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The Structural Basis of Monoclonal Antibody Alz50's Selectivity for Alzheimer's Disease Pathology*

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The epitope on tau protein recognized by the monoclonal antibody Alz50 was defined through internal deletion mutagenesis and quantified by affinity measurements. The epitope is discontinuous and requires both a previously identified N-terminal segment and the microtubule binding region for efficient binding of Alz50. The interaction between these regions is consistent with an intramolecular reaction mechanism, suggesting that Alz50 binding depends on the conformation of individual tau monomers. The results suggest that tau adopts a distinct conformation when polymerized into filaments and that this conformation is recognized selectively by Alz50.

Alz50 is an IgM-class monoclonal antibody that stains the fibrillar pathology (dystrophic neurites, neurofibrillary tangles, and neuropil threads) commonly observed in postmortem histological analysis of Alzheimer's disease (AD)[1] brain (1, 2). Because of these properties, it has emerged as an important tool for gauging the temporal and spatial severity of Alzheimer's disease pathology (3, 4). The major components of the fibrillar pathology are straight and paired helical filaments (PHF) (5), which themselves are comprised largely of hyperphosphorylated forms of the microtubule-associated protein tau (6–10). Previous studies have shown that Alz50 reacts with tau and that its epitope is located at the N terminus (11–13) in a region conserved in all known splice variants of human tau (14). Indeed, Alz50 has been shown to react with tau proteins isolated from normal brain (15), recombinant sources (12), and PHFs (8) by Western analysis. Nonetheless, the ability of Alz50 to label distinct populations of neurons in normal human brain (16), fetal brain (17), and early stage neurofibrillary degeneration (4, 18) suggests that Alz50 selectively recognizes a distinct subset of tau proteins.

To place the many observations on Alz50 immunocytochemistry into a structural context, we reinvestigated its epitope selectivity *in vitro*. The results suggest that individual tau monomers adopt a specific conformation preceding or during filament formation that is selectively recognized by Alz50.

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[1] The abbreviations used are: AD, Alzheimer's disease; PHF, paired helical filament; TBS, Tris-buffered saline; kb, kilobase; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

EXPERIMENTAL PROCEDURES

Materials—All monoclonal antibodies were prepared from supernatants of hybridoma cells grown in serum-free medium. Supernatants containing Alz50 were pooled, precipitated with 45% ammonium sulfate, resuspended in TBS (50 mM Tris HCl, pH 7.5, 50 mM NaCl, and 1 mM EGTA), and dialyzed twice against 100 volumes of TBS. The dialysate was clarified by centrifugation and redialyzed against 5 mM sodium phosphate, pH 7.5, to precipitate the IgM. The resultant fine precipitate was resuspended in S300 buffer (50 mM Tris HCl, pH 7.5, 700 mM NaCl, and 1 mM EGTA), dispersed with 30 strokes of a glass-Teflon homogenizer, and loaded onto a 400-ml (2.6 × 100-cm) S300HR gel filtration column equilibrated and run in S300 buffer. The IgM fraction emerging in the void volume was pooled, dialyzed against storage buffer (10 mM HEPES, pH 7.4, 50% glycerol, and 150 mM NaCl), and stored at −20 °C until used. IgGs were purified by protein A-Sepharose chromatography (19), dialyzed against storage buffer, and stored at −20 °C until used. These included monoclonals Tau-1 (20), Tau-2 (21), Tau-5 (22), 5E2 (23), and Tau46.1 (24), which were raised against bovine tau, and TG5 and MC1, which were raised against human PHF (25). PHFs were prepared by affinity chromatography (26) and supplied by P. Davies (Albert Einstein College of Medicine, Bronx, NY).

Strains—Escherichia coli strains RZ1032 (HfrKL16 PO/45 [*lysA*(61–62)] *dut1 ung1 thi1 relA1* Zbd-279::Tn10 *supE44*) (27), BL21(DE3) (F^- *hsd*S *ompT [lon] lac*UV-5-T7 gene 1) (28), and MM294 (F^- *supE44 hsdR17 endA1 pro thi1*) (29) were used for mutagenesis, expression, and routine transformations, respectively.

Recombinant tau—An expression plasmid for tau was constructed from full-length four-repeat tau (htau40) (14) by first adding useful restriction sites to its cDNA (*Nde*I at the initiation codon and *Eco*RI just after the termination codon) using PCR methodology as described previously (30). For expression in bacteria, the resultant 1.3-kb PCR fragment was digested with *Nde*I/*Eco*RI and ligated into the *Nde*I/*Eco*RI sites of the *E. coli* expression vector pT7C. This derivative of pT7B (31) drives the overproduction of proteins fused to a polyhistidine tag derived from pET15B (32).

Deletion Mutagenesis—A library of tau deletion mutants was built from pT7C-htau40 by either nuclease digestion or oligonucleotide-directed mutagenesis as described in Table I. The resultant constructs were confirmed by DNA sequence analysis.

Epitope Mapping—Antibody reactivity of individual tau deletion mutants was determined in a solid-phase expression assay (31). BL21(DE3) cells containing tau expression plasmids were grown in a grid pattern on nitrocellulose filters, lysed over chloroform, and processed as described (31). Antibody binding was detected by enhanced chemiluminescence using sheep anti-mouse IgG/IgM conjugated to horseradish peroxidase (33). Images were collected on x-ray film and quantified by laser densitometry (Bio-Rad).

