δ2–295		19 ± 1 (n = 2)

B

Figure 1. Microtubule-binding affinities of full-length and amino-truncated tau polypeptides. (A) The microtubule-binding affinities (K_d values) of in vitro-translated tau polypeptides were determined by using a microtubule cosedimentation assay (see MATERIALS AND METHODS). The K_d values are the average of values obtained from multiple experiments, with the SEM and the number of times each construct was assayed shown to the right. In each experiment, truncated tau polypeptides were directly compared with full-length tau as an internal control. In the schematic diagram of tau, 18-amino acid repeats (repeats 1–4) are represented by open boxes and the [215]KKVAVVR[221] sequence in the proline-rich region is shown as a solid box. (B) Microtubule-binding affinity for sequential amino-terminal truncations in 3Rtau and 4Rtau. Note the nonlinear patterns through the proline-rich region and repeat 1, which suggests structural and functional complexity in this region of tau.

between amino acids 215 and 246 caused a 7- to 10-fold reduction in microtubule-binding affinity in both 3R and 4Rtau. These results suggest that the major functional activities of the proline-rich region are located between Lys[215] and Asn[246].

As noted above, there was a small but perhaps significant functional contribution made to microtubule-binding affinity by amino acids 2–214 in 4Rtau but not 3Rtau. The isoform-specific nature of this effect raises the possibility that 3R and 4Rtau may assume distinct structural conformations upon binding to microtubules, a phenomenon that we are investigating in greater detail.

Identification of a Sequence, [215]KKVAVVR[221], that Strengthens Microtubule-binding Affinity in both 3R and 4Rtau

Comparison of constructs Δ2–214 and Δ2–221 (Figure 1) reveals that amino end deletion of the seven amino acids [215]KKVAVVR[221] from both 3Rtau and 4Rtau leads to a three- to fivefold reduction in microtubule-binding affinity. This effect is equal to or stronger than the effect of carboxyl truncation of any of the 18-amino acid repeats in 4Rtau and only slightly less than the effect of truncation of the R1–R2 IR (Goode and Feinstein, 1994). These data demonstrate that [215]KKVAVVR[221] enhances microtubule-binding affinity in both 3R and 4Rtau. However, from a mechanistic point of view, these data do not distinguish between direct binding of [215]KKVAVVR[221] to microtubules versus an indirect mechanism of [215]KKVAVVR[221] action.

Sequential Amino End Truncation Analyses in Tau Suggest Structural Complexity Underlying Tau Function

Further amino end deletion of amino acids 222–246 in the proline-rich region and amino acids 247–295 in the repeat region led to an unexpectedly complex pattern of loss and gain of microtubule-binding function (Figure 1B). Although a precise structural and functional interpretation of these data is not yet possible, the simplest interpretation suggests that the microtubule-bound conformation of tau involves interactions between the proline-rich and repeat regions. This view of tau structure (see DISCUSSION and model in Figure 7) differs considerably from previous models of tau structure (Butner and Kirschner, 1991; Goode and Feinstein, 1994; Gustke et al., 1994), which suggested that the microtubule-binding region is composed of linearly arranged independent microtubule-binding sites.

Lys^{215}/Lys^{216} and Arg^{221} Are Important for Microtubule Binding in both 3R and 4Rtau

Among the interesting features of the ^{215}KKVAVVR^{221} sequence is its possible similarity with the R1–R2 IR microtubule-binding domain, ^{265}KVQIINKK^{272} (Goode and Feinstein, 1994). The microtubule-binding activity of the R1–R2 IR is derived primarily from its three lysine residues, which are separated by five uncharged residues that do not appear to contribute directly to microtubule binding (Goode and Feinstein, 1994). This sequence alone is sufficient to bind to microtubules, to promote microtubule assembly (Goode and Feinstein, 1994), and to suppress microtubule dynamics (Panda *et al.*, 1995). The ^{215}KKVAVVR^{221} sequence also contains two adjacent lysine residues separated from a third positively charged residue (Arg^{221}) by several uncharged amino acids, raising the possibility that it may contribute to microtubule binding in tau by a mechanism similar to that of the R1–R2 IR.

