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Alz-50 and MC-1, a New Monoclonal Antibody Raised to Paired Helical Filaments, Recognize Conformational Epitopes on Recombinant Tau

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Using a series of recombinant tau and FAC1 mutant proteins, this study demonstrates by Western and dot blot analysis that 1) shared epitopes between tau and FAC1 are responsible for Alz-50 binding; 2) Alz-50 reactivity is dependent on two discontinuous portions of the tau molecule; 3) Alz-50 reactivity is most likely the result of a conformational alteration of tau monomers in Alzheimer's disease; and 4) the epitope for MC-1, a novel monoclonal antibody, maps to similar regions of tau but does not react with FAC1. These data raise questions regarding previous studies which have suggested that tau lacks a specific conformation and illustrate the utility of the Alz-50 and MC-1 antibodies in recognizing a distinct pathological conformation of the tau molecule in Alzheimer's disease. J. Neurosci. Res. 48:128–132, 1997. © 1997 Wiley-Liss, Inc.

Key words: FAC1; Alzheimer's disease; conformation

INTRODUCTION

Alz-50 is a monoclonal antibody raised to Alzheimer's disease (AD) basal forebrain homogenates (Wolozin et al., 1986), while MC-1 is a monoclonal antibody raised to Alz-50 immunoaffinity purified paired helical filaments (PHF) from Alzheimer brain tissue. Alz-50 has been shown to react with neurofibrillary tangles and dystrophic neurites in the AD brain (Wolozin and Davies, 1987). In addition, Alz-50 has been shown to react with a subset of neurons in the fetal brain (Wolozin et al., 1988) and a novel Alz-50 positive protein, FAC1, has been cloned from a fetal brain library (Bowser et al., 1995). Comparisons of the sequences of FAC1 and tau revealed two small homologous regions that may be responsible for the cross reactivity of Alz-50 seen in these otherwise distinct proteins. One of these regions corresponding to amino acids 7–9 of tau contains a phenylalanine residue that has previously been shown to be a determinant of Alz-50 binding in synthetic tau peptides (Ksiezak-Reding et al., 1995).

Previous studies have demonstrated the specificity and utility of Alz-50 in the postmortem diagnosis of AD and have illustrated its usefulness as a tool to study the pathological alterations seen in the AD brain (Wolozin and Davies, 1987; Braak et al., 1994). Although it has been shown that Alz-50 recognizes tau in PHF (Ksiezak-Reding et al., 1988; Goedert et al., 1991), to date there has been much controversy over the exact nature of the Alz-50 epitope. Several researchers have argued that the Alz-50 epitope resides in the extreme N-terminus of tau (Ksiezak-Reding et al., 1988; Goedert et al., 1991), while others have argued that Alz-50 recognizes a phosphorylated epitope in the C-terminus of tau (Ueda et al., 1990). If indeed the Alz-50 epitope recognizes a pathological modification of tau, then it is imperative that the nature of the epitope be positively identified and the modifications of tau leading to Alz-50 reactivity be assimilated into our theories of the pathogenesis of AD.

To address these issues, a series of bacterially expressed site-directed and deletion mutants were used to definitively map the Alz-50 and MC-1 epitopes in tau and investigate the cross reactivity of Alz-50 with FAC1. This study demonstrates that the Alz-50 epitope is most likely the result of a conformational alteration of tau monomers in AD and introduces a novel monoclonal antibody, MC-1, whose epitope maps to similar regions of tau but does not react with FAC1.

MATERIALS AND METHODS

Preparation of Recombinant Proteins

Clone htau40 was obtained from M. Goedert (Goedert et al., 1989), digested with Ndel, blunted with Klenow, and excised with HindIII. This fragment was ligated into SmaI/HindIII digested pQE-31 expression vector (Qia-

