Identification of a Novel Microtubule Binding and Assembly Domain in the Developmentally Regulated Inter-repeat Region of Tau

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Abstract. Tau is a developmentally regulated microtubule-associated protein that influences microtubule behavior by directly associating with tubulin. The carboxyl terminus of tau contains multiple 18-amino acid repeats that bind microtubules and are separated by 13-14-amino acid inter-repeat (IR) regions previously thought to function as "linkers." Here, we have performed a high resolution deletion analysis of tau and identified the IR region located between repeats 1 and 2 (the R1-R2 IR) as a unique microtubule binding site with more than twice the binding affinity of any individual repeat. Truncation analyses and site-directed mutagenesis reveal that the binding activity of this site is derived primarily from lys^{265} and lys^{272}, with a lesser contribution from lys^{271}. These results predict strong, discrete electrostatic interactions between the R1-R2 IR and tubulin, in contrast to the distributed array of weak interactions thought to underlie the association between 18-amino acid repeats and microtubules (Butner, K. A., and M. W. Kirschner. *J. Cell Biol.* 115:717-730). Moreover, competition assays suggest that the R1-R2 IR associates with microtubules at tubulin site(s) distinct from those bound by the repeats. Finally, a synthetic peptide corresponding to just 10 amino acids of the R1-R2 IR is sufficient to promote tubulin polymerization in a sequence-dependent manner. Since the R1-R2 IR is specifically expressed in adult tau, its action may underlie some of the developmental transitions observed in neuronal microtubule organization. We suggest that the R1-R2 IR may establish an adult-specific, high affinity anchor that tethers the otherwise mobile tau molecule to the tubulin lattice, thereby increasing microtubule stability. Moreover, the absence of R1-R2 IR expression during early development may allow for the cytoskeletal plasticity required of immature neurons.

TAU is a developmentally regulated family of microtubule-associated proteins (MAP)[1] specifically expressed in neurons (Binder, 1985; for recent reviews see Lee, 1990; Wiche et al., 1991). One-dimensional gel electrophoresis reveals two bands of apparent molecular mass 48–50 kD in fetal brain and four to six bands (48–67 kD) in adult brain (Cleveland et al., 1977*a*; Mareck et al., 1980; Francon et al., 1982; Ginzburg et al., 1982; Drubin et al., 1984*a*; Couchie and Nunez, 1985; Goedert et al., 1990). In addition, several high molecular mass forms of tau (119–125 kD) are expressed in the peripheral nervous system and in some neuronally derived cell lines (Drubin et al., 1985, 1988; Georgieff, 1991; Oblinger et al., 1991; Couchie et al., 1992; Goedert et al., 1992*a*). Both alternative splicing (Lee et al., 1988; Goedert et al., 1989*b*; Himmler, 1989; Kosik et al., 1989) and differential phosphorylation (Butler and Shelanski, 1986) have been shown to contribute to tau heterogeneity (see below for more details). Indeed, when higher resolution two-dimensional gel analysis is employed, as many as 60 different tau isoforms have been detected in adult brain (Larcher et al., 1992).

Biochemical and cell culture analyses have begun to reveal the functional roles of tau. In vitro, tau promotes tubulin polymerization and stabilizes microtubule dynamics (Cleveland et al., 1977*a*,*b*; Dreschel et al., 1992). Upon microinjection into cultured nonneuronal cells, tau co-localizes with microtubules and promotes net microtubule accumulation (Drubin et al., 1984*b*). Moreover, transfection and subsequent expression of tau in non-neuronal cells leads to microtubule bundle formation, which has raised the possibility that tau may form physical cross-bridges between adjacent microtubules (Kanai et al., 1989; Lewis et al., 1989; Lewis and Cowan, 1990). However, it is not yet clear whether these cross-bridges form as a direct result of tau binding (Hirokawa et al., 1988; Lewis et al., 1989; Kanai et al., 1989, 1992; Scott et al., 1992) or as an indirect

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1. *Abbreviations used in this paper*: IR, inter-repeat; MAP, microtubule-associated protein.

consequence of microtubules being stabilized by tau (Chapin et al., 1991; Lee and Rook, 1992).

In a neuronal cell context, studies have suggested an important role for tau in process outgrowth. In NGF-treated PC12 cells, the induction of tau expression levels has been shown to correlate precisely with the induction of microtubule mass and the extent of neurite outgrowth (Drubin et al., 1985). More recently, sense and anti-sense transfection studies have extended these findings, more directly implicating tau in the establishment of axonal polarity, neuronal process elongation and process stability (Caceres and Kosik, 1990; Caceres et al., 1991, 1992; Hanemaaijer and Ginzburg, 1991; Kosik and Caceres, 1991; Shea et al., 1992; Esmaeli-Azad et al., 1994). Consistent with these roles, expression of tau in Sf9 insect cells induces the outgrowth of long cellular processes (Knops et al., 1991) that contain microtubules arranged with axon-like polarity (Baas et al., 1991) and axon-like inter-microtubule spacing (Chen et al., 1992). Taken together, these studies suggest that tau serves to promote axonal process formation and maintenance through its abilities to regulate microtubule dynamics and organization.

The cloning of tau cDNAs has made it possible to begin examining the structural basis by which tau regulates microtubule behavior (Drubin et al., 1984*a*; Lee et al., 1988; Himmler et al., 1989; Goedert et al., 1989*a*,*b*; Kosik et al., 1989). In human brain, alternative mRNA splicing generates six different tau proteins ranging from 352 to 441 amino acids in length. These tau isoforms differ from one another by the presence or absence of a 31-amino acid insert in the carboxyl terminus and 0, 1, or 2 × 29-amino acid inserts in the amino terminus (Goedert et al., 1989*a*,*b*). Whereas only a single tau isoform (lacking any of the inserts) is expressed in the fetal brain, all six isoforms are found in adult human brain (Goedert and Jakes, 1990).

Integrating sequence comparisons with a variety of functional analyses, it is now well established that tau proteins are organized into three regions. The amino terminus is highly acidic and projects from the microtubule surface, where it may interact with other cytoskeletal elements (Hirokawa, 1988). The middle region is proline rich and contains target sites for MAP kinase (Drewes et al., 1992; Goedert et al., 1992*b*), proline directed/cdc2 kinase (Vulliet et al., 1992), and glycogen synthase kinase (Ishiguro et al., 1992; Mandelkow et al., 1992). Several of the target sites for these kinases have been shown to be specifically phosphorylated on tau in Alzheimer disease-afflicted neurons (Biernat et al., 1992; Hasegawa et al., 1992; Goedert et al., 1993) and to reduce the in vitro microtubule binding affinity of tau (Biernat et al., 1993; Bramblett et al., 1993). Finally, the carboxyl terminus is highly basic and possesses microtubule binding activity. It contains either three or four copies of a highly conserved 18-amino acid repeat (Lee et al., 1988, 1989; Goedert et al., 1989*b*; Himmler et al., 1989) separated from one another by less conserved 13–14-amino acid inter-repeat (IR) domains. Most notably from a developmental perspective, all of the 4R tau isoforms (possessing the carboxyl 31-amino acid insert; see Fig. 1 for site of insertion) are expressed specifically in adult neurons (Kosik et al., 1989).

Since their initial discovery, it has been suggested that each 18-amino acid repeat might function as an individual tubulin binding domain (Lee et al., 1988). Consistent with this model, experimentally constructed tau polypeptides containing only a single repeat can bind microtubules in vitro, albeit with greatly reduced affinity relative to intact tau (Lee et al., 1989; Butner and Kirschner, 1991). Furthermore, synthetic peptides corresponding to single repeats are sufficient to induce microtubule assembly in vitro (Ennulat et al., 1989), and 4R tau has been shown to bind microtubules with higher affinity (Butner and Kirschner, 1991; Gustke et al., 1992; Lee and Rook, 1992) and promote tubulin polymerization more efficiently (Goedert and Jakes, 1990) than 3R tau. In vivo, it has been shown that the developmentally regulated transition in expression from 3R to 4R tau correlates with increased microtubule stability and decreased cytoskeletal plasticity (Goedert et al., 1989*a*; Kosik et al., 1989). Taken together, these studies suggest that the carboxyl-terminal tau insert establishes additional microtubule binding sites and/or alters the structure of the microtubule binding region such that microtubule affinity is increased.

To assess the contributions to microtubule binding of different regions of tau, Butner and Kirschner (1991) examined a series of tau fragments with an in vitro microtubule binding assay. Fragments were generated by cleavage of a 4R tau cDNA at unique restriction sites within the coding region followed by in vitro transcription and translation. This approach revealed that fragments containing each repeat, as well as sequences flanking the repeat region, contribute to microtubule binding affinity. More detailed analysis of repeat 3 led Butner and Kirschner (1991) to conclude that the 18-amino acid repeats bind to microtubules through a flexible array of distributed weak sites, as opposed to strong, localized effects. Furthermore, based upon analysis of the R3-R4 IR, it was concluded that the IR domains do not contribute to microtubule affinity and that the increased microtubule binding capabilities of 4R over 3R tau are derived from the addition of repeat 2. However, the resolution of these analyses was limited because the only tau deletion mutants that could be generated were those made possible by the presence of unique restriction endonuclease sites in the coding region of the tau cDNA. Consequently, with the exception of the R3-R4 IR, the contributions of IR and repeat domains could not be uncoupled.

