terations, such as mitotic cell rounding, could be responsible for the altered morphology of the transformed cell if they are expressed during interphase. We propose that concentrations of pp39^{mos} that do not prevent cell proliferation or induce CSF "toxicity" can constitutively modify interphase microtubules and could, therefore, induce the transformed phenotype.

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18. Cytosolic extracts (~2 mg of protein per milliliter) of c-*mos*^{Xe}–transformed cells were prepared as described (Fig. 2), and portions were subjected, after incubation at 37° or 2°C for 20 min, to either gel filtration or anion-exchange chromatography. The fractions were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining for total protein and by protein immunoblotting for pp39^{mos} and tubulin. Most pp39^{mos} and tubulin were recovered in the void volume of the gel filtration column after incubation at 37°C, whereas they were more evenly distributed over different fractions after incubation at 2°C (Fig. 2A). Under conditions in which pp39^{mos} and tubulin coeluted as oligomers, or after anion-exchange chromatography, other cellular proteins were well separated. When cytosolic extracts (~2 mg/ml) from control NIH 3T3 cells were analyzed at 2°C, tubulin was present in many fractions, similar to the profile in Fig. 2A.
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21. A goat antibody (Ap335) to the pp39^{mos} peptide VERFLPRDLSPSIDLRPC present near the NH_2-terminus and a rabbit antibody (Ap232) to AEQLLERLEQECAM near the COOH-terminus also coprecipitated tubulin with pp39^{mos} specifically from the cytosol of c-*mos*^{Xe}–transformed cells. Furthermore, tubulin was heavily phosphorylated in in vitro immunocomplex kinase assays with these antibodies. (Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.)
22. The anti–α-tubulin monoclonal antibody (DM 1A; Sigma) precipitated three times more tubulin than the anti–β-tubulin monoclonal antibody (Amersham) when equal dilutions (1:100) were used in immune precipitations of ^{35}S-labeled cytosolic extracts from either c-*mos*^{Xe}–transformed cells or control NIH 3T3 cells. (Correction was made for the associated β-tubulin in the anti–α-tubulin immune complex. Anti–β-tubulin precipitated only β-tubulin.)
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A68: A Major Subunit of Paired Helical Filaments and Derivatized Forms of Normal Tau

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Putative Alzheimer disease (AD)–specific proteins (A68) were purified to homogeneity and shown to be major subunits of one form of paired helical filaments (PHFs). The amino acid sequence and immunological data indicate that the backbone of A68 is indistinguishable from that of the protein tau (τ), but A68 could be distinguished from normal human τ by the degree to which A68 was phosphorylated and by the specific residues in A68 that served as phosphate acceptors. The larger apparent relative molecular mass (M_r) of A68, compared to normal human τ, was attributed to abnormal phosphorylation of A68 because enzymatic dephosphorylation of A68 reduced its M_r to close to that of normal τ. Moreover, the LysSerProVal motif in normal human τ appeared to be an abnormal phosphorylation site in A68 because the Ser in this motif was a phosphate acceptor site in A68, but not in normal human τ. Thus, the major subunits of a class of PHFs are A68 proteins and the excessive or inappropriate phosphorylation of normal τ may change its apparent M_r, thus transforming τ into A68.

PHFs ARE THE PRINCIPAL STRUCTURAL elements of AD neurofibrillary tangles (NFTs) (*1*). Although not restricted to AD, the number of NFTs correlates with the severity of dementia in AD (*1*). PHFs also occur in the neurites surrounding amyloid-rich senile plaque (SP) cores, and in neuropil threads (NTs) that represent altered neuronal processes (*1*). Low M_r microtubule-associated proteins (MAPs) known as τ are major constituents of PHFs (*1*). A soluble form of PHFs may be formed from τ (*2*). Although other neu-

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ronal cytoskeleton polypeptides also may be components of NFTs and the neurites in SP coronas (*1*), only peptide sequences from the COOH-terminal third of τ have been recovered directly from purified AD PHFs (*3*). The 60- to 68-kD polypeptides known as A68 were initially identified with the ALZ50 monoclonal antibody (MAb) and were thought to be AD-specific and present in NFTs (*4*). Despite immunological and biochemical data that imply that A68 is a modified form of τ, this hypothesis is controversial, and the mechanism whereby this modification could occur is unknown (*5, 6*). For example, almost all available antibodies to τ, including ALZ50, react with A68 (*5*). Nevertheless, A68 has a higher M_r, a more acidic isoelectric point, and far lower solubility in nonionic detergents than τ (*7*). Hence, the precise relation of A68 to τ and to PHFs is unknown.