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gen, Inc., Chatsworth, CA). This construct was designated TauNW and encodes for amino acids 1–342 (Fig. 1). Clone htau40 was also digested with NdeI and HincII, blunted with Klenow, and ligated into SmaI digested pQE-31 expression vector (Qiagen, Inc.). This construct was designated TauNH and encodes for amino acids 1–312 (Fig. 1). TauNW was digested with SmaI, excising the DNA fragment encoding for amino acids 221–299 while maintaining the correct reading frame for the C-terminal portion of the protein, and ligated back to itself. This construct was designated TauΔS (Fig. 1). TauNW was digested with PstI, excising the DNA fragment encoding for amino acids 46–241 while maintaining the correct reading frame for the C-terminal portion of the protein, and ligated back to itself. This construct was designated TauΔP (Fig. 1). A Transformer Site-Directed Mutagenesis kit (Clontech, Inc., Palo Alto, CA) was used to introduce either Glu 7,9 → Ala 7,9 or Phe 8 → Ser 8 mutations into TauNW. These constructs were designated Tau79 and Tau8, respectively. All tau constructs were transformed into MC15 cells containing the pREP4 plasmid, expressed and purified over a Ni^{2+} column as described by the manufacturer (Qiagen, Inc.). FAC1 was ligated into Eco RI digested pMal-C2 fusion protein expression vector (New England Biolabs, Beverly, MA) as described previously (Bowser et al., 1995). The FAC1 construct was transformed into DH5α cells, expressed and purified on an amylose affinity column as described by the manufacturer (New England Biolabs). Site-directed mutagenesis of both the Glu → Ala and Phe → Ser EFE motif of FAC1 was performed as described above and the constructs were designated FAC145 and FAC146, respectively. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad, Inc., Cambridge, MA) and standardized by dilution.

Antibodies and Protein Detection

Alz-50 was raised to Alzheimer basal forebrain homogenates as described (Wolozin and Davies, 1987). The MC-1 and TG-5 monoclonal antibodies were raised to immunoaffinity purified PHFs from AD brain homogenates using an Alz-50 column. The FA2 monoclonal antibody was raised to recombinant FAC1 protein as described (Bowser et al., 1995). The Tau-1 monoclonal antibody was the generous gift of Dr. Lester Binder. The RGS-His monoclonal antibody was purchased from Qiagen, Inc., and recognizes the N-terminal histidine tag that allows for affinity purification of the pQE expressed tau proteins used in this study. Antibody binding was detected by incubation with horseradish peroxidase (HRPO)-labeled isotype specific anti-mouse immunoglobulins and visualized by reaction with 4-chloronapthol.

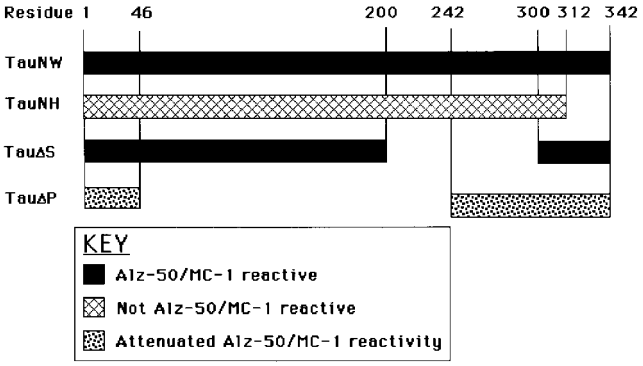

Fig. 1. Schematic diagram illustrating TauNW, TauNH, TauΔS, and TauΔP constructs used to map the C-terminal portion of the Alz-50 and MC-1 epitopes.

Western and Dot Blots

Standardized samples of recombinant proteins were solubilized in sample buffer at 95°C for 5 min, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide gels, and then transferred to nitrocellulose for Western blotting. Protein samples analyzed by dot blot were incubated for 2 hr at 25°C in Tris buffered saline (TBS) and 10 μl of each sample was applied to nitrocellulose and allowed to dry prior to antibody detection. All nitrocellulose bound samples were incubated in 5% milk-TBS to block nonspecific binding for 30 min at 25°C. Primary antibodies were then added at 1:10 dilution in 5% milk-TBS and the blots were incubated for 16 hr at 4°C. Unbound primary antibodies were removed by washing in several changes of TBS. HRPO-labeled secondary antibodies were then added at 1:500 dilution in milk-TBS and incubated for 2 hr at 25°C. The blots were then washed as above and reacted with a 1:4 dilution of 4-chloronapthal (4 mg/ml in methanol) in TBS with the addition of hydrogen peroxide for 5 min at 25°C.