To test this possibility, we used site-directed mutagenesis to generate lysine to alanine substitutions at positions 215/216 and 221 in 3R and 4Rtau and then directly compared the microtubule-binding affinities of the mutant and wild-type polypeptides. As shown in Figure 2, the mutants 4Rtau KK/AA(215/216), 3Rtau KK/AA(215/216), 4Rtau R221A, and 3Rtau R221A bind to microtubules with two- to threefold weaker affinity than their wild-type counterparts. Furthermore, the triple mutant 4Rtau KKK/AAA (215/216/221) binds threefold weaker than wild-type 4Rtau. These data demonstrate that Lys^{215}/Lys^{216} and
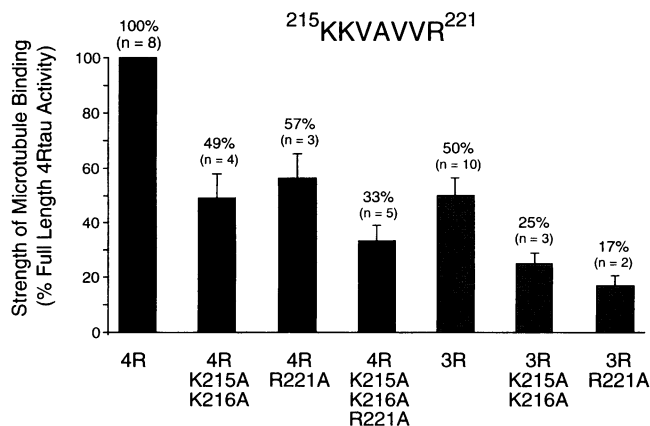
Arg^{221} make important contributions to microtubule binding in tau, approximately equivalent to the contribution of any single 18-amino acid repeat in 4Rtau as measured by carboxyl end truncation (Goode and Feinstein, 1994). These data also agree well with the threefold reduction observed upon amino truncation of ^{215}KKVAVVR^{221} (Figure 1), demonstrating that Lys^{215}/Lys^{216} and Arg^{221} can fully account for the effects of ^{215}KKVAVVR^{221} upon tau microtubule-binding activity.

Lys^{215}/Lys^{216} Are Important for Tau Polypeptide-induced Microtubule Assembly

We next tested whether or not ^{215}KKVAVVR^{221} is important for the ability of tau to promote microtubule assembly. We used the pET bacterial expression system to generate full-length wild-type 4Rtau and mutant 4Rtau KK/AA (215/216) polypeptides and then directly compared their abilities to promote tubulin polymerization at different tau polypeptide concentrations. In this assay, the relative ability of a protein to promote microtubule assembly can be assessed as the concentration of protein required to drive half of the maximally assembled tubulin in the reaction into polymer. As shown in Figure 3, the mutant 4Rtau KK/AA (215/216) protein promotes microtubule assembly approximately twofold less efficiently than the wild-type 4Rtau protein. Thus, together with the data in Figure 2, these data further demonstrate that ^{215}KKVAVVR^{221} is important for both microtubule binding and assembly in the intact tau molecule.

Figure 2. Effects of site-directed mutagenesis of Lys^{215}/Lys^{216} and Arg^{221} on the microtubule-binding affinities of 3R and 4Rtau. A single arginine to alanine mutation at position 221 and double lysine to alanine substitutions at positions 215 and 216 were generated in both 3Rtau and 4Rtau, and a triple mutation KKR/AAA (215/216/221) was generated in 4Rtau. Mutant and wild-type full-length in vitro-translated tau polypeptides were then compared in microtubule-binding affinity assays (see MATERIALS AND METHODS). The relative microtubule-binding affinity of each tau polypeptide is expressed as the percentage of wild-type 4Rtau-binding affinity (K_d). Error bars represent the SEM.