To dissect the microtubule binding activities of tau at high resolution, we have used partial exonuclease III digestion and PCR strategies in conjunction with in vitro transcription and translation to generate tau polypeptides truncated precisely at the borders between repeat and IR domains (as defined by Lee et al., 1988; see also Fig. 1). The functional capability of each polypeptide was assessed in an in vitro microtubule binding assay. In addition to confirming earlier data demonstrating that each repeat contributes to the microtubule binding activity of tau, we have identified a high affinity microtubule binding site in the R1-R2 IR. High resolution truncation and site-directed mutagenesis analyses identify two critical lysine residues responsible for almost all of the binding activity. Competition experiments demonstrate that the R1-R2 IR associates directly with tubulin at a site(s) distinct from the repeat binding site(s). Finally, we have shown that a synthetic peptide corresponding to the active portion of the R1-R2 IR is sufficient to promote stable microtubule assembly from purified tubulin. Since the R1-R2 IR is specifically expressed in adult tau, this microtubule

binding site may regulate tau–microtubule interactions important for the proper maturation of neurons.

Materials and Methods

Subcloning and cDNA Construction

3R and 4R rat tau cDNA clones were generous gifts from Dr. Ken Kosik (Brigham and Women's Hospital, Boston, MA) (described in Kosik et al., 1989). The cDNA encoding the complete coding region of 4R tau, which includes both 29-amino acid amino-terminal inserts, was subcloned into the EcoR1 site of the pGEM-3 vector (Promega Corp., Madison, WI) in the T7 orientation and designated p4Rtau-T7. Since the original 3R tau rat cDNA clone (pKSERT4a-9) lacked amino-terminal coding sequences, sequences corresponding to the amino-terminal half of 4R tau (amino acids 1–163) were joined to sequences corresponding to the carboxyl-terminal half of 3R tau (amino acids 163–401) to reconstitute a full-length 3R tau cDNA encoding both amino-terminal inserts. This was achieved by ligating the BamH1-BamH1 vector/insert-containing fragment of p4Rtau-T7 to the BamH1-BclI insert-containing fragment of pKSERT4a-9. The resulting rat clone (p3Rtau-T7) encodes a 401-amino acid polypeptide homologous to the human Tau39 clone (Goedert et al., 1989*a*). Thus, the polypeptides encoded by p3Rtau-T7 and p4Rtau-T7 are identical except for the presence or absence of the 31-amino acid insert (shown flanked by arrowheads in Fig. 1 *A*) containing repeat 2 and the R1-R2 IR.

Carboxyl-terminal Truncations in 4R Tau

Truncations were made in the carboxyl terminus of 4R tau with the Exonuclease III digestion procedure (Henikoff, 1984), using the "Erase-A-Base" kit from Promega Corp. Plasmids from over 400 clones were rapidly purified as previously described (Goode and Feinstein, 1992), and exact endpoints determined by DNA sequencing (USB Sequenase 2.0) for 120 selected clones. Constructs encoding tau polypeptides truncated from their carboxyl termini at the following amino acid positions were isolated: 213, 216, 217, 218, 228, 230, 231, 235, 251, 256, 263, 264, 265, 267, 272, 274, 279, 281, 283, 287, 291, 293, 295, 301, 303, 304, 306, 308, 309, 310, 313, 315, 316, 317, 319, 324, 326, 327, 328, 329, 331, 333, 334, 337, 338, 339, 340, 342, 343, 349, 350, 354, 356, 357, 358, 359, 361, 366, 373, 381, and 388. Plasmids to be used for in vitro expression were purified by alkaline lysis and cesium chloride banding (Sambrook et al., 1989).

Two carboxyl truncation mutants not obtained by the Exonuclease III procedure were generated by PCR amplification using a primer located 50 bases 5′ of the T7 promoter (5′ GCCTCTCCCCGCGCTTGGC 3′) in p4Rtau-T7 and one of two downstream tau primers. A tau construct truncated at asn270 was generated using the primer 5′-ATTAATTATCTGCACCTTGCC-3′ and a construct truncated at gly264 with the primer 5′-GCCGCCTCCCGGCTGGTG-3′. The PCR fragments were gel purified, reamplified by PCR, phenol/chloroform extracted and ethanol precipitated prior to in vitro transcription. Primers were purchased from Operon Technologies, Inc. (Alameda, CA).

Amino-terminal Truncations in 4R Tau

Tau polypeptides truncated from their amino termini to gly264 and val278 in 4R tau were generated by internally deleting DNA sequences encoding amino acids 2–263 and 2–277, respectively, from pTau4R-T7 using the method of Deng and Nicholoff (1992) and the "Transformer" site directed mutagenesis kit of Clontech (Palo Alto, CA). Briefly, 42′-mer oligonucleotides were designed that bridge 21 bases of the 5′ untranslated region and the initiation codon of 4R tau (Kosik et al., 1989) with 21 bases of internal tau sequence beginning at positions encoding Gly264 or Val278. DNA sequencing of the resulting clones, p4RtauΔ2-263 and p4RtauΔ2-277, verified that the appropriate sequences were deleted.

In Vitro Transcription and Translation of Tau Polypeptides

Carboxyl truncated 4R tau plasmids were digested to completion with SphI (which linearizes precisely 3′ to their coding region endpoints), phenol/chloroform extracted and ethanol precipitated. Transcription reactions were performed in 40 mM Tris Cl (pH = 7.5)/6 mM $MgCl_2$/2 mM spermidine/10 mM NaCl/10 mM DTT/0.5 mM of each ribonucleotide triphosphate. Reactions contained 1–2 μg DNA template and 50 units of T7 RNA polymerase (Boehringer Mannheim Corp., Indianapolis, IN) in a 50-μl volume. Incubation was for 90 min at 39°C. RNA yields were typically ~5–10 μg. 10 μl of the transcription reaction products were then directly added to 35 μl rabbit reticulocyte lysate (Promega) plus 4 μl of [^{35}S]methionine (>1,000 Ci/mMol, 10 μCi/μl NEN) plus 1 μl of a 50X stock of amino acids (minus methionine; final concentration of each amino acid = 20 μM). Translation products were fractionated on 10–15% polyacrylamide gels. Gels were fixed, dried onto filter paper, and autoradiographed. Product yields were calculated from TCA precipitable radioactivity and the specific activity of the isotope. Prior to TCA precipitation, samples were treated with 1 N NaOH/2% H_2O_2 (see Promega Protocols and Applications Guide) to uncouple activated tRNAs carrying labeled methionine. Routinely, specific activities were in the range of 10^5–10^6 cpm/ng tau, and translation products contained ~0.1 ng/μl tau. Translation products were stored at −20°C for up to 8 wk prior to use in binding assays without detectable degradation.

Tubulin Purification

MAP-free tubulin was purified from bovine brain by two cycles of temperature-controlled polymerization/depolymerization and phosphocellulose chromatography (Mitchison and Kirschner, 1984) in LGNPEM buffer (20 mM $NaPO_4$, 100 mM L-glutamate [monosodium salt], 1 mM EGTA, and 1 mM $MgCl_2$; pH 6.8) and stored in 4 mg/ml drop-frozen aliquots at −70°C until use. Protein concentrations were determined by the Bradford assay (Bradford, 1976).

Microtubule Affinity Assays

Microtubules were assembled from PC tubulin at 30°C, in the presence of 1 mM GTP and increasing concentrations of taxol (Calbiochem-Novabiochem Corp., La Jolla, CA) to a final concentration of 20 μM (Butner and Kirschner, 1991). Appropriate dilutions were then made in LGNPEM buffer supplemented with 1 mM GTP and 20 μM taxol. Varying concentrations of polymerized tubulin (0.01–40 μM) were mixed with constant amounts of in vitro translated tau (1 μl; ~0.1 ng) and incubated at room temperature for 15 min. Binding equilibrium between tau and microtubules was achieved in less than 10 min for both full-length and truncated tau polypeptides. Increasing the time allowed for association beyond 10 min had no effect upon the extent of tau binding. Reducing the concentration of tau in the reactions (by 3-, 9-, and 27-fold) also had no effect on the results. Reactions were then loaded over 80-μl sucrose cushions (50% sucrose in LGNPEM buffer supplemented with 20 μM taxol) in 200-μl airfuge tubes (Beckman Instrs. Inc., Fullerton, CA) and centrifuged in a Beckman SW50.1 swinging bucket rotor for 15 min at 100,000 *g* at room temperature. Supernatants were collected, sucrose cushions removed by aspiration, and pellets solubilized in SDS sample buffer (containing 50 mM tris [pH 6.8], 0.5% SDS, 10% glycerol, 0.7 M β-mercaptoethanol). Samples were fractionated on 10–15% polyacrylamide gels, fixed, enhanced with Apex (55% glacial acetic acid, 15% xylenes, 30% ethanol, 0.5% 2,5-diphenyl-oxazole (PPO), dried onto filter paper, and autoradiographed. Tau levels in supernatants and pellets were determined by densitometry, which proved to be consistent with direct measurements of tau levels by band excision and quantitation in a scintillation counter. Percent tau pelleted in each assay is corrected for the slight background level of tau found in the pellet fraction in control reactions lacking any microtubules (Fig. 3 *A*, lane *1*).