Quantitative Analysis of Immunoreactivity

Western blots of TauNW, TauNH, TauΔSma, and TauΔPst were prepared as described above with the exception that immunoreactivity was visualized by chemiluminescent detection. Blots were exposed to film for 5 sec, developed, and immunoreactivity was quantitated by densitometric analysis (Molecular Dynamics, Inc., Sunnyvale, CA). Results were plotted as ratios of Alz-50 or MC-1 reactivity to RGS-His immunoreactivity to further standardize for variations in protein concentrations.

RESULTS

To further define the Alz-50 and MC-1 epitopes on the extreme N-terminus of tau, Tau NW, Tau79, and Tau8 were analyzed by Western blot with these antibodies and

with TG-5 (a monoclonal antibody previously raised in our laboratory that has been epitope mapped to the primary sequence of tau encoding amino acids 220–242; data not shown). Tau NW, Tau79, and Tau8 were all positive for TG-5 staining (Fig. 2). TauNW was also immunoreactive for both Alz-50 and MC-1 staining, whereas Tau79 and Tau8 (N-terminal site-directed mutants) were negative for both antibodies (Fig. 2). These results indicate that amino acids 7–9 (EFE) in tau are essential for Alz-50 and for MC-1 binding.

Site-directed mutants of the corresponding EFE sequence in FAC1 (amino acids 20–22) were created as described above to investigate the cross reactivity of FAC1 with Alz-50. FAC1, FAC145, and FAC146 were standardized by Western blot analysis using the FA2 monoclonal antibody which detected all protein constructs (Fig. 3). Wild-type FAC1 protein was reactive with Alz-50 but not MC-1, indicating differences in the exact epitope requirements for these two antibodies (Fig. 3). FAC145 and FAC146 site-directed mutants of the FAC1 EFE motif were shown to be negative for Alz-50 staining (Fig. 3), further supporting our conclusion that the EFE motif in both tau and FAC1 is essential for antibody binding.

To investigate the possibility that Alz-50 and MC-1 reactivity is also dependent on C-terminal portions of the tau molecule TauNW, TauNH, TauΔS and TauΔP (all containing amino acids 7–9, EFE) were analyzed by Western blot using the RGS-His, Alz-50, and MC-1 monoclonal antibodies. TauNW, TauNH, TauΔS, and TauΔP were all positive for RGS-His staining (Fig. 4). Tau NW was also positive for Alz-50 and MC-1 staining (Fig. 4). Tau NH was negative for Alz-50 and MC-1 staining (Fig. 4). These results indicate that both Alz-50 and MC-1 require a portion of the tau molecule that lies between amino acids 312–342 for binding. TauΔS, like TauNW, was positive for Alz-50 and MC-1 staining (Fig. 4). TauΔP, while positive for Alz-50 and MC-1 staining, exhibited reduced antibody reactivity as shown by densitometric analysis (Fig. 5). These results further confirm our hypothesis that the C-terminal Alz-50 and MC-1 epitopes are located between amino acids 312–342 and additionally demonstrate that a portion of the tau molecule lying between amino acids 46–200 is required for full antibody reactivity.

To determine whether the interaction between N- (amino acids 7–9) and C-terminal (amino acids 312–342) portions of the Alz-50 and MC-1 epitopes in tau could be created by the interaction of two molecules, TauNH (lacking the C-terminal portion of the Alz-50 and MC-1 epitopes) and Tau79 (lacking the appropriate N-terminal portion of the Alz-50 and MC-1 epitopes) were incubated at room temperature for 2 hr under non-denaturing conditions. Molecular weight differences between Tau79 and TauNH precluded Western blotting and necessitated dot blotting reaction mixtures on nitrocellulose membranes. By dot blot analyses TauNW, TauNH, and Tau79 were all positive for Tau-1 staining (Fig. 6). Tau NW was also positive for Alz-50 and MC-1 staining (Fig. 6). TauNH (lacking the C-terminal portion of the Alz-50 and MC-1 epitopes) and Tau79 (lacking the appropriate N-terminal portion of the Alz-50 and MC-1 epitopes) were both negative for both Alz-50 and MC-1 staining confirming our previous results (Fig. 6). TauNH (lacking the C-terminal portion of the Alz-50 and MC-1 epitopes) combined with Tau79 (lacking the appropriate N-terminal portion of the Alz-50 and MC-1 epitopes) failed to show either Alz-50 or MC-1 staining suggesting that the cooperativity between amino acids 7–9 and 312–342 is not an intermolecular phenomenon but rather that it occurs intramolecularly (Fig. 6). These data support our