Synthetic Peptide ^{215}KKVAVVRT^{222} Has Minimal Effects on Microtubule Dynamics

Another potential activity of the ^{215}KKVAVVRT^{222} sequence is its possible effects on microtubule dynamics. Previously, we have shown that synthetic peptides corresponding to either repeat 1 or to the R1–R2 IR alone are sufficient to suppress microtubule dynamics in a sequence-dependent manner and in a manner qualitatively similar to intact tau (Panda *et al.*, 1995). However, as shown in Figure 4 and Table 1, a ^{215}KKVAVVRT^{222} synthetic peptide (designated as P1) did not appreciably affect microtubule dynamics. A scrambled form of P1, P1(S), also had no significant effect on microtubule dynamics (our unpublished results). These observations are in marked contrast to the activities of repeat 1 and R1–R2 IR peptides (Figure 4; see also Panda *et al.*, 1995). The only minor effect of the P1 peptide was a doubling of the time that microtubules spent in an attenuated (pause) state, neither shortening nor growing detectably. However, since this effect represents a rather small increase over background values, it may not be significant. We conclude that the ^{215}KKVAVVRT^{222} sequence alone has little or no effect on microtubule dynamics or assembly.

Synthetic Peptides Corresponding to Subregions of the Proline-rich Region Alone Do Not Promote Significant Levels of Microtubule Assembly

To investigate further the mechanisms by which the proline-rich region strengthens microtubule binding and promotes microtubule assembly in intact tau, we next generated a panel of synthetic peptides corresponding to different portions of the proline-rich region of tau and assayed their abilities to promote microtubule assembly. As shown in Figure 5A, the P123 peptide (Lys^{215}–Asn^{246}) alone promoted only very low levels of tubulin polymerization and only at very high peptide concentrations. Indeed, the activity of this peptide was far too weak to measure a half-maximal concentration. In contrast, a 26-amino acid repeat region peptide (Val^{247}–Lys^{272}; corresponding to repeat 1 plus the R1–R2 IR) promoted effective microtubule assembly with a half maximum of ~175 μM. It is noteworthy that these two peptides are similar in length and have a similar net basic charge. The very high concentration of P123 peptide necessary to exhibit even low activity makes it difficult to distinguish genuine activity from nonspecific activity. Regardless, it is clear that P123 has either little or no inherent microtubule assembly activity.
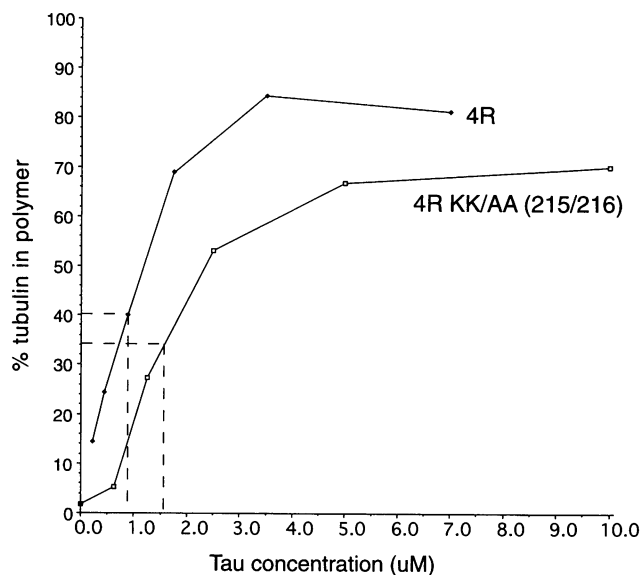
Figure 3. Effects of a 4Rtau KK/AA (215/216) double mutation on the ability of recombinantly expressed tau to induce microtubule assembly. Wild-type and mutant (K215A/K216A) recombinant 4Rtau polypeptides were expressed in *Escherichia coli*, purified, and assayed for their ability to promote microtubule assembly. A polymer mass assay was used to monitor microtubule assembly: purified tubulin (2 mg/ml) was incubated for 30 min at 35°C in the presence of different concentrations of tau polypeptides. Polymerized tubulin was separated from free tubulin by ultracentrifugation, and the pellets and supernatants were analyzed by SDS-PAGE and Coomassie blue staining. Laser densitometry was used to determine the percentage of tubulin in polymer in each reaction.

In addition, various subregions of P123 (P1, residues 215–222; P23, residues 222–247; P3, residues 233–247) were assayed. None exhibited significant microtubule-binding activity, even at 1 mM concentrations (our unpublished results). Thus, neither the P123 region nor subregions within it are sufficient to promote strong microtubule assembly, unlike peptides corresponding to the repeat region.