Translation products occasionally contained a small amount of lower or higher molecular weight tau polypeptides, likely resulting from early translational termination and incomplete template linearization prior to transcription, respectively. However, these products have no effect on the outcome of the microtubule-binding analyses because only the appropriate band is quantitated for each experiment and tubulin is present in vast excess over tau in the microtubule binding reactions. Additionally, tau binding to microtubules has been shown to be noncooperative (Butner and Kirschner, 1991).

Site-directed Mutagenesis

Lysine resides at positions 265, 271, and 272 in full-length 4R tau were converted to alanines by the procedure of Deng and Nicholoff (1992) using the "Transformer" site-directed mutagenesis kit. Initially, single amino acid substitutions were introduced at each of the three positions (K265A, K271A, and K272A). The single mutant K265A was then used as a template for a second round of mutagenesis to generate the double mutant (K265A/K271A), which in turn, served as a template for a third round of mutagenesis

to generate the triple mutant (K265A/K271A/K272A). In all cases, mutagenesis was confirmed by direct DNA sequence analysis. Sequences from −50 of the T7 promoter to amino acid 295 were PCR amplified using the −50T7 primer and the tau295 primer from the mutant plasmid templates. These PCR products were gel purified and transcribed/translated (as described above) to generate two-repeat forms of each mutant (i.e., truncated at gly295).

Peptide Synthesis and Purification

The R1-R2 IR peptide amide (KVQIINKKLD) and R1-R2 IR scramble peptide amide (IKDNVKQILK) were synthesized on a Millipore 9050 Plus peptide synthesizer by DIPCDI/HoBT chemistry (Millipore Corp., Bedford, MA). Following cleavage and deprotection (Solé and Barany, 1992), peptides were purified to >99% purity by reverse phase HPLC, lyophilized, and resuspended in H_2O to generate 10 mM stocks. Peptide concentrations were determined from dry peptide weights and confirmed by A_{215} absorption after resuspension in H_2O. Peptide integrity was confirmed by fast atomic bombardment mass spectrometry (FABMS).

Microtubule Assembly Assays

Microtubule assembly was examined by light scattering in a bulk phase turbidity assay (Gaskin, 1974) and by electron microscopy. MAP-free tubulin was thawed, precleared by centrifugation for 10 min at 20,000 *g*, 4°C, and diluted with cold LGNPEM + 1 mM GTP buffer to 1 mg/ml on ice. 10 μl of peptide (diluted with LGNPEM + 1 mM GTP) was added to 40 μl of 1 mg/ml tubulin, mixed and transferred to wells in a microtiter dish. Final peptide concentration was 300 μM. The final tubulin concentration in these reactions was 0.80 mg/ml, below the critical concentration for spontaneous assembly (which is >1 mg/ml) (Hill and Carlier, 1983). Reactions were initiated by incubation at 30°C in a Ceres 900 UVi spectrophotometer and A_{340} readings taken every 10 s. After 20 min, an aliquot of the reaction was diluted 20-fold in LGNPEM buffer containing 0.5% glutaraldehyde and 30% glycerol, and then negatively stained with uranyl acetate and visualized by electron microscopy as described (Davis et al., 1993).

Other Reagents

The monoclonal antibodies 5E2 (anti-tau) (Nukina et al., 1987) and E7 (anti-β tubulin) (Chu and Klymkowsky, 1987) were kind gifts from Drs. Ken Kosik (Harvard University, Cambridge, MA) and Michael Klymkowsky (University of Colorado, Boulder, CO), respectively. Recombinant 4R tau protein purified from *E. coli* was generously provided by Joe McCarty (University of California, Santa Barbara, CA).

Results

Generation of Carboxy-terminal Truncated Tau Polypeptides

Since it has been demonstrated previously that the microtubule binding activity of tau resides in its carboxyl terminus (Himmler et al., 1989, Lee et al., 1989), we targeted our high resolution analysis to this region of the protein. Beginning with a rat cDNA encoding the full-length 4R tau, we used both partial exonuclease III digestion and PCR amplification procedures to generate deletion endpoints throughout the carboxyl terminus. 63 clones were isolated with carboxyl truncation endpoints between amino acids 213 and 388 (see Materials and Methods). For frame of reference, Fig. 1 shows the primary sequence of rat 4R tau and the locations of the repeats and IR domains (originally deduced from the cDNA sequence in Kosik et al., 1989).

Clones used in the microtubule binding assay were transcribed in vitro, then translated in a rabbit reticulocyte lysate containing [^{35}S]methionine. Fig. 2 shows tau products corresponding to full-length tau (432 amino acids long) and tau polypeptides truncated at several critical positions, including precise borders between each repeat and IR domain. Immunoblotting with the anti-tau monoclonal antibody 5E2 confirmed the identity of these translation products (data not shown).

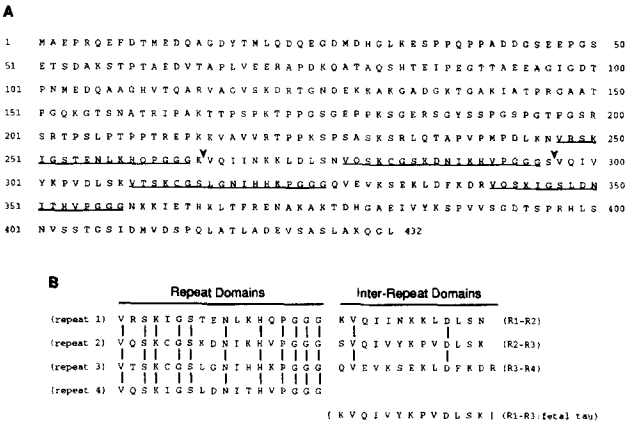

Figure 1. (*A*) Deduced amino acid sequence of rat 4R tau (from Kosik et al., 1989). The four 18-amino acid repeats are underlined and the adult specific 31-amino acid insertion is flanked by arrowheads. (*B*) Primary sequence alignments of the repeat and IR domains. Absolutely conserved amino acids are marked with vertical lines. The R1-R2 IR is specific to 4R tau, while the R1-R3 IR is specific to 3R tau.

The R1-R2 Inter-Repeat Is a Distinct Microtubule Binding Domain with Higher Affinity for Microtubules than Any of the Repeats

To measure the relative microtubule binding affinities of

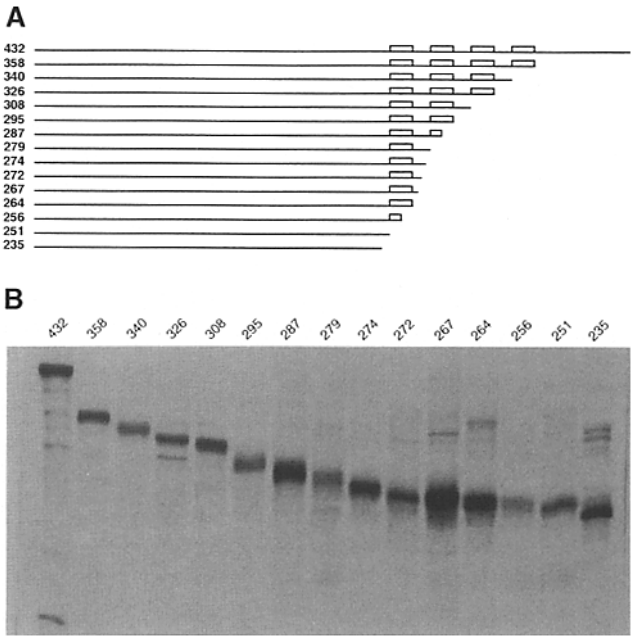

Figure 2. In vitro translated full-length and truncated 4R tau polypeptides. (*A*) Schematic of polypeptides. Each construct is numbered according to its carboxyl-terminal amino acid residue. Boxed regions represent the 18-amino acid repeats. (*B*) Autoradiograph of full-length and truncated tau polypeptides in vitro transcribed and translated in a rabbit reticulocyte lysate containing [^{35}S]methionine.