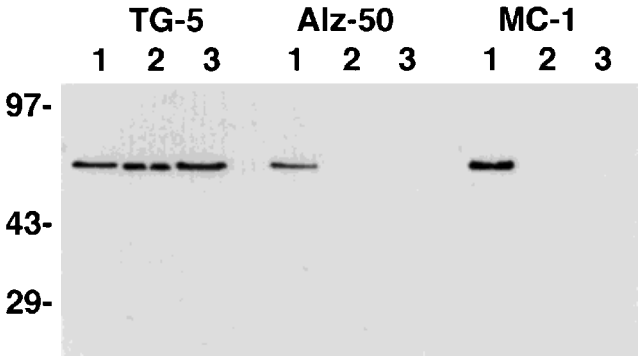

Fig. 2. The Alz-50 and MC-1 epitopes are dependent on amino acids 7–9 of tau. **Lane 1:** Tau NW. **Lane 2:** Tau79. **Lane 3:** Tau8. Standardization of protein concentrations as shown by TG-5 Western blots of TauNW, Tau79, and Tau8. An Alz-50 Western blot detects TauNW but not Tau79 or Tau8 (N-terminal site-directed mutants). A MC-1 Western blot detects TauNW but not Tau79 or Tau8 (N-terminal site-directed mutants).

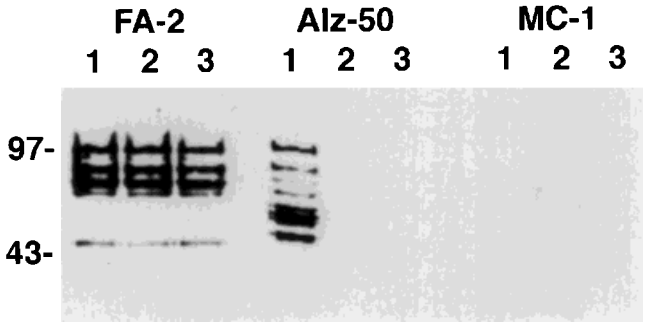

Fig. 3. FAC1 is Alz-50 but not MC-1 reactive. The Alz-50 reactivity of FAC1 is dependent on the EFE motif in FAC1. **Lane 1:** FAC1. **Lane 2:** FAC145. **Lane 3:** FAC146. Standardization of protein concentrations as shown by FA2 Western blots of FAC1, FAC145, and FAC146. An Alz-50 Western blot detects FAC1, but not FAC145 or FAC146. A Western blot shows Alz-50 but not MC-1 reactivity with FAC1.

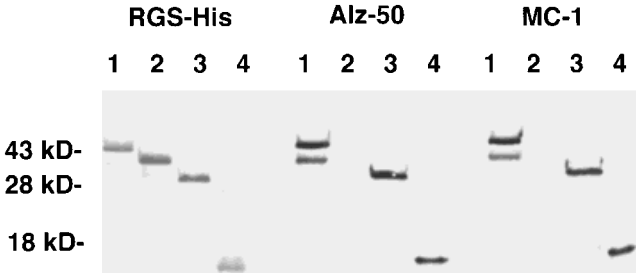

Fig. 4. The Alz-50 and MC-1 epitopes are dependent on amino acids 312–342 of tau. **Lane 1:** TauNW. **Lane 2:** TauNH. **Lane 3:** TauΔSma. **Lane 4:** TauΔPst. Standardization of protein concentrations as shown by RGS-His Western blots of TauNW, TauNH, TauΔS, and TauΔP (all containing amino acids 7–9, EFE). An Alz-50 Western blot detects TauNW and TauΔS but not TauNH, and exhibits attenuated reactivity for TauΔP. A MC-1 Western blot detects TauNW and TauΔS but not TauNH, and exhibits attenuated reactivity for TauΔP.