We addressed this question in a series of biochemical, immunological, and morphological studies of τ, A68, and PHFs. A68 proteins were purified from brains of patients with AD by a modification of a procedure described in (*7*). After sarkosyl (*N*-lauroyl-*N*-methylglycine) extraction and centrifugation, pellets containing A68 were resuspended in buffer [0.1 M MES, 0.17 M NaCl, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.0], boiled for 5 min, and loaded onto a 1.0 to 2.5 M sucrose gradient. Highly purified A68 proteins, which were completely solubilized in SDS sample buffer, were recovered between the 2.25 to 2.5 M sucrose fractions and consisted of three to four polypeptides with an M_r of 60 to 68 kD (Fig. 1A, lane 3). However, the 1.25 to 1.5 M and 1.75 to 2.0 M sucrose fractions also contained A68 that was partially soluble in SDS sample buffer, but it was contaminated with other proteins and some SDS-insoluble material excluded from gels (Fig. 1A, lanes 1 and 2, respectively). Nevertheless, this protocol allowed us to isolate highly purified A68 consistently from NFT-rich cortex from patients with AD (n = 9) and from elderly individuals with Down syndrome (n = 3) (*8*). Material from each of these sources yielded results qualitatively similar to those in Fig. 1A. In contrast, A68 was not detectable in regions of AD brain (that is, cerebellum) devoid of NFTs or in cortex from age-matched controls (n = 4) (*8*).

When the highly purified A68 preparations (as in Fig. 1A, lane 3) were placed on Formvar-coated nickel grids, negatively stained with methanolic uranyl acetate, and viewed by electron microscopy, we observed abundant filaments approximately 10 nm in diameter that were paired and twisted like classical PHFs (Fig. 1, B and C). These filaments were always paired with a helical periodicity of 75 to 80 nm. In paired arrays, these filaments with diameters of 10 nm exhibited diameters of 20 nm at the constrictions; the maximum diameter was 30 to 40 nm. Thus, they were indistinguishable from AD PHFs in situ (*1*). These biochemical and electron microscopic data indicate that