Analytical Methods—Histidine-tagged tau proteins were purified by immobilized metal affinity and gel filtration chromatographies as described for other proteins (32). Protein concentrations of purified htau40 and all antibodies were estimated spectrophotometrically. The extinction coefficient for htau40 was calculated from amino acid content ($A^{\%}_{280\ nm}$ = 1.46) (34), whereas the coefficients for mouse IgG ($A^{\%}_{280\ nm}$ = 13.5) and mouse IgM ($A^{\%}_{280\ nm}$ = 12.0) were taken from the literature (19). Molar concentrations of IgGs and IgMs were estimated assuming molecular masses of 160 and 950 kDa, respectively. The protein concentration of PHF preparations was assayed by the method of Bradford (35) using recombinant tau as standard.

Antibody affinity determinations were performed in duplicate by enzyme-linked immunosorbent assay as described previously (36). Reaction of fixed concentrations of antibody (0.04 nM Alz50, 0.025 nM

TABLE I
Tau deletion mutants used in this study

Mutant	Method[a]	Sites or sequence[b]	Resultant sequence[c]
Δ430–441	S	*Nhe*I	. . .–A^{429}–S* (stop)
Δ395–427	S	*Bsr*GI/*Nhe*I	. . .–Y^{394}–L^{428}–. . .
Δ357–393	S	*Ppu*MI/*Bsr*GI	. . .–S^{356}–Y^{394}–. . .
Δ345–441	S	*Hin*dIII	. . .–L^{344}–N* (stop)
Δ333–441	S	*Pfl*MI/*Nhe*I	. . .–P^{332}–A*–S* (stop)
Δ321–441	S	*Bst*EII/*Nhe*I	. . .–T^{319}–S^{320} (stop)
Δ283–441	O	CAGATAATTAATAAGAAGCTGTAAGAATTCAAA	. . .L^{282} (stop)
Δ231–240	O	GCAGTGGTCCGTAGCCGCCTGCAG	. . .–K^{230}–S^{241}–. . .
Δ241–255	O	GTCTTCCGCCAAGGTCAAGTCCAAGAT	. . .–S^{240}–V^{256}–. . .
Δ256–270	O	CAGACCTGAAGAATGGAGGCGGGAAG	. . .–N^{255}–G^{271}–. . .
Δ271–282	O	AAGCACCAGCCGGATCTTAGCAACGT	. . .–P^{270}–D^{283}–. . .
Δ222–301/344[d]	S	—	. . .R^{221}–V*–E*–G^{302}–. . . . . .–L^{344}–N* (stop)
Δ211–356	S	*Ppu*MI/*Bsr*BI	. . .–S^{210}–L^{357}–. . .
Δ163–355	S	*Sfi*I/*Ppu*MI	. . .–Q^{162}–S^{356}–. . .
Δ162–244	S	*Sfi*I/*Pst*I	. . .–Q^{162}–T^{245}–. . .
Δ162–209	S	*Sfi*I/*Bsr*BI	. . .–G^{161}–H*–S^{210}–. . .
Δ155–393	S	*Sac*II/*Bsr*GI	. . .–P^{154}–Y^{394}–. . .
Δ155–355	S	*Sac*II/*Ppu*MI	. . .–P^{154}–S^{356}–. . .
Δ155–244	S	*Sac*II/*Pst*I	. . .–P^{154}–T^{245}–. . .
Δ155–209	S	*Sac*II/*Bsr*BI	. . .–P^{154}–S^{210}–. . .
Δ155–163	S	*Sac*II/*Sfi*I	. . .–P^{154}–G^{164}–. . .
Δ122–427	S	*PmI*I/*Nhe*I	. . .–H^{121}–L^{428}–. . .
Δ103–334	S	*Pvu*II/*Pfl*MI	. . .–T^{102}–G^{335}–. . .
Δ103–155	S	*Pvu*II/*Sac*II	. . .–T^{102}–G*–G^{156}–. . .
Δ84–393	S	*Sac*I/*Bsr*GI	. . .–G^{83}–Y^{394}–. . .
Δ84–354	S	*Sac*I/*Ppu*MI	. . .–G^{83}–S^{356}–. . .
Δ84–161	S	*Sac*I/*Sfi*I	. . .–G^{83}–G^{164}–. . .
Δ19–121	S	*Bsi*WI/*Pml*I	. . .–Y^{18}–V^{122}–. . .
Δ19–83	S	*Bsi*WI/*Sac*I	. . .–Y^{18}–A^{84}–. . .
Δ9–155	S	*Bst*BI/*Sac*II	. . .–F^{8}–G*–G^{156}–. . .
Δ2–18	O	CGGCAGCCATATGGGGTTGGGGGAC	. . .–M^{1}–G^{19}–. . .