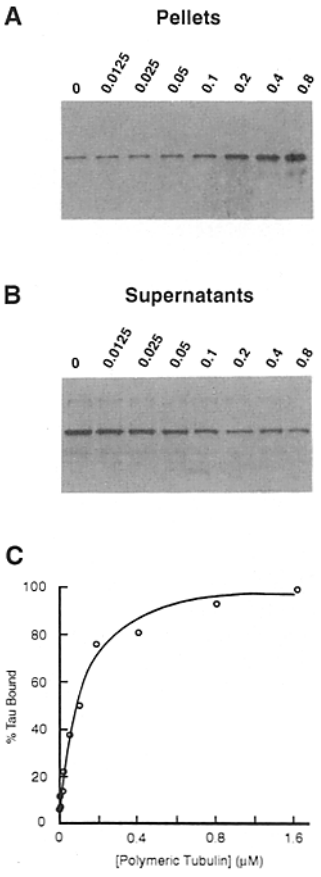

Figure 3. Measurement of microtubule binding affinity (K_d) for full-length 4R tau. A constant amount of ^{35}S labeled in vitro translated tau ($\sim 10^{-10}$ M final concentration) was mixed with variable concentrations of taxol assembled microtubules (concentration in μM tubulin is given above each lane) and allowed to associate to equilibrium. Microtubules were pelleted, then bound tau and unbound tau were isolated and fractionated by PAGE. (*A*) Autoradiograph of tau present in the pellets. (*B*) Autoradiograph of tau in the corresponding supernatants. (*C*) Resulting binding curve. The percent tau bound in each reaction was determined by densitometry (subtracting background) and plotted versus taxol-assembled tubulin concentration. The binding constant (K_d) for a given tau construct is defined as the concentration of tubulin required to cosediment 50% of the tau in the reaction.

different tau polypeptides, we utilized a microtubule cosedimentation assay (see Fig. 3). A constant amount of in vitro translated, [^{35}S]methionine-labeled tau protein was mixed with variable amounts of taxol-polymerized tubulin and allowed to achieve binding equilibrium. In all reactions, tubulin was present in large molar excess of tau (>100:1 tubulin to tau). Bound tau was separated from unbound tau by ultracentrifugation, and the microtubule binding affinity (apparent K_d) for a given tau polypeptide defined as the concentration of polymerized tubulin required to cosediment half of the tau in the reaction.

As seen in Fig. 3, the binding of full-length 4R tau to microtubules is concentration dependent and saturable, with an apparent K_d of 1.6×10^{-7} M tubulin. This value is in close agreement with previous reports (Butner and Kirschner, 1991). The specificity of the tau–microtubule interaction was assessed by binding in the presence of a 10,000-fold excess of unlabeled 4R tau (recombinantly expressed in *E. coli*) or nonspecific protein (BSA). The unlabeled tau effectively competed with labeled tau for binding sites on tubulin, whereas excess BSA had no effect on binding of the labeled tau (data not shown). Additionally, to verify that microtubules remained polymerized after dilution, all gels were Coomassie blue stained to assess tubulin levels in the pellets and supernatants. For microtubule dilutions below the level of Coomassie blue detection, gels were immunoblotted and probed with a β tubulin monoclonal antibody. At microtubule concentrations as low as 0.01 μM tubulin (the lowest used in our assays), >90% of the tubulin was detected in the pellets (data not shown).

Table I. Relative Contributions to Microtubule Affinity of Individual Domains within Tau

Domain in tau	Amino acid residues	Relative contribution to MT affinity (fold effect on K_d)
R1*	236–264	2.4
R1-R2	265–279	6.9/7.6‡
R2	280–295	3.3
R2-R3	296–308	–
R3	309–326	1.7
R3-R4	327–339	–
R4	340–358	1.6
C-term	359–432	1.4

Relative contributions (fold effect on K_d) were determined by calculating the ratios between the microtubule binding affinities (K_ds) of tau polypeptides shown in Fig. 4.
* In addition to repeat 1 (247–264), this domain includes 11-amino acid residues of NH_2-terminal flanking sequence (236–246).
‡ Two values are shown for the contribution made by the R1-R2 IR, as determined by carboxyl and amino end truncation analysis, respectively.

We next determined the microtubule binding affinities of tau polypeptides with carboxyl-terminal endpoints located precisely at the borders between repeat and IR domains originally assigned by Lee et al. (1988) (see Fig. 4). Comparison of the K_d values allows the assessment of the relative contributions made by each repeat and IR region to microtubule affinity (Table I). Repeats 1, 3, and 4 each contribute approximately twofold to microtubule binding affinity. The adult specific repeat 2 is stronger, contributing 3.3-fold to the K_d. The R2-R3 IR and R3-R4 IR make no apparent contributions. The most striking observation, however, comes from comparison of the microtubule binding activities of constructs 4Rtau264 and 4Rtau279. As seen in Fig. 4 and Table I, the R1-R2 IR possesses very strong microtubule binding activity, conferring a 6.9-fold increase in the strength of the tau–microtubule interaction.

There are two general mechanisms which could account for the R1-R2 IR microtubule binding activity. First, the R1-R2 IR could itself associate with tubulin, serving as an additional microtubule binding site. Alternatively, the R1-R2 IR could enhance microtubule binding activity indirectly by influencing the conformation of microtubule binding domains such as adjacent repeats. If the R1-R2 IR interacts directly with tubulin, truncations from the amino end of tau differing only by the presence or absence of the R1-R2 IR would be predicted to exhibit comparable differences in microtubule binding activity to those observed with carboxyl end truncations. On the other hand, if the R1-R2 IR influences the binding activity of other regions of tau, there should be a marked disparity in the apparent R1-R2 IR activity as measured by truncations from the carboxyl versus the amino ends.

To test these predictions directly, we generated two amino-terminally truncated tau polypeptides that each contain repeats 2, 3, and 4, but differ by the presence or absence of the R1-R2 IR (Fig. 4, constructs 4RtauΔ2-263 and 4RtauΔ2-277). Comparison of their respective microtubule binding affinities shows that the R1-R2 IR containing construct 4RtauΔ2-263 binds microtubule 7.6-fold stronger than 4RtauΔ2-277. This is in excellent agreement with the 6.9-fold difference observed using carboxyl end truncations (constructs

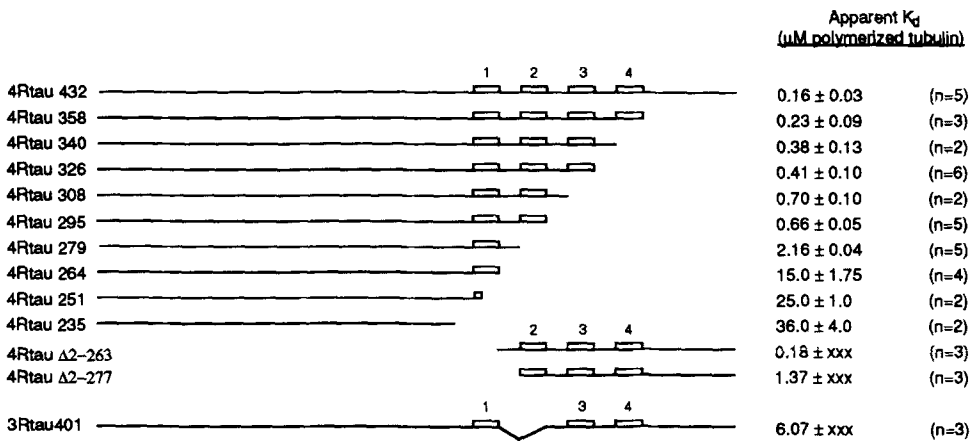

Figure 4. Microtubule binding affinities of full-length and truncated 4R tau polypeptides. The microtubule binding affinities (K_d's) of in vitro translated tau polypeptides were determined as in Fig. 3 and are presented as the average of the measured values (n = the number of times each polypeptide was assayed). Where $n > 2$, standard error of the mean is included and where $n = 2$, the range is given. Each construct is numbered according to its carboxyl terminal amino acid residue. Boxed regions represent the 18-amino acid repeats.

4Rtau264 and 4Rtau279). These data provide strong support for the conclusions that (*a*) the R1-R2 IR is itself a microtubule binding domain and (*b*) it can act independently of the repeat domains.

The Microtubule Binding Activity of the R1-R2 IR Is Contained within an 8-Amino Acid Long Sequence (KVQIINKK)

In order to define the microtubule binding activity of the R1-R2 IR at higher resolution, we compared the microtubule affinities of tau polypeptides truncated at gly263, gly264, lys265, gln267, asn270, lys272, asp274, and gln279 (see Fig. 1 for positions). As seen in Fig. 5, the binding profile for this region indicates that the entire microtubule binding activity is contained within a short stretch of 8 amino acids defined by the sequence KVQIINKK (lys265-lys272). A 2.4-fold change in K_d is observed between constructs ending at gly264 and lys265, identifying lys265 as critical for microtubule binding activity. The 3.1-fold change in K_d observed between constructs ending at asn270 and lys272 identifies lys271 and/or lys272 as critical element(s) for microtubule binding, although this analysis cannot assess their individual contributions. Additionally, the binding activity is not equally distributed throughout this domain as has been reported for repeat 3 (Butner and Kirschner, 1991), but rather, appears to be highly localized in three lysine residues. These observations have a number of structural and functional implications (see Discussion).