Joining of Proline-rich Region Sequences Lys^{215}–Asn^{246} to Repeat Region Sequences Dramatically Enhances Microtubule Assembly

The data in Figure 1 suggest that the repeat region (amino acids 247–358) and/or the extreme carboxyl terminus of tau (amino acids 359–432) might be the target of intramolecular interactions with the proline-rich region. We first tested the hypothesis that the most likely target of the proline-rich region might be the adjacent se-
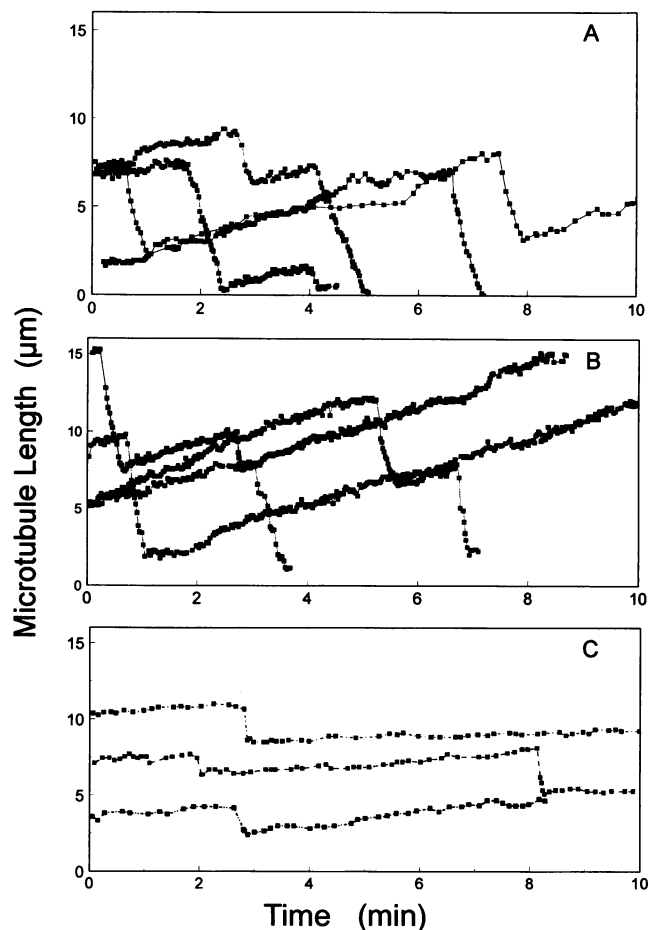
Figure 4. Changes in steady-state plus end microtubule lengths with time in the absence (A) or presence (B) of 1000 μM P1 peptide (^{215}KKVAVVRT^{222}) or of 1000 μM R1–R2 IR (^{265}KVQIINKK^{272}) peptide (C). Data presented in C are from Panda *et al.* (1995).

Table 1. Effects of the P1 peptide (^{215}KKVAVVRT^{222}) and a scrambled P1 peptide, P1(S) (AVKVRTVK) on dynamic instability parameters at the plus ends of microtubules at steady state

	Control (no peptide)	P domain	P(S) domain
Rate (μm/min)			
Growing	0.92	1.03	1.01
Shortening	12.5	14.2	17.3
Phase duration (min)			
Growing	2.30	2.6	2.52
Shortening	0.41	0.45	0.36
Attenuation	0.91	1.76	0.78
% time in phase			
Growing	84.2	83.1	88.2
Shortening	11.2	8.0	7.3
Attenuation	4.6	8.9	4.6
Length (μm/event)			
Growing	2.12	2.70	2.53
Shortening	5.0	6.4	6.2
Transition frequencies (min^{-1})			
Catastrophe	0.32	0.19	0.22
Rescue	2.01	1.52	2.0
Transition frequencies (μm^{-1})			
Catastrophe	0.36	0.21	0.23
Rescue	0.16	0.11	0.10
Dynamicity (μm/min)	2.16	1.99	2.14

Tubulin concentration was 14 μM. All other conditions were as described by Panda *et al.* (1995).

quences in the repeat region. To test this model, we prepared two synthetic tau peptides, Lys^{215}–Lys^{272} and Val^{247}–Lys^{272}, both of which include R1 and the R1–R2 IR, but differ by the presence or absence of the proline-rich region sequence Lys^{215}–Asn^{246}. We then directly compared the abilities of these two peptides to promote tubulin polymerization.