[a] Deletions were created by oligonucleotide-directed mutagenesis (O) or by restriction digests (S).
[b] Pairs of restriction sites were selected that, when digested and religated, would conserve the htau40 reading frame. pT7C-htau40 was cleaved with the selected endonucleases, blunt-ended with either Klenow fragment of DNA polymerase I (for 5′ overhangs) or T4 DNA polymerase (for 3′ overhangs), or both. Resultant fragments were ligated and used to transform MM294 cells.
[c] Asterisks represent residues not normally part of htau40 that were introduced by mutagenesis.
[d] The Δ222–301/344 and Δ345–441 were gifts of G. Jicha and P. Davies. Chimera Δ222–301/344 was created by ligating a *Sma*I–*Hin*dIII fragment encoding to the third microtubule repeat (Gly^{302}–Leu^{344}) to a *Sma*I fragment encoding the N-terminal 221 residues of tau. In the final construct, the two segments are connected via a two residue linker (Val–Glu). Bacterial expression of this and all other constructs was confirmed by SDS-polyacrylamide gel electrophoresis.

Tau-5, 0.01 nM TG5, 0.5 nM MC1, and 0.01 nM Tau46.1 or 0.2 nM Tau-2) with varying concentrations of analyte (htau40 and PHF-tau) proceeded in blocking buffer (0.2% bovine serum albumin, 0.02% NaN_3, and TBS) overnight at 4 °C. For each antibody assayed, the concentration range of analyte varied from a minimum of at least 10-fold above the concentration of antibody binding sites (except where noted) to a maximum of approximately 100 nM. Under these conditions, analyte concentrations approximated a steady state. After this incubation, concentrations of unbound antibodies were determined by enzyme-linked immunosorbent assay performed in 96-well plates precoated with 250 ng of htau40/well and blocked with 5% nonfat milk/TBS. Captured antibodies were detected using horseradish peroxidase-linked anti-mouse IgG and *o*-phenylenediamine dihydrochloride as colorimetric substrate. Reactions were stopped with H_2SO_4 and quantified by absorbance measurements (490 nm).

The resultant data was analyzed graphically as Scatchard plots of ν/a *versus* ν, where ν is the fraction of total antibody binding sites bound at a given concentration of analyte and a is the corresponding free analyte concentration (36). Binding parameters were calculated from linear regression analysis of these plots. Dissociation constants (K_d) were derived from the negative slopes (reported ± 1 S.E. of the estimate), whereas the fractions of antibody binding sites occupied at saturating concentrations of analyte were calculated from the abscissa intercept (37).

Calibration—The *accessible* content of tau in nondenatured PHFs was determined by solution-phase (enzyme-linked immunosorbent assay) immunoassays using pure htau40 as standard and Tau46.1, 5E2, Tau-5, and TG5 as primary antibodies. Because PHFs contain all six splice forms of tau, ranging from 351 to 441 amino acids in length, a mean molecular mass of 45 kDa was assumed for PHF-tau.

RESULTS

Alz50 Reacts Preferentially with PHF-tau—To quantify the binding selectivities of a panel of anti-tau monoclonal antibodies, equilibrium binding experiments with both monomeric tau (htau40) and authentic affinity-purified PHFs were performed as described under "Experimental Procedures." On the basis of SDS-polyacrylamide gel electrophoresis, all protein reagents used in binding experiments were of high purity (Fig. 1). The analysis began with four anti-tau IgGs known to bind continuous epitopes on tau independently of the state of tau phosphorylation: Tau-5 (22), 5E2 (23), Tau46.1 (24), and TG5 (see below). Results for Tau-5 binding to both htau40 and PHFs are illustrated in Scatchard format in Fig. 2. The resultant Scatchard plots are linear, indicating that the Tau-5 antibody interacts with tau via a single noncooperative binding site (37). Furthermore, each plot revealed nearly complete fractional occupancy at saturating concentrations of analyte (tau), proving that essentially all antibody binding sites are accounted for and that the Tau-5 antibodies are fully active. The binding constants (K_d) calculated from these plots and summarized in Table II show that Tau-5 binds both monomeric htau40 and PHFs with nearly identical affinity. These results are consistent with Tau-5 binding a tau epitope that is presented nearly identically in monomeric and PHF-tau.

Binding data for Tau46.1, 5E2, and TG5 are also summarized in Table II. Although these antibodies bind tau with differing affinities, each retains the characteristics summarized above for Tau-5. Each antibody returned linear Scatchard plots and bound monomeric tau and PHFs with nearly identical affinities (K_d) and with essentially full occupancy of available antibody binding sites. Thus, antibodies that bind tau through simple continuous epitopes, such as Tau-5, Tau46.1, 5E2, and

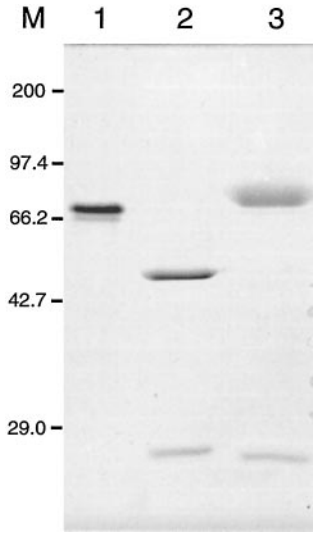

FIG. 1. **Reagent purity.** Anti-tau antibodies and recombinant htau40 were purified as described under "Experimental Procedures," resolved by SDS-polyacrylamide gel electrophoresis (11% acrylamide), and stained with Coomassie Blue. *Lanes 1–3* contain 2-μg aliquots of htau40, the IgG Tau-5, and the IgM Alz50, respectively. *Lane M* contains molecular mass standards myosin (200 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), and bovine carbonic anhydrase (29 kDa). The two bands stained in the Tau-5 and Alz50 lanes correspond to heavy and light immunoglobulin chains. The purity of Tau-5 is typical of all IgGs used in this study.