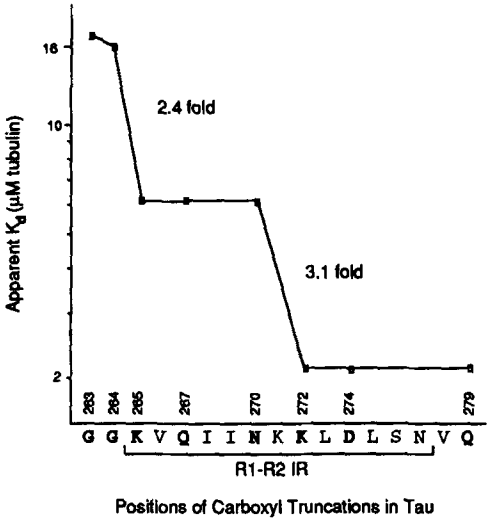

Figure 5. High resolution truncation analysis of the R1-R2 IR. Microtubule binding affinities were determined for tau polypeptides with carboxyl truncations located between amino acids 263 and 279 of 4R tau (gly263, gly264, lys265, gln267, asn270, lys272, 274, 279). The single letter code amino acid sequence of tau 263-279 is shown above the x-axis. Bold faced letters correspond to carboxyl-terminal amino acid positions of the polypeptides assayed.

Lys265 and Lys272 Are Critical for the Microtubule Binding Activity of the R1-R2 IR

To test the importance of each lysine residue more directly, we used site-directed mutagenesis to convert lysines to alanines at positions 265, 271, and 272, singly and in concert. Since the microtubule binding assay is most accurate (i.e., smallest standard deviations) in the range of ~1–10 μM taxol-stabilized tubulin, the effects of these substitutions were assessed with tau polypeptides truncated at the end of repeat 2 (i.e., extending from the amino terminus to amino acid gly295). From the binding data presented in Fig. 6, it can be seen that lys265 and lys272 are the most important elements in the R1-R2 IR–microtubule binding interaction. The lys265 mutation (K265A) reduces microtubule binding affinity by 2.3 ± 0.48-fold and the lys272 mutation (K272A) by 3.6 ± 0.42-fold. Thus, each of these individual lysine residues makes a contribution to microtubule binding affinity that is comparable to an entire repeat. The lys271 mutation (K271A) makes a lesser but still significant contribution to the strength of interaction, causing a 1.4 ± 0.42-fold reduction in K_d. The triple mutant reduces binding activity by 5.6 ± 0.74-fold. Consistent with the truncation analysis of the R1-R2 IR (Fig. 5), the site directed mutation analysis (Fig. 6) demonstrates that the microtubule binding activity in the R1-R2 IR is mediated primarily by lys265 and lys272, with a lesser contribution from lys271.

wild type	K	V	Q	I	I	N	K	K
K265A	A	-	-	-	-	-	-	-
K271A	-	-	-	-	-	-	A	-
K272A	-	-	-	-	-	-	-	A
triple	A	-	-	-	-	-	A	A

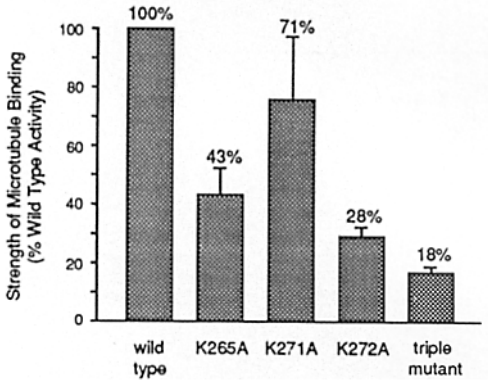

Figure 6. Site-directed mutagenesis of the R1-R2 IR. Lysines at positions 265, 271, and 272 were converted to alanines by site directed mutagenesis of 4R tau. Mutant and nonmutant polypeptides with carboxyl-terminal truncations at gly295 (i.e., containing repeats 1 and 2) were compared. Microtubule binding affinities (K_d's) were determined as in Fig. 3. Locations of amino acid substitutions between positions lys265 and lys272 are given above the bar graph. The bar graph is a comparison of the microtubule binding affinities (expressed as % of nonmutant binding activity [K_d]) of mutant and nonmutant tau. For each polypeptide, $n = 2$ and the range is represented with an error bar.

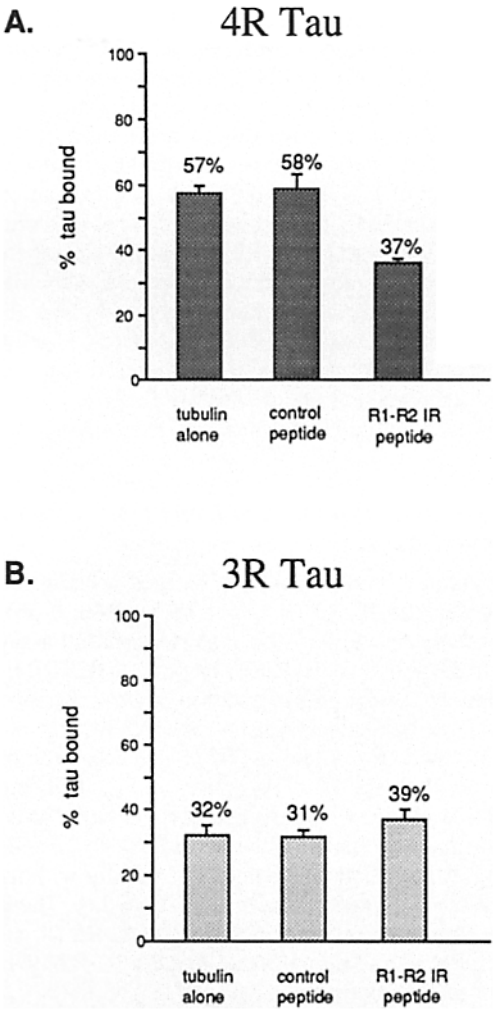

Figure 7. Microtubule binding competition assay. A synthetic peptide corresponding to the active portion of the R1-R2 IR was assayed for its ability to compete with 4R tau and 3R tau for microtubule binding. The % full-length 4R and 3R tau bound at constant microtubule concentration (0.2 μM tubulin for 4R tau and 4 μM tubulin for 3R tau) was determined in the presence or absence of 300-μM R1-R2 IR peptide (IVQIINKKLD) or control peptide (IKDNVKQILK). (*A*) Peptide effects on 4R tau binding to microtubules. (*B*) Peptide effects on 3R tau binding to microtubules. Numbers above bars correspond to percent tau bound. For each polypeptide, $N = 4$ and the range is represented with an error bar.

Comparison of the Microtubule Binding Affinities of 3R Tau and 4R Tau

To better understand the developmental role played by the R1-R2 IR in tau, we compared the microtubule binding affinities of full-length 4R and 3R tau. As mentioned earlier, 4R and 3R tau differ by the presence or absence of a 31-amino acid insert containing the R1-R2 IR and repeat 2. As seen in Fig. 4, we observed a large difference (~40-fold) in the binding affinities of 3R and 4R tau. This is in marked contrast to the observations of other workers, who observed only ~4.5-fold differences (Butner and Kirschner, 1991; Gustke et al., 1992). Possible explanations for these differences are presented in the discussion section.

The R1-R2 IR Microtubule Binding Site is Specific to 4R Tau and Binds to Distinct Site(s) on Tubulin Relative to Those Bound by the Repeats

A question that naturally arises from these results is whether the microtubule binding site defined by the R1-R2 IR is unique to 4R tau or if there is a homologous site present in 3R tau. Since two of the three active lysine residues (lys265 and lys271) in the R1-R2 IR of 4R tau are present in the corresponding positions of the R1-R3 IR of 3R tau (Fig. 1), it seemed possible that the R1-R3 IR might bind similarly to microtubules. On the other hand, it seemed equally possible that the active structure required for R1-R2 IR microtubule binding might be disrupted by the substitutions of a rigid proline residue at position 272 and/or a tyrosine for asparagine at position 270 in the R1-R3 IR.

To test for the presence of a 3R tau microtubule binding site homologous to the R1-R2 IR, we compared the ability of a synthetic peptide corresponding to the first 10 amino acids of the R1-R2 IR (KVQIINKKLD) to compete for microtubule binding with 4R tau and 3R tau. As seen in Fig. 7, the synthetic peptide effectively competes with 4R tau, but not 3R tau, suggesting that homologous binding sites are either not present or not accessible in 3R tau. To control for competition due to nonspecific charge effects, another peptide with the identical amino acid composition as the R1-R2 IR peptide presented in a scrambled order (IKDNVKQILK) was assayed in parallel reactions. As seen in Fig. 7, the control peptide failed to compete with either tau isoform,

demonstrating the sequence specificity of the R1-R2 IR-microtubule interaction. Interestingly, the R1-R2 IR peptide had a slight but reproducibly positive effect upon the strength of the fetal tau–microtubule interaction (see discussion).

There are several implications of these results. First, the fact that the R1-R2 IR peptide competes with the binding of 4R tau to microtubules is consistent with our truncation results indicating that the R1-R2 IR binds directly to microtubules. Second, the failure of the R1-R2 IR peptide to compete with 3R tau suggests that the site on tubulin bound by the R1-R2 IR is distinct from the site on tubulin bound by any sequences found in the 3R tau, including the repeats. Finally, the sequence specificity of the peptide competition suggests that the proper arrangement and presentation of the lysines in the R1-R2 IR is essential for its successful interaction with microtubules.

