



Alzheimer Disease Tangles and Threads Display Multiple Tau Phosphorylation Sites

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Materials and Methods

- Synthesis of 11-mer overlapping spots of full chain tau (440 amino acids) and its corresponding phosphorylated threonine or serine site peptides** : These were synthesized on membranes using an AutoSpot robotic instrument based on previously described methodology (Guo et al, Virology 324:251-6, 2004). The overlapping peptides were shifted from N-terminal to C-terminal by 1 amino acid. The synthesized membranes were stored in sealed bags at 4 °C until used.
- Recombinant Tau40 expression and purification**: Recombinant human tau 40 (2N4R, T40) was expressed in E. coli and purified as described previously (Arai et al JBC 280; 5145-5153, 2005). Purified T40 proteins were stored at -80 °C prior to use. Protein concentration was measured using a modification of the Lowry assay.
- Rabbit anti-phospho-Tau antibodies and their paired anti-Tau antibodies**: Rabbit anti-phospho-Tau antibodies were raised against synthetic phosphopeptides corresponding to human full length tau at the following sites: Thr181, Ser198, Ser202, Thr205, Thr212, Ser214, Thr217, Ser262, Ser356, Ser396, Ser400, and Ser404. Rabbit paired antibodies (non-phospho-Tau specific) were raised against synthetic peptides covering the following sites of full length tau : Thr181, Ser198-Thr205, Thr202, Thr212-Thr217, Ser262, Ser356, Ser396-Ser404, and Ser404.
- Mapping of rabbit anti-phospho-Tau antibodies and the paired anti-Tau antibodies on peptide array membranes**: 20µg/ml purified recombinant tau in 10mM PBS was incubated with peptide array membranes at 37°C overnight. After blocking with 5% skim milk in TBS-T 0.2 at 37 °C for 2 hr, the membranes were incubated with primary antibodies diluted 1:500–1000 at 37 °C for 2 hr. Then, after washing with TBS-T 0.2, the membranes were incubated in 1:4000 HRP-labeled goat anti-rabbit IgG at 37 °C for 1 hr. Finally, the membranes were analyzed with a Bio-Rad Fluorescent Imager after developing with ECL WB detection reagent.
- Immunohistochemistry Detection of antibodies**: The rabbit anti-phospho-Tau antibodies and their non-phosphorylated matching pairs were detected on the 30µm thick sections. To remove endogenous peroxidase activity, the sections were first incubated in 0.5% hydrogen peroxide for 30 min. The sections were preincubated with 5% skim milk for 30 min, then incubated with the primary antibody diluted from 1:500 to 1:2500 in PBST containing 3% skim milk overnight at room temperature or for 72 hr at 4 °C. After washing 3 x 5 min with PBST, the sections were incubated for 2 hr at room temperature with goat-anti-rabbit biotinylated secondary antibody diluted at 1:2000, followed by incubation in a mixture of avidin and biotinylated HRP for 1 h at room temperature. Finally, the sections were stained with DAB and nickel ammonium sulfate which produced a dark-purple reaction product. After development of staining, the sections were rinsed in distilled water, dehydrated in graded ethanol, passed 2 x 5 min in xylene and were mounted in Entellan.

Abstract

Insoluble tau accumulations are characteristic of AD and many other tauopathies. They occur primarily in the form of neurofibrillary tangles(NFTs) and threads. Tau is known to be phosphorylated in these abnormal accumulations but it is still uncertain as to which phosphokinases are primarily involved. To explore this question, we epitope mapped a series of anti-tau antibodies directed at specific sites of phosphorylation and compared their ability to detect NFTs and threads in paraformaldehyde fixed sections of AD brain. We synthesized on cellulose membranes an array of 11-mer tau peptides shifted by one amino acid and phosphorylated at key threonine and serine sites. We found that specific antibodies raised against peptides phosphorylated at threonine 181, 205, 212, and 217 and at serine 198, 214, and 396 each recognized the specific phosphorylated peptides but not the corresponding non-phosphorylated peptides. By immunohistochemistry, each antibody strongly recognized both NFTs and threads in AD tissue with no apparent qualitative differences. We conclude that each of these threonine and serine sites of tau is abundantly phosphorylated in the threads and NFTs that develop in AD brain. The phosphokinase GSK-3 β has been reported to phosphorylate recombinant tau at each of these sites and cdk5 has been reported to phosphorylate all but serine 198, 262 and 400. These phosphokinases could be playing a key role in the development of tau pathology in AD.

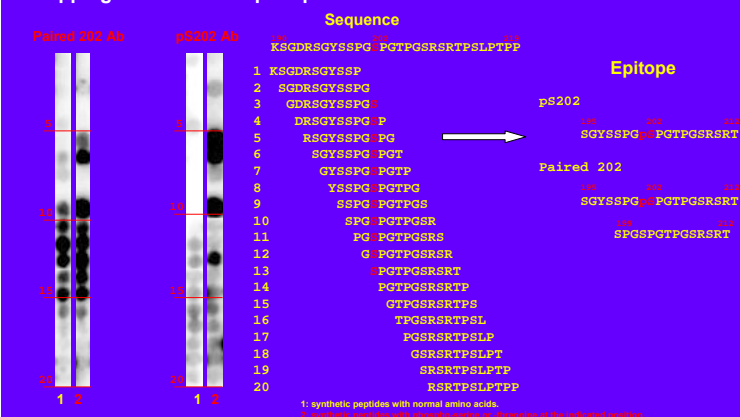
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Conclusions

- Threonine and serine sites of human Tau at T181, S198, S202, T205, T212, S214, T217, S262, S356, S396, and S400 are abundantly phosphorylated in the threads and NFTs that developed in AD brain.
 - pS356 and pS262 antibodies stained only a limited number of neurofibrillary threads(NFT), unlike other antibodies staining strongly NFT, NFT and plaque dystrophic neurites (DN).
 - Phosphokinase cdk5 can phosphorylate recombinant tau protein at the same sites as GSK-3 β except it cannot phosphorylate S198, S262 and S400.
 - Phosphokinases GSK-3 β and cdk5 play a key roles in the development of tauopathy in AD.
3. Immunohistochemical Detection of NFTs, DNs, and NPTs with Rabbit Phospho-Tau and Non-Phospho-Tau Antibodies

Results

1: Mapping of Rabbit Anti-phospho-Tau and Paired Anti-Tau Antibodies



2: Phosphorylation of Human Recombinant Tau Protein by Phosphokinases

Confirmed Kinases for Phosphorylated Serine or Threonine Sites in Human Tau protein												
	T181	S198	S199	S202	T205	T212	S214	T217	S262	S356	S396	S400
GSK-3β	+	+	+	+	+	+	+	+	+		+	+
cdk5	+		+	+	+	+	+	+			+	
MAPK	+		+	+	+			+			+	
CaMKII									+	+		
JNK	+			+	+	+		+			+	
PKA							+	+	+	+		
p38	+			+	+	+				+	+	