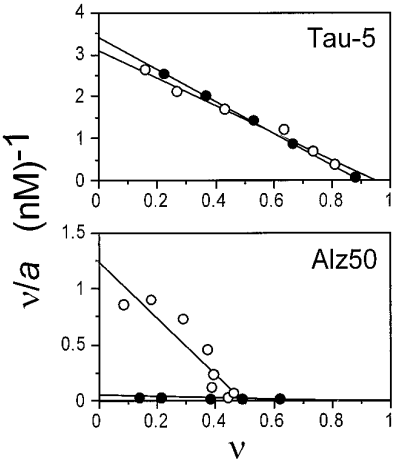

FIG. 2. **Alz50 is selective for PHF-tau.** The affinity of Tau-5 (*top*) and Alz50 (*bottom*) for purified recombinant htau40 (●) and PHF-tau (○) was determined as described under "Experimental Procedures." The results are depicted as Scatchard plots in which ν is the fraction of bound analyte and a is the concentration of free analyte at equilibrium (25). Tau-5 binds both forms of tau with high affinity and complete saturation of available antibody binding sites (*i.e.* $\nu \approx 1$ at saturating analyte concentration). In contrast, Alz50 binds PHF-tau more tightly than it binds htau40 and with occupation of only 50% of available antibody binding sites (see text for details).

TG5, comprise a single class of reagent capable of detecting tau equally well in its monomeric or pathologically aggregated states.

Like the Tau-5 class of antibody described above, the interaction of Alz50 with tau derived from normal brains, AD brains, and recombinant sources is well documented (13, 14). To quantify its selectivity for tau isoforms in solution relative to other tau antibodies, Alz50 was subjected to affinity measurements against both recombinant human tau and authentic PHFs as described above. The resultant Scatchard plots are illustrated in Fig. 2. Like the antibodies introduced above,

TABLE II
Affinity measurements

Antibody	htau40 K_d	PHF K_d	Ratio[a]
	nM	*nM*	
Tau46.1	5.13 ± 0.50	7.69 ± 0.30	0.67 ± 0.09
5E2	0.44 ± 0.06	0.34 ± 0.05	1.30 ± 0.26
Tau-5	0.26 ± 0.01	0.35 ± 0.02	0.74 ± 0.05
TG5	0.084 ± 0.011	0.070 ± 0.011	1.20 ± 0.25
Alz50	25.0 ± 3.8	≤0.32 ± 0.11	≥76.0 ± 27.8
Tau-2	90.9 ± 24.8	6.67 ± 0.89	13.6 ± 4.1
MC1	40.2 ± 3.4	5.66 ± 0.71	7.1 ± 1.1

[a] Ratio = K_d of tau binding/K_d of PHF-tau binding. Values less than unity reflect selectivity for recombinant htau40, whereas values greater than unity reflect selectivity for PHF-tau.

Alz50 bound recombinant tau ($K_d \cong 20$ nM) with nearly complete saturation of available binding sites. Yet Alz50 differed by displaying a clear selectivity for PHF-tau, which it bound with an observed affinity of 0.32 nM (Fig. 2; Table II). Because this value approximates the concentration of antibody binding sites in the assay, it must be considered an upper limit for K_d (38, 39), so its true affinity for PHF may be even greater. The results indicate that the interaction of Alz50 with PHF-tau is nearly 2 orders of magnitude greater affinity than its interaction with recombinant monomeric tau. In addition, the stoichiometry of binding is reduced to ≈0.5 PHF-tau molecules/antibody binding site, perhaps reflecting steric hindrance between the decavalent Alz50 (an IgM) and the PHFs (which average 20 nm in width and 0.25 μm in length) (26).

The behavior of Alz50 was paralleled by that of Tau-2, an IgG raised against bovine tau (21). Tau-2 is known to react poorly with recombinant human tau (40) but strongly with the fibrillar pathology found in AD brains (41). As summarized in Table II, Tau-2 also is selective for the PHF conformation of human tau, which it binds with >10-fold higher affinity than it binds recombinant htau40. Similar behavior also is exhibited by MC1, a new IgG raised against PHF (25). These results suggest that Alz50, Tau-2, and MC1 form a second class of anti-tau antibody that reacts preferentially with tau epitopes when they are presented in the context of PHF.

Alz50 Recognizes a Conformational Epitope on tau—The affinity data confirms that nonphosphorylated recombinant tau contains the sequences necessary for Alz50 binding but suggests that unlike Tau46.1, 5E2, and Tau-5, factors other than primary structure mediate the high affinity interaction between PHF-tau and Alz50. To examine this possibility, the ability of anti-tau antibodies to bind either htau40 or htau40 denatured via boiling in dilute SDS was assessed. As shown in Fig. 3, antibodies that recognize continuous epitopes on tau, such as Tau46.1, react similarly with htau40 regardless of whether this analyte was subjected to SDS-mediated denaturation. Alz50 staining, however, is nearly destroyed by this treatment. Similar results were obtained when PHF-tau was substituted for htau40 as analyte. In this case, however, total Tau46.1 reactivity seems to increase after PHF denaturation (Fig. 4). Because the binding affinity of Tau46.1 for tau is insensitive to denaturation (Fig. 3), this increase in Tau46.1 reactivity probably results from more tau being accessible to it. These results suggest that a portion of tau molecules packed into PHFs are inaccessible to antibody until released by denaturation. Despite the increase in accessible tau, PHF denaturation still leads to a dramatic decrease in Alz50 reactivity (Fig. 4). Together these data suggest that the Alz50 epitope on human tau and PHF is denaturation sensitive, and therefore probably conformational in nature.

