Sequential phosphorylation of Tau by glycogen synthase kinase-3β and protein kinase A at Thr212 and Ser214 generates the Alzheimer-specific epitope of antibody AT100 and requires a paired-helical-filament-like conformation

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AT100 is a monoclonal antibody highly specific for phosphorylated Tau in Alzheimer paired helical filaments. Here we show that the epitope is generated by a complex sequence of sequential phosphorylation, first of Ser199, Ser202 and Thr205 (around the epitope of antibody AT8), next of Thr212 by glycogen synthase kinase (GSK)-3β (a proline-directed kinase), then of Ser214 by protein kinase A (PKA). Conversely, if Ser214 is phosphorylated first it protects Thr212 and the Ser-Pro motifs around the AT8 site against phosphorylation, and the AT100 epitope is not formed. The generation of the AT100 epitope requires a conformation of tau induced by polyanions such as heparin, RNA or poly(Glu), conditions which also favor the formation of paired helical filaments. The Alzheimer-like phosphorylation can be induced by brain extracts. In the extract, the kinases responsible for generating the AT100 epitope are GSK-3β and PKA, which can be inhibited by their specific inhibitors LiCl and Rp, respectively. A cellular model displaying the reaction with AT100 is presented by SF9 insect cells transfected with Tau. Knowledge of the events and kinases generating the AT100 epitope in cells might allow us to study the degeneration of the cytoskeleton in Alzheimer’s disease.

Keywords: Tau; microtubule-associated protein; phosphorylation; Alzheimer; epitope mapping.

Alzheimer’s disease is a neurodegenerative disorder characteristic of advanced age. Its causes are not well understood. In the brain of Alzheimer’s disease patients two types of abnormal protein deposits are found, the amyloid plaques (consisting mostly of the Aβ peptide) and neurofibrillary tangles, consisting of aggregated paired helical filaments (PHF), which are built from the neuronal microtubule-associated protein Tau. Abnormal Alzheimer Tau is highly phosphorylated, and it is thought that the events leading to hyperphosphorylation and aggregation are related to the breakdown of neuronal microtubules, the axonal transport that depends on them, and cell death (Kosik and Greenberg, 1994; Mandelkow et al., 1995; Johnson and Jenkins, 1996).

To monitor the Alzheimer-like state of Tau a number of antibodies have been characterized, which are of diagnostic value for post-mortem autopsies, may provide an early diagnosis, and could be used to study cellular models of Alzheimer’s disease. Some of these antibodies depend on conformation [e.g. Alz50 (Carmel et al., 1996, Jicha et al., 1997)], others on phosphorylation at certain sites [e.g. AT8, AT270, PHF-1 and SMI31 (Lee et al., 1991; Biernat et al., 1992; Mercken et al., 1992; Hasegawa et al., 1993; Goedert et al., 1994; Ishiguro et al., 1995)], and others on phosphorylation and a folded conformation [e.g. SMI34 (Lichtenberg-Kraag et al., 1992)].

Hyperphosphorylation of Tau in Alzheimer occurs primarily at (Ser or Thr)-Pro motifs, suggesting that proline-directed kinases are responsible [e.g. mitogen-activated protein kinase (MAP kinase), glycogen synthase kinase (GSK)-3 and cyclin-dependent kinase (Cdk)5 (Drewes et al., 1992; Mandelkow et al., 1992; Hanger et al., 1992; Vulliet et al., 1992; Paudel et al., 1993; Baumann et al., 1993; Ishiguro et al., 1993)], but other sites affected by other kinases [e.g. calmodulin-dependent protein kinase, protein kinase A (PKA) and protein kinase C (PKC)] occur as well (Steiner et al., 1990; Correas et al., 1992; Scott et al., 1993; Morishima-Kawashima et al., 1995). Phosphorylation weakens the interaction with microtubules to various degrees, consistent with the observation that microtubules become more labile in Alzheimer’s disease after loss of their microtubule-associated proteins (Köpke et al., 1993; Bramlett et al., 1993; Yoshida and Ihara, 1993). Several phosphorylation sites of Tau have been implicated in the loss of microtubule binding, for example Ser262 in human Tau [phosphorylated by microtubule-affinity-regulating kinase (Biernat et al., 1993; Drewes et al., 1995)], while proline-directed kinases have a comparatively mild effect (Drechsel et al., 1992; Trinczek et al., 1995).