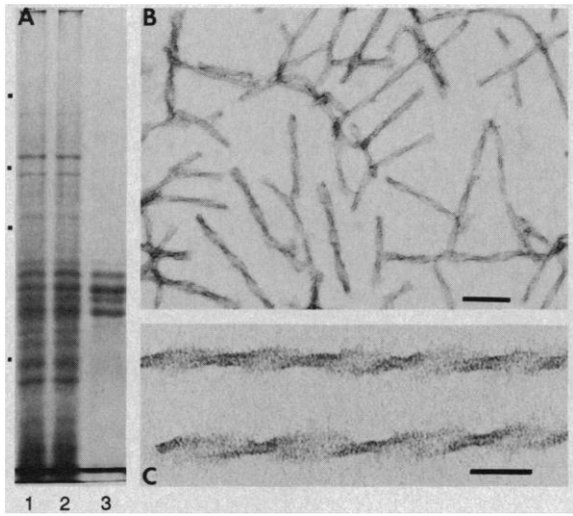

Fig. 1. (**A**) Coomassie brilliant blue–stained gel of A68 and other fractions containing A68 from an AD brain after sarkosyl extraction. The crude A68 preparation was loaded onto a stepwise sucrose gradient and centrifuged in a SW50 rotor at 175,000*g* for 16 hours. A thick, dark brown band was recovered at 1.25 to 1.5 M sucrose (fraction 1, lane 1), a thick, light brown band was found at 1.75 to 2.0 M sucrose (fraction 2, lane 2), and a small amount of pale brown material was recovered at 2.25 to 2.5 M sucrose (fraction 3, lane 3). Each sucrose fraction was washed once, solubilized in sample buffer, and run on a 7.5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gel. Only material in fraction 3 was completely soluble in sample buffer containing SDS whereas fractions 1 and 2 contained insoluble material after boiling in SDS sample buffer. In lanes 1 and 2, 40 μg of protein was loaded, and in lane 3, 10 μg of protein was loaded so that the amount of A68 would be similar to that in lanes 1 and 2. This was established by quantitative immunoblotting with ^{125}I-labeled second antibody in parallel experiments. Nevertheless, we noted slight variations from one AD brain to another in the intensity of the A68 proteins stained with Coomassie blue (compare lane 3, Fig. 1A, with lane 4, Fig. 2A). M_r markers (dots to left) were 205,000, 116,000, 77,000, and 46,5000 kD. Quantitative immunoblotting showed that fraction 3 contained less than 20% of the total A68 recovered from all three fractions (*9*). (**B** and **C**) Negatively stained A68 proteins from AD brains. An A68 preparation like that shown in lane 3 of (A) was placed on Formvar-coated nickel grids, stained with 2% methanolic uranyl acetate, and viewed by electron microscopy. The A68 filaments shown here are identical to classical PHFs, and their diameters fall within the upper range of measurements reported for in situ PHFs (*1, 20*). (**B**) Low magnification (bar = 100 nm) views showed abundant PHFs uncontaminated with the amorphous material often associated with NFT and PHF isolates from AD brains (*3*). (**C**) At higher magnification (bar = 50 nm), the A68-derived PHFs have "fuzzy" margins where these filaments are the widest (30 to 40 nm), but they are smoother along the "twisted" segments where the diameter of the PHFs is constricted (20 nm).

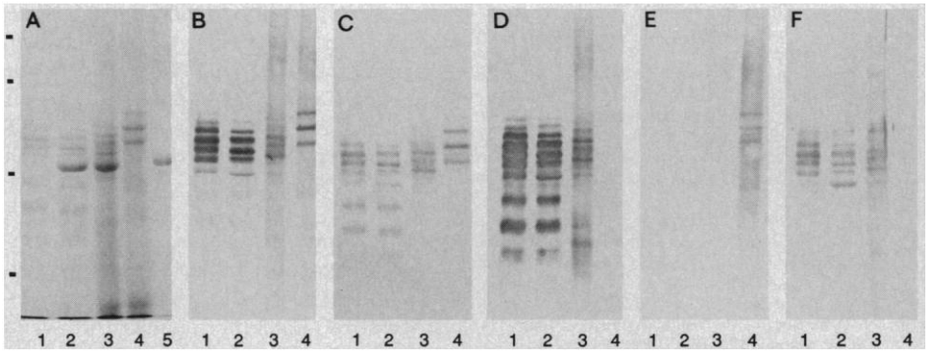

Fig. 2. Biochemical and protein immunoblot data for A68, normal human τ, and enzymatically dephosphorylated A68 and normal human τ. (**A**) Coomassie brilliant blue–stained 10% SDS-PAGE gel. (**B** through **F**) Nitrocellulose replicas of gels identical to the one in (A) except that lane 5 (showing the M_r of *E. coli* alkaline phosphatase that also is visible in lanes 2 and 3) was omitted in the immunoblots. In (A) through (F): lane 1, human τ; lane 2, enzymatically dephosphorylated human τ (using Type IIIN *E. coli* alkaline phosphatase from Sigma) as described (*15*); lane 3, A68 dephosphorylated as in lane 2; and lane 4, A68. M_r markers were 116,000, 77,000, 465,000, and 33,000 kD, as indicated to the left of lane 1 in (A). The blots in (B), (C), and (D) were probed with three MAbs that bind different τ epitopes (T46, ALZ50, and Tau-1, respectively) (*4–6, 10, 12*). The gel replicas in (E) and (F) were immunoblotted with the antisera to τ peptides (10 μg/ml anti-T3P and 10 μg/ml anti-T3, respectively), both of which were affinity-purified. The purity of the T3 and T3P peptides, and the location of the phosphate in T3P, were demonstrated by gas-phase microsequencing, fast atom bombardment mass spectrometry, and phosphate analysis. We purified human τ by cycling the first high-speed brain supernatant in the presence of taxol with exogenous phosphocellulose-purified bovine tubulin (1 mg/ml) (*21*).

PHFs are composed of A68 and that no other proteins are required for the formation of PHFs from the A68 proteins prepared as described here.