Epitope-mapping Experiments—To determine the amino acid sequences on tau that serve as epitopes, a recombinant

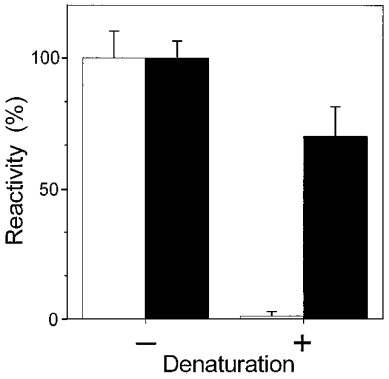

FIG. 3. **Alz50 binds a conformational epitope on tau.** Htau40 (3 ng) was spotted on nitrocellulose paper before (−) or after (+) incubation under denaturing conditions (5 min in boiling 0.1% SDS). The resultant filters were then probed with 0.1 nM Alz50 (□) or 1 nM Tau46.1 (■) and developed using chemiluminescent detection as described under "Experimental Procedures." The signal obtained for each analyte/antibody pair in the absence of denaturation was defined as 100% reactivity for purposes of normalization. *Bar*, mean ± range of duplicate assays. The results show that Alz50 reactivity toward denatured tau is reduced dramatically relative to that of Tau46.1, an antibody that recognizes a continuous epitope on tau.

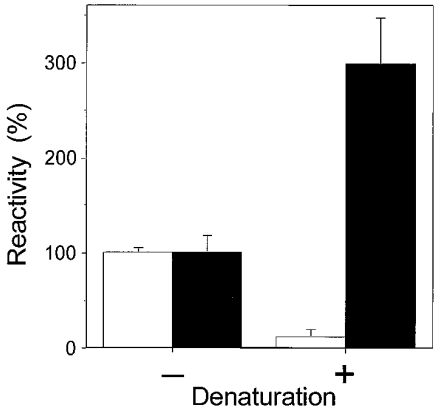

FIG. 4. **Alz50 binds a conformational epitope on PHF.** PHFs (3 ng) were spotted on nitrocellulose paper before (−) or after (+) incubation under denaturing conditions (5 min in boiling 0.1% SDS). The resultant filters were then probed with 0.1 nM Alz50 (□) or 1 nM Tau46.1 (■) and developed using chemiluminescent detection as described under "Experimental Procedures." The signal obtained for each analyte/antibody pair in the absence of denaturation was defined as 100% reactivity for purposes of normalization. *Bar*, mean ± range of duplicate assays. The results show that Tau46.1 reactivity increases approximately 3-fold upon PHF denaturation, suggesting that a third of tau molecules packed into PHFs are inaccessible to antibody until the structure is disrupted by denaturation. Despite the increase in accessible tau molecules, Alz50 reactivity toward PHF drops dramatically after denaturation.

library containing random deletions in the htau40 coding sequence was constructed and screened for an ability to bind various monoclonal antibodies as described under "Experimental Procedures." The approach of using deletions to map epitope boundaries was adopted because of its ability to identify both continuous and discontinuous epitopes on proteins of low secondary structure content such as tau. In addition, all tau deletion mutants made to date can be overexpressed solubly in *E. coli* at high levels, which facilitates analysis (data not shown).

To validate this mapping approach, the library was first screened with two antibodies whose epitopes on tau are known: Tau46.1 (24) and Tau-1 (24, 42–44). Results are illustrated graphically in Fig. 5 and summarized in Table III. For Tau-1, the segment Pro^{162}–Gly^{210} identified by deletion mapping encompasses the previously described Tau-1 epitope (Pro^{189}–Gly^{207}) and its essential core sequence Gly^{192}–Ser^{199} (42, 44).

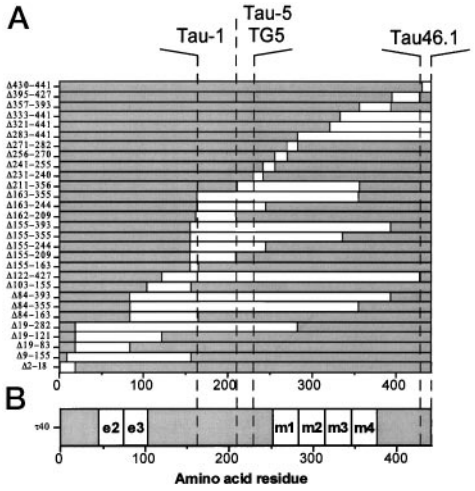

FIG. 5. **Epitope mapping.** The ability of monoclonal antibodies Tau-1, Tau-5, Tau46.1, and TG5 to interact with deletion mutants of htau40 was assessed as described under "Experimental Procedures." Epitopes (*vertical dashed lines*) were defined as segments on tau that, when deleted, completely attenuated antibody reactivity. Deletion of segments outside those identified as epitopes had no observable effect on antibody binding. *A*, mutants containing deletions in the segments illustrated lose the ability to bind Tau-1, Tau-5, Tau46.1, and TG5. The regions deleted are shown graphically in *white* for each mutant. *B*, a schematic of htau40 is shown for comparison, including the positions of alternatively spliced exons 2 and 3 (*e2* and *e3*) (62) and the four microtubule binding repeats (*m1–m4*). These results confirm that Tau-1, Tau-5, Tau46.1, and TG5 bind continuous epitopes within the tau molecule.