As shown in Figure 5, B and C, the Val^{247}–Lys^{272} peptide (R1 plus R1–R2 IR) has a max of ~175 μM and the Lys^{215}-Lys^{272} peptide (P123 plus R1 plus R1–R2 IR) has a max of ~17 μM, demonstrating that the addition of Lys^{215}–Asn^{246} improves microtubule assembly activity by ~10-fold. Thus, even though P123 (Lys^{215}–Asn246) alone has minimal inherent microtubule assembly activity, it strongly enhances microtubule assembly in the presence of adjacent repeat region sequences.

DISCUSSION

Identification of a Sequence in the Proline-rich Region, ^{215}KKVAVVR^{221}, that Strengthens the Microtubule-binding Affinities of both 3R and 4Rtau

Previous studies have shown that the proline-rich region of tau strengthens the microtubule-binding affinity and microtubule assembly-promoting capabilities of tau (Lewis *et al.*, 1989; Kanai *et al.*, 1992; Lee and Rook, 1992; Brandt and Lee, 1993; Gustke *et al.*, 1994); however, the specific location of these activities and

the mechanisms of their action have remained unclear. By using a series of amino end truncations (Figure 1), we have localized the majority of the microtubule-binding activity of the proline-rich region to a 30-amino acid sequence, Lys^{215}–Asn^{246}. Furthermore, we have identified a short sequence within this region, ^{215}KKVAVVR^{221}, that enhances microtubule binding by three- to fivefold in both 3Rtau and 4Rtau. In identifying this region, we have likely refined the location of a previously reported microtubule-binding enhancing activity of the proline-rich region. Lee and co-workers have previously shown that a 20-amino acid sequence in tau (amino acids 154–173 in fetal human tau, which correspond to residues 203–222 in the rat tau cDNA used in this study) is important for the abilities of tau 1) to bind to microtubules in transfected non-neuronal cells (Lee and Rook, 1992); 2) to promote microtubule assembly in vitro (Brandt and Lee, 1993); and 3) to induce neurite outgrowth in PC12 cells (Leger *et al.*, 1994). Our analyses have identified a 7-amino acid sequence, and particular amino acids within it (Lys^{215}, Lys^{216}, and Arg^{221}), that can account for much of the activity (Figures 2 and 3). It also is interesting to note that this region is highly conserved between tau and MAP2 (Lewis *et al.*, 1988; Ferralli *et al.*, 1994) and reasonably well conserved between tau and MAP4 (Aizawa *et al.*, 1991; Chapin and Bulinski, 1991; West *et al.*, 1991), both in terms of sequence and position relative to the repeat region (Figure 6). Thus, it is possible that this region has a conserved function in all three MAPs, a view strengthened by the observation that Lys^{215} marks the amino end boundary of high sequence homology among the three MAPs (Figure 6).

Actions of the Proline-rich Region Require the Presence of Sequences in the Repeat Region

It is important to note that the proline-rich region strengthens microtubule binding and assembly only in the context of a larger tau molecule that includes the repeat region. No significant effects on microtubule assembly or dynamics are observed for the ^{215}KKVAVVRT^{222} synthetic peptide, (Figure 4 and Table 1). In addition, no strong effects on microtubule assembly are observed for synthetic peptides corresponding to other parts of the proline-rich region (peptides P123, P23, and P3; our unpublished results; see Figure 5 for peptide positions on map of tau). This lack of inherent activity in the proline-rich region peptides is consistent with previous studies (Butner and Kirschner, 1991; Brandt and Lee, 1993; Goode and Feinstein, 1994; Gustke *et al.*, 1994) and is in marked contrast to peptides corresponding to R1 and the R1–R2 IR, both of which are sufficient to bind to microtubules, promote microtubule assembly (Ennulat *et al.*, 1989; Joly *et al.*, 1989; Joly and Purich, 1990; Goode

and Feinstein, 1994), and suppress microtubule dynamics (Panda et al., 1995) in a sequence-specific manner. However, the joining of the P123 sequence to the R1/R1–R2 IR sequence in the form of a composite peptide (Lys^{215}–Lys^{272}) causes an ~10-fold increase in the efficiency of peptide-induced microtubule assembly relative to the R1/R1–R2 IR peptide alone (Figure 5). Thus, the ability of the proline-rich region to enhance microtubule binding and assembly appears to require the presence of additional sequences in the repeat region.