A R1-R2 IR Synthetic Peptide Is Sufficient to Promote Tubulin Polymerization

Given its microtubule-binding abilities, we next investigated the possibility that the R1-R2 IR might be capable of promoting microtubule assembly. A bulk phase turbidity assay was used to test the ability of the R1-R2 IR peptide (KVQIINKKLD) to promote tubulin polymerization in vitro. Purified tubulin, below its critical concentration, was incubated in the presence or absence of peptide at 30°C and assayed for changes in turbidity every 10 s. As shown in Fig. 8 *A*, the R1-R2 IR peptide alone efficiently promotes tubulin polymerization, whereas the control peptide (IKDNVKQILK) is ineffective (Fig. 8 *B*). Reactions containing tubulin without peptide (Fig. 8 *C*) also show no increase in turbidity. These results confirm the sequence specificity of the R1-R2 IR activity shown in Fig. 7 and demonstrate that the R1-R2 IR is sufficient to promote microtubule assembly.

To complement the turbidity assays and ensure that the polymerization effects of the R1-R2 IR peptide do not result from association of tubulin into nonmicrotubule aggregates, we examined the polymerization products by electron microscopy. Control reactions where no polymerization was detected (Fig. 8, *B* and *C*) showed no apparent tubulin aggregates or other discernible structures (Fig. 9 *A*). The R1-R2 IR peptide reactions contained abundant quantities of tubulin polymers, most with morphologies indistinguishable from normal microtubules (Fig. 9, *B–D*). Many of the microtubules were also found to be closely associated in parallel bundles (Fig. 9 *B*), suggesting a possible role for the R1-R2 IR in microtubule bundling in addition to microtubule binding and assembly. Finally, some of the tubulin polymers in these samples appeared as sheets partially rolled up into microtubules. These may represent intermediates in the R1-R2 IR peptide-induced microtubule assembly process. It is possible that the R1-R2 IR may initially promote the lateral association of tubulin into sheets which roll up into microtubules.

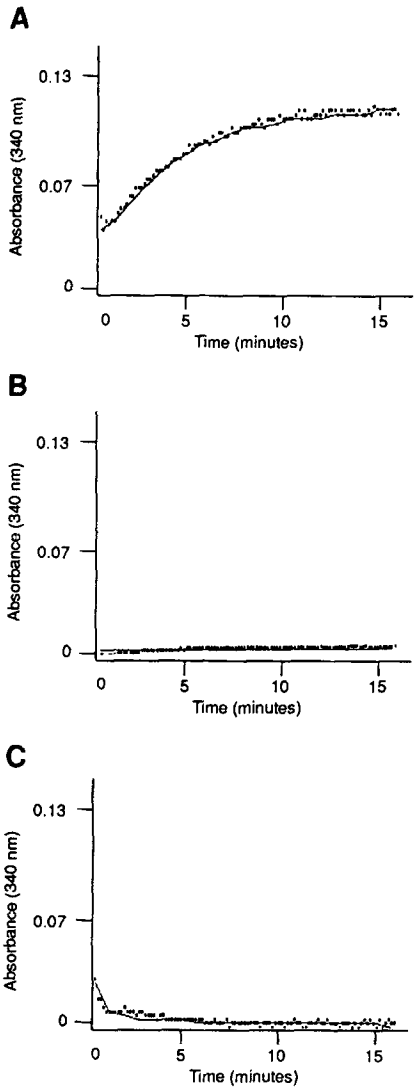

Figure 8. R1-R2 IR peptide-induced tubulin polymerization. A constant concentration of purified tubulin (0.8 mg/ml) was incubated at 30°C in the presence or absence of tau peptides and monitored for changes in bulk phase turbidity (increased A_{340}). Absorbency readings were taken every 10 sec and are plotted versus time of incubation for (A) 300 μM R1-R2 IR peptide (KVQIINKKLD), (*B*) 300 μM control peptide (IKDNVKQILK), and (*C*) no peptide.

Discussion

We have used several lines of evidence to demonstrate that the tau R1-R2 IR domain: (*a*) contains a high affinity microtubule binding site with at least twice the binding affinity of any individual 18-amino acid repeat; (*b*) is sufficient to promote tubulin polymerization into normal appearing microtubules; and (*c*) binds to a distinct site (or sites) on microtubules than bound by the repeats. Since the R1-R2 IR tubulin binding site is specifically expressed in adult tau isoforms, these results have important implications for the developmental regulation of microtubules. Our data demonstrate that the transition from fetal to adult tau isoforms is accompanied by qualitative as well as quantitative changes in tau–microtubule interactions. In addition to increasing the microtubule binding affinity of tau, expression of the 31-amino acid insert introduces a unique type of tubulin binding site (the R1-R2 IR) that may enable adult tau to regulate microtubules differently than can fetal tau.

The Functional Roles of IR Domains

These results require that our perspectives regarding the

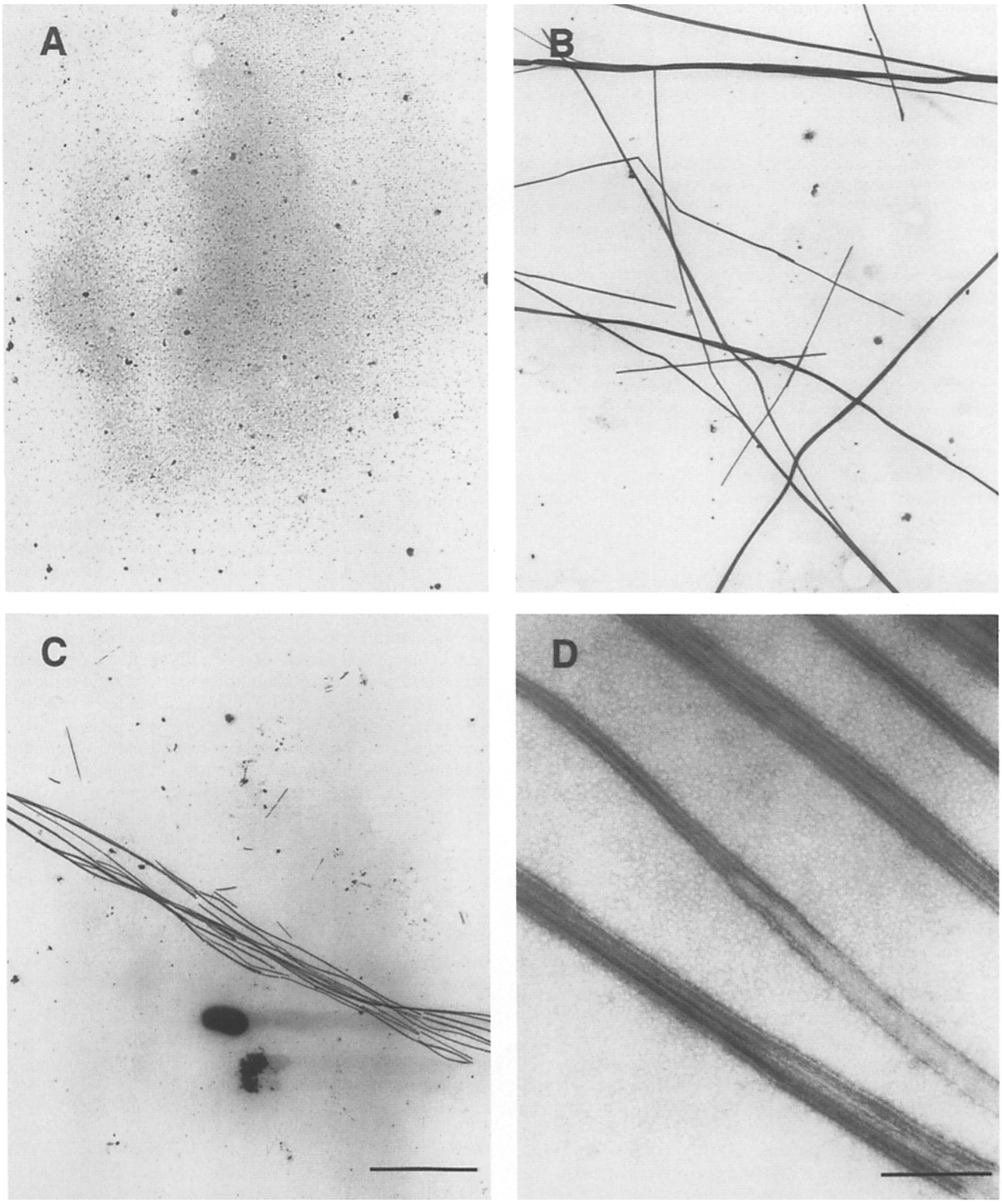

Figure 9. Electron micrographs of microtubules assembled by the R1-R2 IR peptide (KVQIINKKLD). Samples of the tubulin polymerization reactions shown in Fig. 8 were removed 20 min after initiation at 30°C and diluted 20-fold in 30% glycerol, negatively stained with uranyl acetate, and visualized at low and high magnification. (*A*) Low magnification of tubulin incubated with control peptide (IKDNVKQILK). (*B* and *C*) Low magnification of microtubules assembled with the R1-R2 IR peptide. Bar, 1 μM; bar shown in C also applies to panels A and B. (*D*) High magnification of microtubules assembled with the R1-R2 IR peptide. Bar, 100 nM.