TABLE III
Tau epitopes

Antibody	Mapped previously?	Reference no.	This study[a]
46.1	Yes: Ser^{404}–Leu^{441}	24	Leu^{428}–Leu^{441}
Tau-1	Yes: Pro^{189}–Gly^{207}	24, 42, 43, 44	Pro^{162}–Gly^{210}
Tau-5	No		Ser^{210}–Arg^{230}
TG5	No		Ser^{210}–Arg^{230}

[a] Data are from Fig. 5.

For Tau46.1, the segment Leu^{428}–Leu^{441} (Fig. 5) identified by deletion mapping lies within the 38-residue region Ser^{404}–Leu^{441} identified previously (24) but improves the resolution of the epitope to just 14 residues.

Deletion mapping was extended to two previously uncharacterized antibodies as well: Tau-5, an IgG raised against bovine tau (22), and TG5, an IgG raised against PHF (25). The results, again illustrated graphically in Fig. 5 and summarized in Table III, reveal that both antibodies recognize essential residues located within the segment Ser^{210}–Arg^{230}. In this respect Tau-5 and TG5 closely resemble not only 5E2 (Ser^{214}–Pro^{233}) (24) but also AT120 (Pro^{218}–Lys^{224}), an antibody raised against PHF that is useful in premortem diagnosis of AD (45, 46). Thus, four monoclonal antibodies (Tau-5, TG5, AT120, and 5E2) raised independently from two different tau antigens (bovine tau and human PHF) all bind within the same ≈20-residue segment of tau. In addition to demonstrating the utility of deletion mapping, these data suggest that the region Ser^{210}–Arg^{230} is both highly antigenic and accessible on the surface of at least some of the tau molecules aggregated into PHFs.

Identification of the Alz50 Epitope on tau—The amino acid sequences necessary for Alz50 binding were determined by screening the deletion library described above. The results, shown graphically in Fig. 6, reveal that Alz50 binding is mediated by two segments on tau. The first lies within the N-terminal 18 residues of tau and corresponds to the epitope identified previously (13, 14). It is essential for Alz50 binding

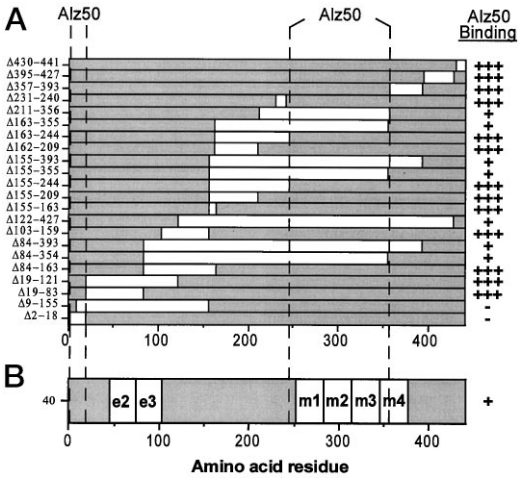

FIG. 6. **The Alz50 epitope is discontinuous.** The ability of Alz50 (0.1 nM) to interact with deletion mutants of htau40 was assessed as described under "Experimental Procedures." Epitopes (*vertical dashed lines*) were identified on the basis of two criteria: deletion of sequences within any portion of the epitope must attenuate Alz50 binding, whereas deletions completely outside the epitope must retain a strong positive interaction with Alz50. A strong positive interaction (+++) is defined as a signal ≥50% of full-length htau40, whereas a weak positive interaction (+) is defined as a signal ≤10% of htau40. Complete absence of Alz50 binding is symbolized as −. *A*, mutants containing deletions in either of the two segments illustrated do not bind Alz50 efficiently. The regions deleted are shown graphically in *white* for each mutant. *B*, a schematic of htau40 is shown for comparison, including the positions of alternatively spliced exons 2 and 3 (*e2* and *e3*) and the four microtubule binding repeats (*m1–m4*).

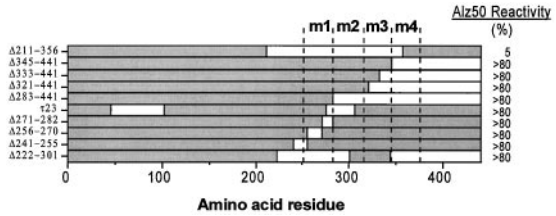

FIG. 7. **Alz50 and the microtubule repeat region of tau.** The ability of Alz50 (0.1 nM) to interact with deletion mutants spanning the microtubule binding domain of htau40 was determined as described in the legend to Fig. 6. Alz50 reactivity is shown as the percentage of reactivity compared to full-length htau40 control. The results show that a single repeat is sufficient to mediate efficient Alz50 binding (see text).

because any mutant that contains a deletion in this region cannot bind Alz50. The second epitope consists of sequences located within the microtubule repeat region. As shown in Fig. 6, deletion of the entire microtubule repeat region, such as in mutant Δ155–393, results in >90% loss of Alz50 reactivity. Smaller deletions that leave only a portion of one repeat, such as mutant Δ210–355, show the same loss of reactivity. These results suggest that Alz50 is sensitive to the conformation of tau because the epitope it recognizes is discontinuous. The full epitope is comprised of sequences located in the N-terminal and microtubule binding regions of tau.