There are at least two general mechanisms that could underlie these functional capabilities that are consistent with our data. First, there could be intramolecular interactions between the proline-rich and repeat regions that enhance microtubule binding. Alternatively, the proline-rich region could promote intermolecular tau–tau oligomerization to strengthen microtubule binding affinity. However, this intermolecular oligomerization model might be expected to produce cooperative binding behavior, which has not been observed for tau (Butner and Kirschner, 1991; Goode and Feinstein, 1994; Gustke et al., 1994). Thus, the available data favor a model invoking intramolecular interactions between the proline-rich and repeat regions (such as is shown schematically in Figure 7). However, it remains uncertain whether sequences in the proline-rich region form direct interactions with tubulin and/or enhance the tubulin-binding capabilities of the repeat region.

A Model for How the Proline-rich Region May Regulate Microtubule-binding Affinity

It is especially interesting to integrate the model in which efficient microtubule binding requires structural interactions between the proline-rich and repeat regions with the well-known role of phosphorylation in the regulation of tau function. An extensive literature demonstrates that phosphorylation of tau can markedly reduce microtubule-binding affinity (Lindwall and Cole, 1984; Drechsel et al., 1992; Gustke et al., 1992; Biernat et al., 1993; Bramblett et al., 1993; Goedert et al., 1993; Drewes et al., 1995). Notably, many of the phosphorylation sites in tau are located within the proline-rich region (for review, Goedert et al., 1994; Trojanowski and Lee, 1994). There is also a serine

Figure 5. Relative abilities of different tau domain synthetic peptides to promote microtubule assembly. A polymer mass assay was used to monitor microtubule assembly: MAP-depleted tubulin (2 mg/ml) was incubated for 30 min at 35°C in the presence of different concentrations of synthetic peptide. Polymerized tubulin was separated from free tubulin by ultracentrifugation, and the pellets and supernatants were analyzed by SDS-PAGE and Coomassie blue staining. Laser densitometry was used to determine the percentage of tubulin in polymer in each reaction. All reactions were performed in duplicate, and all assays were repeated on separate days.
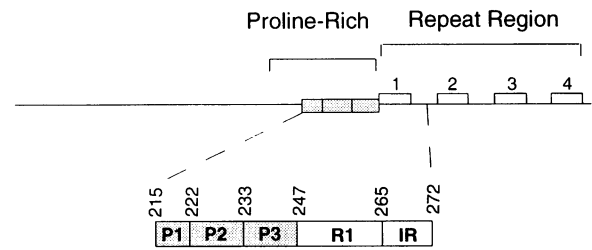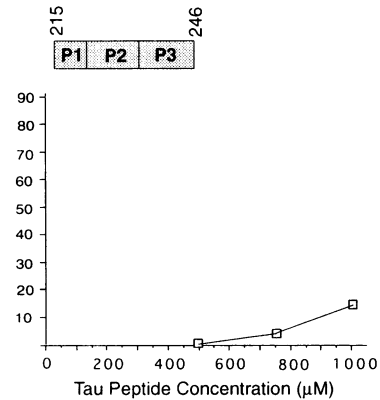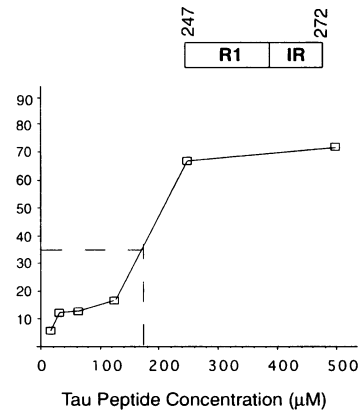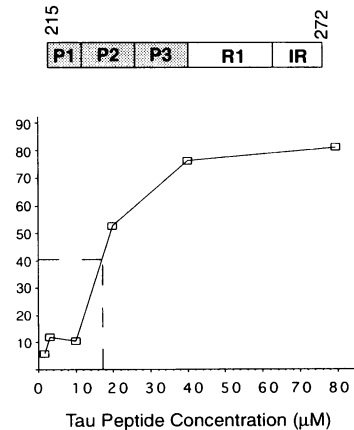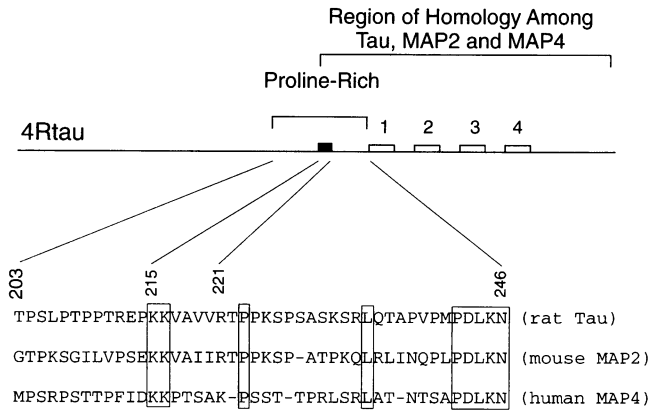