functional role(s) of the tau IR domains be re-evaluated. The previous assignment of mere linker function to IR domains has been based upon two arguments. First, microtubule binding analysis of the R3-R4 IR domain revealed no detectable binding activity (Butner and Kirschner, 1991). Our data corroborate this observation (see Fig. 4 and Table I). However, until the present work, no other IR domains had been examined sufficiently. Second, interpretations of sequence data have focused upon the extensive sequence homology among the repeats and the minimal sequence homology among the IR regions (see Fig. 1 *B*), all within single tau polypeptides. At the same time, much less attention has been given to the fact that the sequence of each individual IR region is identical across species lines (human, bovine, rat, murine) (Goedert et al., 1989*a*,*b*; Himmler et al., 1989*a*; Kosik et al., 1989; Lee et al., 1988). Such extraordinary evolutionary conservation would not be expected if the IR regions served only a linker function. Considering both the sequence data and the work presented here, we suggest that the variation in IR sequences may reflect the diversity of their functional roles within the microtubule binding region. Our data demonstrate roles for the R1-R2 IR domain in microtubule binding and assembly, leaving the possible functions of the R2-R3, R3-R4, and R1-R3 (in 3R tau) IR domains yet to be determined.

Comparison of Truncation Analysis Results with Previous Reports

That the R1-R2 IR possesses strong microtubule binding activity is, in fact, consistent with previous work. Butner and Kirschner (1991) reported that a fragment containing repeat 1 and the R1-R2 IR (corresponding to amino acids 239–273) in rat tau) possesses a disproportionately strong microtubule binding affinity compared to fragments containing each of the other three repeats. These authors noted that the added microtubule affinity must be contained either in the specific amino acid residues that distinguish repeat 1 from the other three repeats or in the sequences that flank repeat 1. However, the methods employed could not further localize this unusual activity. Our analyses now make it clear that the added microtubule binding activity in that tau fragment is derived from the R1-R2 IR, not the first repeat.

Further comparison of our data with that of Butner and Kirschner (1991) reveals good agreement with respect to the overall patterns of binding behavior but some quantitative differences in K_d values. Several possible explanations for these differences exist. First, while the constructs used here contain intact amino termini, the truncation strategy employed by Butner and Kirschner (1991) required deletion of the amino-terminal third of the protein (152 amino acids). Although the amino terminus is not sufficient to bind microtubules at a detectable level (Lee et al., 1989), it has been shown to exert a modulatory influence upon the microtubule binding ability of the carboxyl half of tau (Butner and Kirschner, 1991), which might explain some of the observed differences. Second, tau polypeptides assayed in our study were synthesized in a rabbit reticulocyte lysate, whereas those generated by Butner and Kirschner (1991) were translated in a wheat germ extract. There could be differences in the phosphorylation states of tau polypeptides translated in these two systems that affect tau conformation and microtubule binding capability. However, since we have determined that the full-length 4R tau has the same K_d when synthesized in either translation system (Goode, B. L., and S. C. Feinstein, unpublished data), and both sets of deletion analyses were performed on 4R tau, this seems an unlikely explanation. Finally, the binding assays were performed in different buffer systems. Again however, the fact that our K_d for 4R tau is in close agreement with that of Butner and Kirschner (1991) also makes this seem an unlikely explanation. For these reasons, the most likely explanation for the quantitative differences in K_d values for some of the tau polypeptides is the presence or absence of the amino third of the protein. Understanding the modulatory influences of the amino terminus on the carboxyl microtubule binding region will required further analysis of these regions.

The R1-R2 IR and Repeats Interact with Microtubules via Different Molecular Mechanisms

Although both the R1-R2 IR and the repeats bind to microtubules, their microtubule association characteristics differ. Quantitatively, the contribution of the R1-R2 IR to the microtubule binding affinity of tau is significantly stronger than the contribution of any single repeat. Qualitatively, the R1-R2 IR and repeat 3, which was examined in detail by Butner and Kirschner (1991), appear to interact with microtubules via different molecular mechanisms. This is most clearly illustrated by comparing the linear, gradual loss of binding affinity observed as amino acids are deleted from repeat 3 (Butner and Kirschner, 1991; Fig. 8) to the nonlinear, abrupt changes in microtubule affinity we observed in a similar analysis of the R1-R2 IR (Fig. 5). Whereas the repeats have been suggested to derive their binding energies from a distributed array of weak sites involving van der Waals and/or highly shielded ionic forces (Butner and Kirschner, 1991), the R1-R2 IR derives most of its binding energy from two well defined lysine residues, implicating strong ionic interactions between the R1-R2 IR and tubulin. Assuming that all of the repeats associate with microtubules by a similar mechanism to repeat 3, these data suggest that the R1-R2 IR and the repeats bind to microtubules through distinct molecular mechanisms.

The R1-R2 IR–Microtubule Interaction Is Adult Specific

The peptide competition experiments shown in Fig. 7 suggest that a microtubule binding site homologous to the R1-R2 IR is absent in 3R tau. The role of lys272, which appears to be especially important to R1-R2 IR function (Fig. 6), may be critical in distinguishing the functional capabilities of the R1-R2 IR in 4R tau from those of the R1-R3 IR in 3R tau. Comparison of the R1-R2 IR active sequence (KVQIINKK) with the corresponding R1-R3 IR sequence (KVQIVYKP) reveals that lys265 and lys271 are present in both, but lys272 is replaced by a proline in 3R tau. In addition to markedly diminished microtubule binding affinity in the absence of lys272, the introduction of a structurally rigid proline in the R1-R3 IR may disrupt the binding capability of lys265 and/or lys271. This could explain the observation that the R1-R2 IR peptide successfully competes for microtubule binding with 4R tau, but fails to compete with 3R tau (Fig. 7 *B*). It is also

possible that the substitution of asn270 in 4R tau for tyr270 in 3R tau may facilitate the strong binding effects of lys272.

Structural Considerations for the Microtubule Binding Regions of 3R and 4R tau

An apparent discrepancy exists between the results of our 4R tau deletion analysis and our comparison of full-length 3R tau and 4R tau. Truncation and mutagenesis analyses indicate that the developmentally regulated 31-amino acid insert contributes ~10-fold to the microtubule binding affinity of 4R tau (see Fig. 4, 5, and 6). If the overall structures of the microtubule binding regions of 3R and 4R are similar, this predicts that the difference between the K_d's of full-length 3R and 4R tau should also be ~10-fold. In striking contrast, we have found that they differ by ~40-fold (Fig. 4).

The explanation for this paradox may lie in functionally significant structural differences between the microtubule binding regions of 3R and 4R tau. Recent evidence shows that 3R and 4R tau can be differentially phosphorylated at common sites (Kanemaru et al., 1992; Ksiezak-Reding et al., 1992; Bramblett et al., 1993; Goedert et al., 1993), suggesting the presence of conformational differences between 3R and 4R tau. Consistent with this model, Wille et al. (1991) have determined that the microtubule binding regions of 3R and 4R tau have equivalent molecular lengths, despite a large difference in their respective primary sequence lengths. Thus, insertion of the 31-amino acid insert may not simply be additive from a structural point of view, but rather could alter the overall structure of the microtubule binding region. In this manner, the contribution of a particular domain to the microtubule binding affinity could vary depending on its structural context (3R or 4R tau). Consequently, understanding the domain contributions to the binding microtubule affinity of 3R tau may require a separate deletion analysis of that isoform.

A second issue to address is the difference in results between our 3R versus 4R tau comparison and those of two other groups. As mentioned earlier, we find ~40-fold difference between the K_d of 3R tau and 4R tau. In contrast, Butner and Kirschner (1991) and Gustke et al. (1992) found only approximately four- to five-fold differences. However, a major difference between these studies is that each group used different translation systems. Butner and Kirschner (1992) employed a wheat germ extract, Gustke et al. (1992) expressed tau in *E. coli* and we used a rabbit reticulocyte lysate. To assess potential effects of translation systems, we have compared directly the microtubule binding affinities of 3R and 4R tau translated in wheat germ extract and reticulocyte lysates. Consistent with the findings of Butner and Kirschner (1991) and our work described in this paper, we observed differences of approximately four- to five-fold and ~40-fold, respectively (our unpublished data). While the binding of 4R tau was very similar in both translation systems, 3R tau bound ~10-fold lower when synthesized in the reticulocyte lysate relative to the wheat germ extract. One likely explanation for this difference is that each translation system possesses different kinase/phosphatase activities, some of which specifically reduce the MT binding affinity of 3R tau (Goedert et al., 1993; Bramblett et al., 1993). Elucidating the molecular mechanisms underlying these observations may be of great use in understanding the regulation of tau structure and function.