The microtubule repeat region of htau40 consists of four ≈31-residue repeats arranged in tandem, with pairwise identities that range from 55–60% (47). When conservative substitutions are taken into account (conservative substitutions are defined in Ref. 48), the repeats share as much as 70% similarity. To determine whether the four repeats of htau40 contribute to Alz50 binding individually or in aggregate, mutants containing deletions spanning the microtubule repeat region were assayed for Alz50 reactivity as described above. The results show that mutants containing just three repeats retain full Alz50 reactivity (Fig. 7). This is most clearly seen with mutant Δ345–441 (which retains repeats m1, m2, and m3 but not m4) and the naturally occurring tau isoform htau23 (which retains repeats m1, m3, and m4 but not m2). Partial deletion of the first repeat leaving m2, m3, and m4 is also fully reactive (*e.g.* Δ241–255 and Δ256–270). Sequential deletion of C-terminal residues to leave two (Δ333–441 and Δ321–441) or just one (Δ283–441) repeat are similarly reactive with Alz50. These results suggest that the minimal reactive unit of the second Alz50 epitope is a single microtubule binding repeat. To confirm this assignment, a chimera was constructed containing the full sequence of the third microtubule binding repeat fused to the N-terminal 221 residues of htau40 and analyzed for reactivity with Alz50. Like Δ283–441, this mutant also retains full Alz50 reactivity. These results confirm that the presence of a single microtubule repeat is sufficient to enhance the binding affinity of Alz50 for tau.

The Reaction of Alz50 with tau Is Consistent with an Intramolecular Mechanism of Action—In some studies, PHFs appear as two hemifilaments wound helically around one another (5, 49), suggesting they consist of a multidimensional lattice of tau monomers, each in contact with several nearest neighbors (50). Yet, when PHFs are prepared for microscopy by freeze-drying and vertical platinum-carbon replication, no ordered substructure is resolved (51, 52), suggesting that tau monomers are arranged randomly within the PHF. Thus, Alz50 may interact preferentially with PHF-tau because individual tau monomers adopt an ordered structure, thereby stabilizing an intramolecular epitope (*i.e.* the epitope consists of different regions on a single tau polypeptide) or because amorphous aggregation of tau facilitates an intermolecular reaction (*i.e.* the epitope spans two regions of neighboring tau monomers). Similarly, the interaction of Alz50 with recombinant tau may be mediated by one or multiple tau molecules. To determine whether the interaction of Alz50 with recombinant tau was intra- or intermolecular, equal amounts of purified tau deletion mutants that lacked complementary portions of the Alz50 epitope were mixed, spotted on nitrocellulose paper, and examined for restoration of Alz50 binding. The mutants employed for this experiment were Δ2–18, an N-terminal deletion containing an intact microtubule repeat region, and Δ211–356, which has an intact N terminus but a defective microtubule repeat region. The results confirmed that deletion of either component of the Alz50 epitope eliminates or greatly reduces Alz50 binding relative to wild type (Figs. 6 and 7). Mixing of Δ2–18 and Δ211–356 before incubation with antibody, however, did not further augment Alz50 binding or restore it to wild-type levels. These data suggest that reaction of Alz50 with recombinant tau mutants cannot be reconstituted by trans-complementation of adjacent molecules, which is consistent with an intramolecular reaction mechanism.

DISCUSSION

Because Alz50 was raised against a crude Alzheimer brain homogenate (1), the precise nature of its immunogen has remained elusive. Although initial characterization suggested that Alz50 bound a novel Alzheimer's disease-associated protein (ADAP) (1, 53), subsequent work proved that it reacted with tau (12, 14), the principal component of neurofibrillary tangles (10). Nonetheless, the basis of its selective immunohistochemical properties remained unclear. The results presented here suggest that Alz50 is selective for neurofibrillary pathology in part because it binds a conformational epitope on tau that is stabilized or prevalent in PHFs. Thus, although most anti-tau antibodies stain the fibrillar pathology, Alz50 is among the most robust. Selective binding may also underlie the efficiency of PHF immunopurification using P42, an IgG class switch of Alz50 (26).

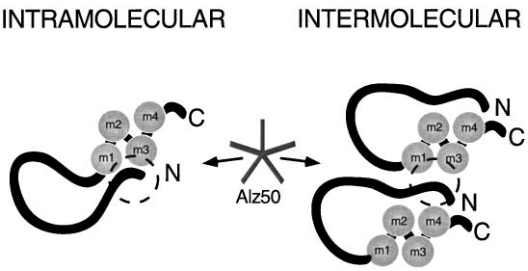

FIG. 8. **Model of Alz50 selectivity.** The Alz50 epitope is comprised of sequences from the N-terminal and microtubule repeat regions of tau (*dashed circle*). Although a single repeat is capable of promoting Alz50 binding, it is not clear which of the four present in htau40 actually does. To illustrate this point, the model shows the epitope overlapping more than one repeat. Although epitope formation theoretically could be intra- or intermolecular, evidence indicates the reaction is intramolecular. See "Discussion" for details.