A caveat in interpreting the antibody reactivities is the fact that some Alzheimer-like sites occur under normal circumstances, albeit to a much lesser extent, especially in fetal brain tissue (Kanemaru et al., 1992; Bramlett et al., 1993; Brion et al., 1993; Seubert et al., 1995), and in biopsy samples (Matsuo et al., 1994). In this respect, antibody AT100 appears to be much more specific and is the only antibody that recognizes just Alzheimer Tau. This provided the motivation for this investigation of the epitope requirements and the responsible kinases. Furthermore, while the epitopes of many antibodies are contiguous and can be phosphorylated by purified kinases, the epitope of AT100

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Abbreviations. GSK, glycogen synthase kinase; PKA, protein kinase A; PHF, paired helical filament; ISPK, insulin-stimulated protein kinase; MAP kinase, mitogen-activated protein kinase; PKC, protein kinase C; Cdk, cyclin-dependent kinase.
appeared to be more complex, depended on more than one kinase, and depended on a folded conformation. We show here that the epitope can be generated by two kinases (GSK-3β and PKA) in a sequential manner, affecting two nearby residues (Thr212 and Ser214). It also depends on the prior phosphorylation of (Ser or Thr)-Pro motifs around residue 200 (including the AT8 epitope). Thirdly, it requires a conformation of Tau that is conducive to PHF formation and can be generated in vitro by polyanions such as heparin, RNA, or poly(Glu). The epitope can be generated by kinases present in the brain extract, and it occurs in certain cellular environments. These results offer the prospect of monitoring Alzheimer-like processes of degeneration in cell models.

**MATERIALS AND METHODS**

**Monoclonal antibodies and kinases.** Antibodies AT8, AT180, AT270, and AT100 were obtained from Innogenetics (Mercken et al., 1992), monoclonal antibody 12E8 was from Athena Neurosciences (Seubert et al., 1995), monoclonal antibody PHF-1 was a gift from P. Davies (Albert Einstein College, Bronx NY; Greenberg et al., 1992), and Tau-1 was a gift from L. Binder (Northwestern University, Chicago IL; Binder et al., 1985). For epitopes see Fig. 2. As a control for total Tau we used the polyclonal human Tau antibody from DAKO. Reconstitubinant MAP kinase activator was prepared by K. Stamer, and Cdk5 by K. Baumann. Insulin-stimulated protein kinase (ISPK; dase. Bound antibody was detected by the enhanced chemiluminescence System (Amersham).

**Phosphorylation by different kinases.** All phosphorylation reactions were carried out at 37°C in 40 mM Hepes, pH 7.2, 3 mM MgCl₂, 5 mM EGTA, 2 mM ATP and a cocktail of protease inhibitors. The reaction was initiated by the addition of 10 mM, 20 mM or 50 mM LiCl (to inhibit GSK-3) or Rb subunit (to inhibit PKA) for 10 min at room temperature and then added to Tau protein.

**Phosphopeptide mapping.** Mapping was performed following Boyle et al. (1991). After phosphorylation reactions using [γ-³²P]ATP, the kinase was removed by boiling the sample in 0.5 M NaCl, 10 mM dithiothreitol and centrifugation. The heat-stable Tau was precipitated with trichloroacetic acid. Cysteine residues were oxidized by performic acid treatment. The protein was digested overnight with trypsin (Promega, sequencing grade) using 20 pmol of enzyme in a ratio of 1:20 (by mass). Two-dimensional phosphopeptide mapping by thin-layer electrophoresis/chromatography was performed on thin-layer cellulose plates (Macherey & Nagel). The identification of spots was described previously (Drewes et al., 1995; Illenberger et al., 1996). Ser214 was identified by peptide sequencing and by comparison with defined phosphorylated peptides.