We then performed amino acid composition and sequencing analyses on A68 preparations like those shown in lane 3 of Fig. 1A. The composition of all A68 isoforms revealed a large number of Pro, Lys, and Gly residues (*9*), which is characteristic of human τ (*10*). However, because the complete amino acid sequence of each human τ isoform is still unknown, we compared the smallest A68 isoform with the smallest τ isoform (that is, τ lacking alternatively spliced inserts) (*10*). These two forms had similar amino acid compositions (*9*). The NH_2-terminus of A68 could not be sequenced because it was blocked. However, three fragments with M_r of 18, 21, and 23 kD were recovered from cyanogen bromide digests of A68, and each was sequenced. The first 14 amino acids of the 18-kD fragment [that is, PDLKNVKSKIGSTE (*11*)] were identical to residues 251 to 264 of normal human τ [numbering system as in (*10*)]. Identical sequences from the 18-kD fragments were obtained with an A68 preparation from a single AD brain and from pooled gray matter dissected from five different AD brains. The 21-kD fragment was sequenced through 14 cycles, and this sequence was identical to residues 128 to 141 of human τ (that is, VSKSKDGTGSDDKK). However, the recovery of Ser^{137} was unusually low, suggesting that it may be phosphorylated. Finally, the 23-kD fragment included sequences from both the 18- and 21-kD fragments, suggesting incomplete cyanogen bromide digestion. Because the sequences derived from these fragments corresponded to regions in τ separated by more than 100 residues, it is likely that A68 contains the entire τ molecule.

We then extended comparisons of the properties of A68 and τ to include immunoblot and immuno–electron microscopic studies using seven MAbs specific for identified epitopes distributed along the entire length of τ (*5–7, 12, 13*). All of these MAbs to τ, including T46 (which binds to the COOH-terminus of τ) and ALZ50, recognized all A68 isoforms in immunoblots except for the Tau-1 MAb (Fig. 2, B, C and D). However, Tau-1 did recognize dephosphorylated A68 (lane 3, Fig. 2D) as noted earlier (*7, 13, 14*). In the immuno–electron microscopic studies, PHFs from purified A68 fractions reacted with the same group of MAbs to τ in addition to ALZ50. This is significant because the epitopes recognized by these MAbs extend over almost the entire length of τ (*13*), and none of these τ epitopes are absent from A68-derived PHFs. Immunodecoration patterns of the A68-derived PHFs were obtained with the MAbs T14 (which binds an epitope in the NH_2-terminal third of τ) (Fig. 3A) and T46 (which binds the COOH-terminus of τ) (Fig. 3B). In contrast, antibodies to other putative PHF components, that is, ubiquitin, neurofilament (NF), and β-amyloid proteins never recognized A68 either in protein immunoblots or as PHFs on grids (*9*). These data provide further evidence that PHFs are composed of A68 and that A68 is derived from τ.

To distinguish τ from A68, we developed antisera to a motif in τ, that is, the single KSPV at residues 395 to 398 (*10*). We