A model of Alz50 selectivity is illustrated in Fig. 8. It predicts that the N terminus of PHF-tau is in close association with the microtubule repeat region. Together, the two regions comprise the Alz50 epitope. Because of an emphasis on N-terminal deletion or expression screening methods better suited for analysis of continuous epitopes (12, 26), earlier mapping strategies successfully identified the first but not the second component of the Alz50 epitope. The reaction of Alz50 with human tau isoforms in adult and fetal brain is consistent with the conservation of the epitope in all known splice variants of the tau gene (11). Although the presence of a single repeat promotes Alz50 binding, it is not clear which of the four repeats present in htau40 actually forms the epitope when the tau molecule is packed into the PHF. It is conceivable that, due to the flexibility of the tau molecule and the conserved nature of each microtubule repeat, that individual tau molecules within the PHF form the epitope from different repeats.

Although the Alz50 epitope could result from either intra- or intermolecular interactions, the inability to reconstitute it from mixed deletion mutants, its appearance before neurofibrillary tangle formation in postmortem brain sections (4), and its existence on monomeric recombinant tau as seen on Western blots suggest that the mechanism of interaction is intramolecular. Presumably, monomeric tau can adopt the Alz50 conformation due to its flexibility (54), but the energy required to do so is reflected in a higher K_d value.

It is important to emphasize that Alz50 is selective but not specific for tau conformation. As shown here, selectivity can be demonstrated at low concentrations of antibody and tau. High concentrations of either reagent can drive the reaction and mask the selectivity (13). Thus, Alz50 reacts with recombinant tau on Western blots after denaturation in SDS-polyacrylamide gels (12) and can be blocked by incubation with high concentrations of N-terminal peptide (14). The antibody also can cross-react with proteins unrelated to tau, including bovine serum albumin (55) and $p125^{fac1}$ (56). The selectivity properties of Alz50 probably contributed to the early controversies surrounding the relationship between PHFs and tau (1, 10, 53).

Early reports suggested that the Alz50 epitope was phosphorylation-sensitive (57). Although it is clear that phosphorylation is not required for epitope recognition, it may contribute to high affinity binding of Alz50 by stabilizing certain conformations of tau. Indeed, the development of Alz50 immunoreactivity parallels that of hyperphosphorylated tau as measured by the AT8 antibody during onset of AD (3). Both AT8 and Alz50 immunoreactivity seem to precede the occurrence of neurofibrillary tangles. In addition, phosphorylation may promote the assembly of tau monomers into straight and paired helical filaments, which then locks tau into the conformation selectively bound by Alz50 (58, 59).

The first antibody selective for tau conformation characterized was Tau-2, which recognizes a continuous epitope ≈16 residues in length located in the N-terminal third of the molecule. It has been suggested that as a result of differences in the structures of bovine and human tau in this region, Tau-2 reacts poorly with human tau until it adopts a conformation similar to that of bovine tau (40). Presumably, this conformation is adopted by human tau in PHFs, so that like Alz50, Tau-2 is useful in immunohistochemical studies of AD (41). On the basis of affinity measurements, we predict that MC1 will share histochemical selectivity with Alz50 and Tau-2.

The results presented herein suggest that the many anti-tau antibodies raised to date can be organized into at least three categories. The first, exemplified by Tau-5, consists of reagents that bind continuous epitopes on tau independently of tau conformation or state of postranslational modification. Antibodies of this class have proved valuable as capture antibodies for tau-based immunoassays (44, 45). The second class, exemplified by Alz50, selectively binds specific conformations of tau. These antibodies can identify populations of tau adopting filament-like conformations before PHFs are sufficiently developed to view microscopically (4). The third class of antibody, exemplified by AT8, is dependent on tau phosphorylation for binding (44). These agents are particularly useful for selectively detecting hyperphosphorylated forms of tau that accumulate in neurofibrillary tangles (58, 59). We predict that a potential fourth class of antibody, simultaneously selective for both tau conformation and phosphorylation state, will provide the most sensitive probes for neurofibrillary tangles yet discovered.

The existence of conformation-selective antibodies described herein provides additional evidence that tau, a protein that contains little ordered structure when isolated in monomeric form, is capable of adopting a more organized structure under certain circumstances. For example, in AD and other neurodegenerative diseases, tau assembles into two regular repeating pathological structures termed paired helical and straight filaments. When so assembled, tau acquires the ability to bind thioflavine-S (54), a fluorescent dye that preferentially interacts with proteins that adopt an amyloid conformation (60). Furthermore, when bound to fatty acids, soluble tau acquires the ability to activate phospholipase C-γ *in vitro* (61). This suggests that tau can adopt specific conformations in solution as well as upon polymerization.

We conclude that progressive modification and polymerization of tau proteins into filaments is preceded or accompanied by conformational changes that can be identified and quantified by a class of conformation-selective monoclonal antibody exemplified by Alz50. Currently, we are applying this approach to other anti-PHF/tau antibodies to correlate molecular structure with early events in the development of the fibrillar pathology.

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The Structural Basis of Monoclonal Antibody Alz50's Selectivity for Alzheimer's Disease Pathology

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