**Preparation of biopsy Tau, fetal Tau and PHF tau.** Brain tissue was boiled in a microwave oven for 2 min and homogenized with 5 ml 50 mM Pipes, pH 6.9, 0.5 M NaCl, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 1 mM Na₂VO₃, 5 mM dithiothreitol, 1 mM PhMeSO₄F, 10 μM microcystin, and a cocktail of other protease inhibitors. The homogenate was centrifuged for 1 h at 40000 g. The supernatant was concentrated according to the method of Wessel and Flügge (1984) and used for immuno blotting. PHF Tau was prepared as described by Greenberg and Davies (1990).

**Preparation of crude brain extracts.** Extracts from mouse brain were prepared as described (Lichtenberg-Kraag et al., 1992) by homogenizing the brain tissue in 50 mM Tris/HCl pH 8.0, 5 mM EGTA, 2 mM dithiothreitol, 50 mM NaF, 1 mM Na₂VO₃, and a cocktail of protease inhibitors (leupeptin, aprotinin, pepstatin A at 10 μg/ml each and 1 mM PhMeSO₄F) and centrifuged at 100000 g for 10 min at 4°C. The supernatant was used for the phosphorylation. The reaction was carried out in 40 mM Hepes, pH 7.2, 3 mM MgCl₂, 5 mM EGTA, 2 mM ATP, 10 μM okadaic acid and a cocktail of protease inhibitors. 1 μl extract was added to 20 μl Tau solution (0.5 μg/μl) at 37°C for 16 h. For phosphorylation experiments with the brain extract in the presence of kinase inhibitors the brain extract was first incubated with 10 mM, 20 mM or 50 mM LiCl (to inhibit GSK-3) or Rb subunit (to inhibit PKA) for 10 min at room temperature and then added to Tau protein.

**Immunoblotting.** For immunoblot analysis, the proteins or peptides were transferred to PVDF membranes (Millipore) and the residual protein-binding sites were blocked with 5% milk in 10 mM Tris/HCl pH 7.2, 150 mM NaCl, 0.05% Tween 20. The blots were incubated for 1–2 h at room temperature with primary antibodies AT8 (dilution 1:4000), AT100 (1:500), AT180 (1:1000), AT270 (1:4000), PHF-1 (1:500), and 12E8 (1:4000). The secondary antibody was labelled with horseradish peroxidase. Bound antibody was detected by the enhanced chemiluminescence System (Amersham).
The AT100 epitope is highly specific for Tau from Alzheimer tissue. A number of antibody epitopes have been described that distinguish Alzheimer Tau from Tau in normal brain tissue and are thus of diagnostic value. Many of these depend on phosphorylation, especially at Ser-Pro or Thr-Pro motifs, indicating an overactivity of proline-directed kinases or an underactivity of constructs KXGS motifs of repeats 1 and 4; Seubert et al., 1995) and Tau-1 (around residue 200; Biernat et al., 1992; Szendrei et al., 1993) are indicated. The deletion constructs contain at least one of the P domains (P2) and variable extensions on either side.

The AT100 epitope contains phosphorylated Thr212 and Ser214. To determine the epitope we used a set of recombinant constructs of Tau expressed in E. coli, some of which are shown in Fig. 2. On the basis of amino acid character, Tau can be subdivided into several domains (Gustke et al., 1994). For the present work, the important ones are the repeat (R) domains (Fig. 2) and the extensions on either side, the basic regions P1 and P2, and the basic C-terminal tail. P1 and P2 form essentially one domain,
but they can be distinguished by chymotryptic cleavage after Tyr197, which also separates the C-terminal assembly fragment of Tau and the N-terminal projection fragment (Steiner et al., 1990). The constructs shown here have P2 in common, with variable extensions on either side, e.g. K17 is composed of P2 and the repeats, while K43 consists of P2 preceded by P1 and followed by the repeats.