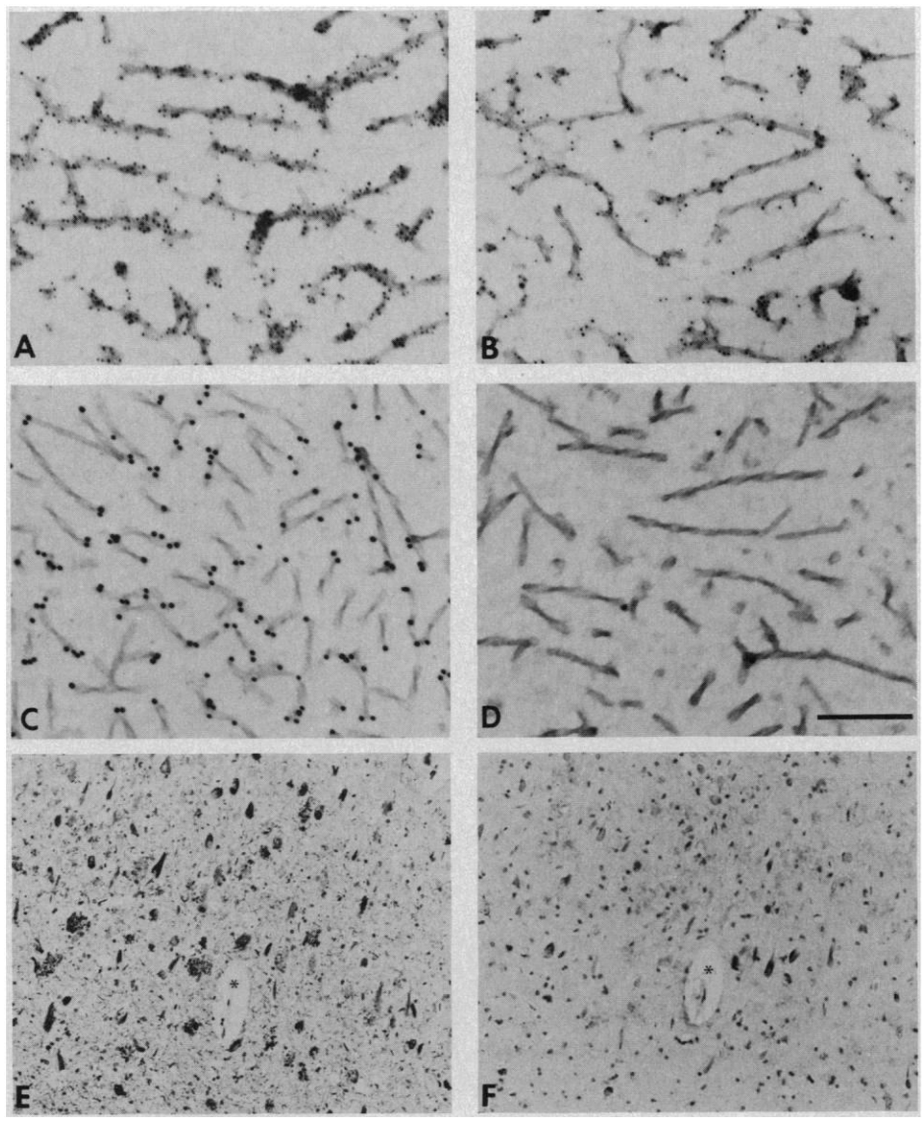

Fig. 3. Immunolabeled A68-derived PHFs probed on nickel grids with MAbs to τ or with the antipeptide antibodies. (**A**) T14, (**B**) T46, (**C**) anti-T3P, and (**D**) anti-T3. We immunolabeled A68 by absorbing purified A68 onto carbon, Formvar-coated grids and blocking for 30 min with 2% newborn calf serum plus 1% cold water fish gelatin in tris-buffered saline. The grids were incubated with primary MAbs (undiluted spent supernatants) or with the peptide-specific antibodies (diluted 1:200:) for 30 min. After extensive blocking and washing, the grids were incubated for 1 hour with goat antibody against mouse or rabbit immunoglobulin G conjugated to 5- or 10-nm gold particles, respectively. At the end of the incubation, the grids were washed extensively, negatively stained in 2% methanolic uranyl acetate for 10 min, and viewed by electron microscopy. All micrographs are enlarged to the same extent, and the bar in (D) = 200 nm. (**E** and **F**) Light microscopic immunohistochemical staining patterns produced with anti-T3P (E) and anti-T3 (F) antisera in adjacent 6-μm-thick sections of AD hippocampus. The tissue sections were cut from paraffin-embedded blocks fixed in 70% ethanol with 150 mM NaCl (*8*). Note the position of the same blood vessel (asterisk) in the center of the field in (E) and (F). The anti-T3P antibody labels NFTs, neurites in SPs, and numerous NTs that are not well visualized at this magnification. The anti-T3 antibody labels only a few NFTs. The magnification in (E) and (F) is ×250.

focused on this KSPV sequence because our KSPV-specific MAbs recognized all τ isoforms without enzymatic dephosphorylation in protein immunoblots, but they only reacted with AD NFTs in tissue sections after enzymatic dephosphorylation (*15*). The KSPV sequence in τ could be an abnormal phosphate acceptor site; therefore, the transformation of τ into A68 might involve the abnormal phosphorylation of Ser^{396}. To test this possibility, we synthesized the peptide (the T3 peptide) based on residues 389 to 402 (GAEIVYKSPVVSGD) in human τ. A phosphorylated form of this T3P peptide also was prepared by selective phosphorylation of the first Ser (that is, Ser^{396} in human τ) as described (*16*). Antisera were prepared to each peptide (*8*), and the specificity of these two antibodies for τ and A68 was assessed in immunoblots. The antiserum to T3P recognized A68 but not normal human τ, and the antibody to T3 recognized τ but not A68 (Fig. 2, E and F). This result implies that a phosphate at Ser^{396} distinguishes A68 from normal human τ, that Ser^{396} in the τ KSPV motif is not a normal phosphate acceptor site in τ, and that the conversion of normal human τ to A68 could be in part due to the phosphorylation of Ser^{396}. To test the ability of these two antisera to bind other neuronal cytoskeletal proteins with KSPV motifs, that is, NF proteins (*15*), we probed protein immunoblots of brain homogenates enriched in these proteins with both antisera and found that no other proteins reacted with these antibodies. Thus, unique sequences flanking the KSPV motif of τ, A68, and the two peptides described here may specify two distinct conformations that are differentially recognized by the antibodies to T3 and to T3P.