What Is the Nature of the Cognate Tau Binding Sites in Tubulin?

A question that naturally arises from these observations is the identity of the tau-binding sites on tubulin. It has been shown that subtilisin digestion of tubulin produces a glutamic acid-rich 4-kD fragment, derived from the carboxyl terminus of tubulin, that binds both tau and MAP 2 (Serrano et al., 1984*a*; Littauer et al., 1986; Maccioni et al., 1988; Cross et al., 1991). This region is believed to be exposed on the surface of tubulin and to suppress tubulin self-association through acidic charge repulsion (Luduena, 1993). Consistent with this model, subtilisin removal of the carboxyl tail dramatically lowers the critical concentration required for tubulin polymerization (Serrano et al., 1984*b*). Given the importance of lysines 265, 271, and 272 in the interaction between the tau R1-R2 IR domain and microtubules, it is attractive to speculate that the R1-R2 IR binds to the acidic carboxyl terminus of tubulin, masking its negative ionic influence on tubulin polymerization and promoting microtubule assembly. This model is consistent with our observation that the R1-R2 IR peptide, which is only 10 amino acids in length, is sufficient to promote tubulin polymerization.

On the other hand, this model also requires that the carboxyl terminus of tubulin contains two distinct classes of tau-binding sites (one for the R1-R2 IR and one for repeats) since the repeat-binding site has previously been mapped to this region (Farias et al., 1992). At first glance, this appears to contradict reports that the carboxyl terminus of tubulin contains only one class of tau-binding site (Maccioni et al., 1988; Farias et al., 1992). However, these investigations of the tau–tubulin interaction were limited to a short carboxyl-terminal tubulin peptide corresponding to just one of the six β tubulin isotypes. Since the carboxyl terminus of β tubulin is highly variable among different isotypes (reviewed in Luduena, 1993), it is possible that only a subset of β tubulin isotypes contain binding sites for the R1-R2 IR. It is also possible that sequences in α tubulin are required to form the R1-R2 IR tau binding site or that posttranslational attachment of glutamate residues in the carboxyl terminus of tubulin (reviewed in Luduena, 1993) is required. Our suggestion that the R1-R2 IR binds the carboxyl tail of tubulin is merely a speculation; it is equally possible that it binds elsewhere in tubulin. Another potential R1-R2 IR-binding site worth noting is the amino terminus of α tubulin, as suggested by Littauer et al. (1986).

Developmental Implications of the Adult Specific Expression of the R1-R2 IR

During the developmental window of time from P8 to P15 in rat, neuronal migration and axonal pathfinding are largely completed and neurons become actively engaged in synapse formation. The cytoskeletal plasticity associated with immature neurons is significantly diminished and microtubule arrays are stabilized. In the search for molecular mechanisms underlying these developmental changes, it has been shown that the transition from 3R to 4R tau expression occurs within this same time frame (Kosik et al., 1989). As has been suggested (Bramblett et al., 1993), 3R tau may facilitate

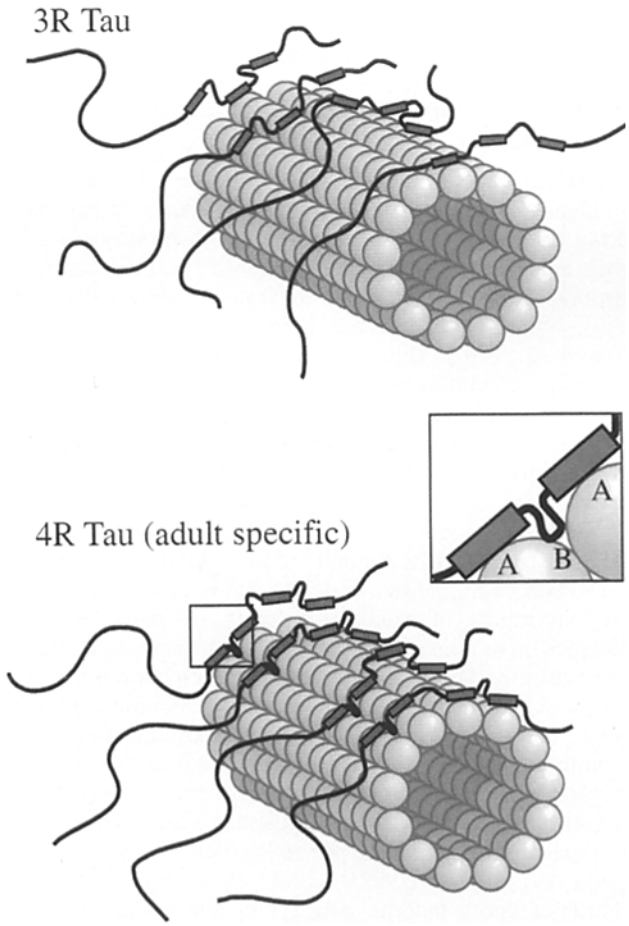

Figure 10. Model depicting how 3R and 4R tau may associate differently with microtubules. 3R tau may migrate along the microtubule surface through an array of reversible weak interactions, as suggested by Butner and Kirschner (1991). Incorporating the findings of this study, 4R tau is shown tightly tethered to a position on the tubulin lattice through the interaction of its adult-specific high affinity binding site (see discussion). In this way, 4R tau may more efficiently hold its position on the microtubule lattice and prevent tubulin depolymerization. This model is consistent with reports that 4R tau binds and stabilizes microtubules more efficiently than 3R tau. The 4R tau–microtubule interaction is enlarged in the inset to illustrate that the R1-R2 IR likely binds to distinct site(s) (site B) on tubulin from the 18–amino acid repeats (site A), as suggested from the microtubule binding competition assays in Fig. 7.

more rapid shifts in the equilibrium of microtubules between the polymerized and depolymerized states, which may enhance the ability of dendrites and axons to reposition themselves as they establish contacts with their targets. Our demonstration that 4R tau interacts with microtubules qualitatively as well as quantitatively differently than 3R tau provides insight into a possible molecular mechanism underlying the transition in microtubule state and suggests a unique microtubule-associated function for the carboxyl tau insert.

Based upon the distributed array of weak binding energies observed for the interaction between repeat 3 of tau and microtubules, Butner and Kirschner (1991) suggested that tau polypeptides "can assume multiple conformations and can pivot and perhaps migrate on the surface of the microtubule." While this may accurately describe the binding behavior of 3R tau, the strength and highly localized nature of the R1-R2 IR interaction with microtubules suggests that 4R tau likely assumes a more defined conformation. We suggest that the high affinity R1-R2 IR domain–tubulin interaction may anchor the otherwise flexible and mobile tau molecule to a unique binding site on microtubules (see Fig. 10). This anchor may also increase the efficiency of repeat–microtubule interactions by tethering adjacent repeats (repeat 1 and 2) closer to the microtubule surface. In doing so, the adult tau-specific anchoring event could more efficiently inhibit the loss of tubulin subunits from the ends of microtubules, providing additional stability to the microtubule array. Another consequence of the R1-R2 IR–microtubule interaction might be to induce a conformational change in tubulin favoring self association and net assembly. This could explain the positive effect upon 3R tau binding to microtubules seen in the presence of the R1-R2 peptide (Fig. 7 *B*). Clearly, these possibilities are not mutually exclusive.

Summary

We have identified and characterized a developmentally regulated microtubule binding and assembly domain located in the R1-R2 IR of tau. We have shown that the R1-R2 IR interacts directly with tubulin at a site(s) distinct from those utilized by the 18–amino acid repeats. Since the developmental switch from 3R tau to 4R tau expression coincides with the maturation of the neuronal cytoskeleton, the additional capabilities conferred on tau by the R1-R2 IR may figure prominently in cytoskeletal maturation and proper neuronal development.

We are extremely grateful to Les Wilson and Mary Ann Jordan and the members of their labs for their expertise and generosity throughout this project. In addition, we especially acknowledge Ashley Davis for assistance with the EM; Andy Hunter for assistance with DNA sequencing; Herb Miller for his patience and abundant supplies of tubulin; Monte Radeke for synthesizing and purifying synthetic peptides; and Rob Toso for help with video enhanced microscopy. We thank Hanna H.-Reding for providing an initial aliquot of the R1-R2 IR synthetic peptide; the UCSB Chemistry Department FABMS facility for establishing the molecular integrity of our peptides; and Margie Jess for assistance with computer graphics. We are also grateful to Joe McCarty for recombinant tau protein; Ken Kosik for tau cDNAs and the 5E2 anti-tau mAb; and Michael Klymkowski for the E7 anti-β tubulin mAb. Finally, we are grateful to Debbie Blumberg, Ashley Davis, Kathy Foltz, Beth Hinkle, Vicky Makrides, Joe McCarty, and Monte Radeke for critical comments on the manuscript.

This work was supported by grants from the California Department of Health Services (Alzheimer's Disease Program) and the University of California, Santa Barbara Academic Senate to S. C. Feinstein.

Received for publication 8 September 1993 and in revised form 10 December 1993.

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