Figure 5.

Figure 6. Sequence homology in the proline-rich regions of tau, MAP2 and MAP4. The primary sequences of these three MAPs are aligned and conserved residues boxed. The ^{215}KKVAVVR^{221} sequence is represented by a solid box in the schematic of tau. Homology in the proline-rich region of these three MAPs is limited to the 25–30 amino acids adjacent to repeat 1. The sequences are especially highly conserved at positions corresponding to Lys^{215}, Lys^{216}, and Arg^{221} in tau, raising the possibility that these may play a conserved functional role in all three MAPs.

residue located in repeat 1 (Ser^{262}) that causes an especially strong reduction in microtubule-binding affinity upon phosphorylation (Biernat *et al.*, 1993;

Drewes *et al.*, 1995). If structural interactions between the proline-rich and repeat regions are required for maximal microtubule-binding activity, it is possible that Lys^{215}/Lys^{216} and Arg^{221} might interact with acidic residues in the tau repeat region or tubulin to establish an optimal microtubule-binding conformation. If that is the case, then phosphorylation events in the proline-rich region, or at Ser^{262} in repeat 1, might serve to weaken or even disrupt these productive interactions by competing for key ionic interaction sites (see model in Figure 7). In this manner, the proline-rich region may function in a regulatory capacity, modulating tau structure and function in response to phosphorylation. Many examples of intramolecular regulatory mechanisms have been described, including DNA-binding transcription factors (Godowski *et al.*, 1987; Lefstin *et al.*, 1994; Jonsen *et al.*, 1996) and many regulated kinases (Soderling, 1990; Hubbard *et al.*, 1990).

Structural Possibilities for the Microtubule-bound Conformation of Tau

It is widely held that tau has an extended rod-like structure in which tubulin-binding domains are distributed sequentially along the primary structure, each acting independently of one another—a "linear"
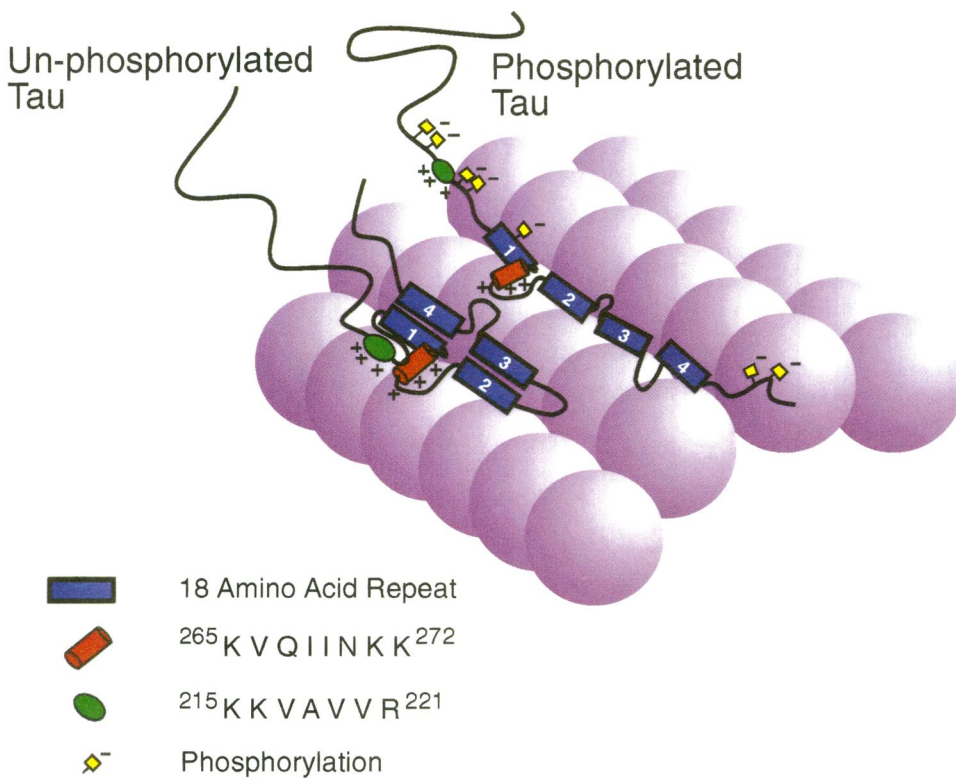