We next asked if one or both antibodies would bind to PHFs derived from purified A68 and in situ NFTs. The antiserum to T3P (Fig. 3C), but not the antibody to T3 (Fig. 3D), decorated isolated A68-derived PHFs, suggesting that these PHFs contain A68 but not normal τ. When we probed sections (frontal cortex, hippocampus) of AD ($n = 5$) and Down syndrome ($n = 2$) brains rich in lesions composed of PHFs (that is, NFTs, SPs, and NTs) with these same two antisera using the immunoperoxidase method (*8, 12, 15*), the antibody to T3P stained abundant NFTs, SP coronas, and NTs (Fig. 3E). By contrast, the antibody to T3 failed to stain SP coronas and NTs, but it did stain a few NFTs, especially in cases with very large numbers of NFTs (Fig. 3F). This may reflect a redistribution of normal τ from the axons to the perikarya of neurons at risk for NFT formation in AD (*1, 8, 17*). Only the antibody to T3P consistently stained the occasional hippocampal NFTs in age-matched controls ($n = 3$) (*9*). Thus, these observations are consistent with the notion that A68 is present in most, if not all types of pathology associated with the accumulation of PHFs (that is, NFTs, SP neurites, and NTs).

To assess the role inappropriate phosphorylation might play in the transformation of τ to A68, we dephosphorylated preparations of both τ and A68 with *Escherichia coli* alkaline phosphatase (*15*). The enzymatic dephosphorylation of normal τ resulted in a slight increase in the electrophoretic mobility of most τ isoforms. In contrast, all A68 isoforms showed a significant drop in M_r after the same treatment (Fig. 2A). The dephosphorylated A68 proteins all migrated to positions in the gel very close to those of dephosphorylated τ (Fig. 2A). More importantly, A68 was detectable by the antibodies to both Tau-1 and T3 after enzymatic dephosphorylation (Fig. 2, D and F), and dephosphorylation abolished the immunoreactivity of A68 with the antiserum to T3P (Fig. 2E). These data confirmed that two sites, that is, the KSPV motif and the Tau-1 epitope, are phosphorylated in A68 but not in normal τ. Finally, since the dephosphorylation of A68 proteins reduced their M_r such that the gel migration pattern of these polypeptides was similar to that of dephosphorylated τ, we infer that the abnormal phosphorylation of endogenous normal τ plays a major role in the conversion of τ to A68.

Two major conclusions emerge from this report: (i) A68 proteins are major subunits of an SDS-soluble form of PHFs and (ii) A68 contains amino acid sequences identical to those in spatially separate regions of normal human τ. This establishes that A68 is derived from τ itself and that regions extending from the NH_2- to the COOH-terminal domains of τ are present in PHFs, albeit in a modified form.

Our data also suggest that abnormal phosphorylation plays a major mechanistic role in the sequence of events leading to the formation of PHFs from normal τ, and we identified one potential abnormal phosphate acceptor site as Ser^{396} in the normal human τ KSPV motif. It is significant that this KSPV motif is present in all τ isoforms and is located near the microtubule binding repeats (residues 244 to 368) (*10, 18*). The phosphorylation of Ser^{396} may account for the inability of A68 to bind to microtubules, and this may result from a change in the secondary structure of the residues contained within the T3 peptide as measured by circular dichroism (*9*). However, the aberrant phosphorylation of other Ser residues also could play a role in the transformation of τ into A68. Two candidates are the Ser residues in the Tau-1 epitope (within amino acid 189 to 207 of τ) (*13*), and in the recently identified KESP motif (amino acid 44 to 47 of τ) (*19*).

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11. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D. Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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