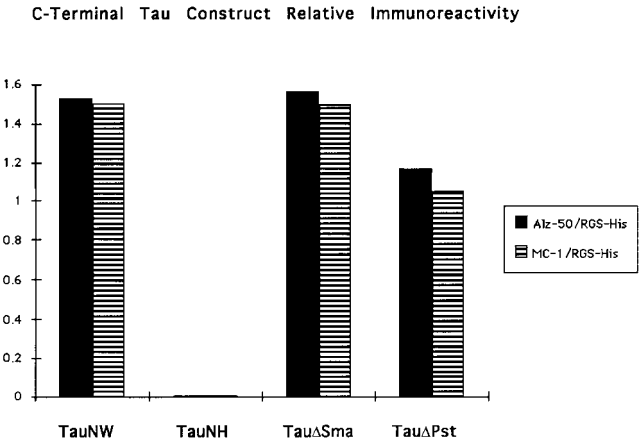

Fig. 5. Quantitative analysis of Alz-50 and MC-1 immunoreactivity standardized by comparison with RGS-His immunoreactivity. TauNW and TauΔSma exhibit full Alz-50 and MC-1 reactivity, TauNH is devoid of immunoreactivity, and TauΔPst shows a reduction in Alz-50 and MC-1 binding.

hypothesis that monomeric tau can and does assume a pathological conformation that allows for both Alz-50 and MC-1 binding.

DISCUSSION

In this study it has been shown that Alz-50 and MC-1 binding was dependent on sequences at the extreme N-terminus of tau, confirming the results of previous studies (Ksiezak-Reding et al., 1988; Goedert et al., 1991). We have also further defined the role of amino acids 7–9 in the creation of the Alz-50 and MC-1 epitopes. FAC1 also contains the EFE motif we have found important for the Alz-50 binding of tau and is responsible for the cross reactivity of Alz-50 with FAC1.

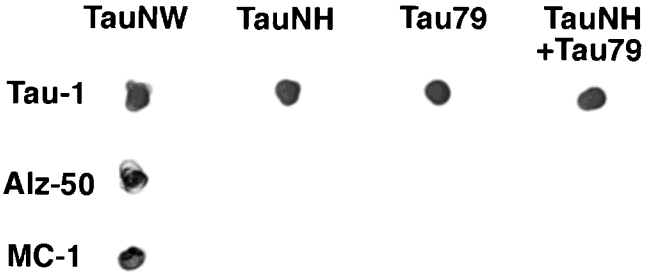

Fig. 6. Intermolecular interactions between TauNH (lacking the C-terminal portion of the Alz-50 and MC-1 epitopes) and Tau79 (lacking the appropriate N-terminal portion of the Alz-50 and MC-1 epitopes) do not form the Alz-50 or MC-1 epitopes. Standardization of protein concentrations as shown by Tau-1 dot blots of TauNW, TauNH, Tau79, and TauNH + Tau79. An Alz-50 dot bot detects TauNW but not TauNH, Tau79, or TauNH + Tau79. A MC-1 dot blot detects TauNW but not TauNH, Tau79, or TauNH + Tau79.

In addition to the N-terminal portion of the Alz-50 and MC-1 epitopes, a C-terminal portion of the tau molecule lying between amino acids 312–342 has been identified that is required for Alz-50 and MC-1 binding. Our analysis of the sequence homology between tau and FAC1 also identified a conserved 5 amino acid sequence corresponding to amino acids 326–330 of tau. This suggests that the FAC1 protein may have similar C-terminal epitope requirements for Alz-50 binding. It is noteworthy that at least 6 isoforms of the tau molecule are found in AD brain homogenates and all have been shown to be reactive with the Alz-50 antibody (Ksiezak-Reding et al., 1988; Goedert et al., 1991). The epitope mapping of the Alz-50 and MC-1 antibodies falls within regions of the tau molecule that are conserved in all 6 isoforms of the tau molecule (Goedert et al., 1989), further supporting our conclusions. FAC1 is reactive with Alz-50 but not with MC-1. These data suggest that the Alz-50 and MC-1 epitopes are very closely related but not identical. Immunocytochemical data (not shown) also indicate differences between these two antibodies.