Figure 7. One possible model for how the proline-rich region regulates microtubule-binding affinity in tau. Thus, the data in this article suggest that there is a nonlinear relationship between structure and function in the proline-rich and repeat regions of tau (Figure 1B) and that the abilities of the proline-rich region to enhance microtubule binding and assembly involve intramolecular interactions with adjacent sequences in the repeat region (Figures 4 and 5). Furthermore, site-directed mutagenesis in the proline-rich region suggests that electrostatic interactions may be important for these activities (Figures 2 and 3). We have incorporated these criteria into a schematized model, in which phosphorylation events in the proline-rich region (which occur in early neuronal development and in Alzheimer's diseased neurons) may compete for, and disrupt, ionic interactions that are important for structural–functional interactions between the proline-rich and repeat regions. The model suggests one explanation for how phosphorylation outside of the repeat region can strongly influence overall microtubule-binding affinity.

view of tau structure and function. This view evolved primarily from a combination of 1) the inability of physical methods to detect appreciable secondary or tertiary structure in purified tau preparations (Cleveland et al., 1977a; Schweers et al., 1994); 2) the extended appearance of individual tau molecules visualized by electron microscopy (Hirokawa et al., 1988; Wille et al., 1992); and 3) deletion analyses of tau revealing a processive loss of microtubule-binding affinity with sequentially larger carboxyl-terminal deletions (Butner and Kirschner, 1991; Goode and Feinstein, 1994). However, the data presented in Figures 1 and 5 conflict with the linear perspective. These data suggest that there may be considerable structural and functional complexity in tau, specifically in the proline-rich and adjacent repeat region sequences.

How might these different data be reconciled? It is important to consider the possibility that the structure of tau in solution may differ greatly from the structure of tau bound to microtubules. Although tau in solution may behave as a "Gaussian coil," with little secondary or tertiary structure, as suggested by Schweers et al. (1994), microtubule-bound tau may have a complex structure with intramolecular folding. Previous studies provide support for such a model. For example, Lichtenberg-Kraag et al. (1992) showed that the epitope for the tau antibody SMI34 requires sequences on either side of the repeat region (Lichtenberg-Kraag et al., 1992), suggesting that the repeat region may fold back on itself. Kanemaru et al. (1992) showed that although 3R and 4Rtau differ in sequence by only a 31-amino acid insertion in the repeat region, they can be differentially phosphorylated in their proline-rich regions when exposed to the same kinases and phosphatases. This suggests that the addition of the 4Rtau-specific insertion can lead to structural changes outside of the repeat region, a view that is consistent with a recent comparison of functional microtubule-binding domains in 3R and 4Rtau (Goode, Denis, and Feinstein, unpublished data). Finally, Gustke et al. (1994) have shown that the stoichiometry of saturated tau–microtubule binding does not change between full-length tau and tau constructs in which repeats and/or the proline-rich region have been deleted. This observation is inconsistent with linear models in which each repeat in tau is hypothesized to interact with a different tubulin subunit along the microtubule lattice (Butner and Kirschner, 1991; Goode and Feinstein, 1994). Thus, the data suggest a new and more complex view of microtubule-bound tau structure, one involving intramolecular folding and domain interactions.

ACKNOWLEDGMENTS

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