The Alz-50 and MC-1 epitopes lie in discontinuous portions of the tau molecule. These data show that the creation of the Alz-50 and MC-1 epitopes involves an association between two portions of tau that are separated by more than 300 amino acids. The aggregation of tau in PHF, with which Alz-50 and MC-1 are both reactive (Wolozin and Davies, 1987), suggests that the interaction between the N- and C-terminal portions of the Alz-50 and MC-1 antibodies occurs in PHF in the AD brain. In addition, previous studies have shown that normal tau is able to form antiparallel dimers in solution (Wille et al., 1992), suggesting that the Alz-50 and MC-1 epitopes may be the result of an intermolecular conformation. Physical analysis of the tau molecule has failed to show that tau monomers have any significant thermodynamically stable

intramolecular conformation (Schweers et al., 1994), again supporting the hypothesis that intermolecular conformations may play an important role in creation of the Alz-50 and MC-1 epitopes. However, these analyses do not rule out the possibility that the tau molecule may adopt an intramolecular fold that might bring the N- and C-terminal portions of the Alz-50 and MC-1 epitopes into contact.

In this study, we have been unable to create the Alz-50 and MC-1 epitopes intermolecularly using the complementary deletion mutants TauNH and Tau79. Our data instead suggest that the Alz-50 and MC-1 positive conformation of tau is most likely an intramolecular phenomenon. In addition, certain deletions of intervening sequence (TauΔP, deleted for amino acids 46–241) in tau between the C- and N-terminal portions of the Alz-50 and MC-1 epitopes attenuate antibody reactivity, suggesting that this region of the tau molecule is required for such a conformation to occur. While it is still possible that the Alz-50 and MC-1 reactive conformations of the tau molecule represent an intermolecular association of tau monomers that is attenuated by deletions of intervening sequence (amino acids 46–199) between the N- and C-terminal portions of the Alz-50 and MC-1 epitopes, our data argue against this possibility. TauNH and Tau79 both contain amino acids 46–199 and yet are not reactive with either Alz-50 or MC-1 when combined. If this portion of the tau molecule were required for intermolecular associations between tau monomers one would have expected to observe Alz-50 and MC-1 reactivity using this combination of tau mutants, which is clearly shown not to be the case in this study.

The use of unmodified recombinant proteins in this study demonstrates that posttranslational modifications, i.e., phosphorylation, glycosylation, glycation, and proteolysis, of the tau molecule are not required for Alz-50 and MC-1 reactivity by Western and dot blot analyses. The lack of Alz-50 and MC-1 reactivity with tau in normal adult brain tissue by immunocytochemical analysis (Wolozin and Davies, 1987; Braak et al., 1994) suggests that either the epitopes are not exposed or that normal tau lacks the intramolecular conformation required for Alz-50 and MC-1 reactivity in vivo, but that this conformation is artificially created during Western blot analysis. It is interesting to speculate that the pathological alterations of tau in AD may impose conformational restraints on tau monomers in vivo, enhancing Alz-50 and MC-1 reactivity by immunocytochemical analysis, and may further play a role in the development of neuritic pathology in AD.

These data represent a significant step forward in our understanding of the Alz-50 epitope and introduce a new monoclonal antibody, MC-1, which is similar but not identical to Alz-50. Additionally, these data dispute previous studies which have suggested that tau lacks a specific conformation and illustrate the utility of the Alz-50 and MC-1 antibodies in recognizing a distinct pathological conformation of the tau molecule in AD, providing useful insights into our understanding of the disease process that occurs in AD and our interpretation of Alz-50 and MC-1 staining. Future analysis of the posttranslational modifications or factors leading to the creation of the Alz-50 epitope in vivo may help to define the temporal sequence of tau alterations leading to the development of neuritic pathology in AD.

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