To generate Alzheimer-like phosphorylated epitopes we used a kinase activity from brain extract; as shown earlier, normal mammalian brain tissue contains the protein kinases that can phosphorylate Tau efficiently provided that phosphatases are inhibited, e.g. by okadaic acid (Lichtenberg-Kraag et al., 1992; Gustke et al., 1992). All the deletion constructs of Tau (Fig. 2) were phosphorylated by the brain extract. This usually caused an increase of the apparent molecular mass in the SDS gel and a splitting into several bands, depending on the type and extent of phosphorylation (Butler and Shelanski, 1986; Fig. 3A). However, the constructs differ in terms of their reaction with AT100 (Fig. 3B): human Tau23 and K35 (containing domains P1 and P2, the repeats, and the C-terminal tail) display a strong reaction; K23 and K43 (where the repeats or the tail are missing) have an intermediate reaction; and with K17, K25 and K33 (where domain P2 lacks one of its immediate neighbors, either P1 or the repeats) the reaction is almost abolished. The comparison of the domains of these and other constructs shows that the epitope of AT100 lies in domain P2 (Ser198-Leu243) and is enhanced by the presence of neighboring domains.

With the aim of defining the epitope of AT100 more precisely we constructed a set of single or double point mutants where Ser or Thr residues were replaced by Ala (Fig. 2), thus preventing their phosphorylation. Judging by the shift in the SDS gel, all point mutants were phosphorylated by the brain extract (Fig. 4a). Most constructs reacted with AT100, except when Thr212 or Ser214 were mutated to Ala, showing that these two residues (when phosphorylated) are part of the AT100 epitope.

The AT100 epitope requires sequential phosphorylation of Thr212 by GSK-3β and of Ser214 by PKA, phosphorylation around the AT8 epitope, and a PHF-like conformation of Tau. Several groups have shown that the proline-directed kinases MAP kinase, GSK-3 (α or β) or Cdk5 can phosphorylate Ser-Pro or Thr-Pro motifs in Tau. Moreover Ser214 can be phosphorylated by several non-proline-directed kinases, such as PKA, PKC and ISPK (data not shown; Mandellkow et al., 1995). These kinases were therefore considered candidates for generating the AT100 epitope. We used them singly or in combination to phosphorylate Tau, but the AT100 epitope did not appear. Since the activity of kinases can be modified by polyanions, such as heparin, heparin was added to the solution, but again the AT100 epitope failed to form. We noted that Thr212 could be

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**Fig. 3. SDS gel and immunoblot with AT100 of different Tau constructs after phosphorylation with the brain extract.** (A) SDS gel (15%) showing the phosphorylation of different constructs with the brain extract (Biernat et al., 1992). Lanes 1, 3, 5, 7, 9, 11 and 13, before phosphorylation; lanes 2, 4, 6, 8, 10, 12 and 14, after phosphorylation. The upward shift in molecular mass is a measure of the extent of phosphorylation. All constructs become phosphorylated and are shifted. Due to heterogeneous phosphorylation sites the proteins are distributed over several closely juxtaposed bands. Lanes 1 and 2, Tau23; lanes 3 and 4, K17; lanes 5 and 6, K23; lanes 7 and 8, K25; lanes 9 and 10, K33; lanes 11 and 12, K35; lanes 13 and 14, K43. (B) Immunoblot of different constructs with AT100 after phosphorylation with the brain extract. Lane 1, unphosphorylated human Tau23. Lanes 2–8, Tau23, K17, K23, K25, K33, K35 and K43, respectively, after treatment with brain extract.

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**Fig. 4. SDS gel and immunoblot with AT100 of different point mutants (Ser—Ala) of Tau after phosphorylation with brain extract.** (a) The SDS gel (10%) shows that all mutants are shifted upwards after phosphorylation. Lane 1, unphosphorylated human Tau23. The protein in lane 9 runs at a higher position because this construct was derived from human Tau40 (containing two N-terminal inserts and repeat 2). Some constructs show split bands due to bacterial proteolysis. (b) Immunoblot with AT100, showing a reaction for most constructs, except when Thr212 and Ser214 are mutated to Ala.
Table 1. Search of kinases capable of generating the AT100 epitope.

<table>
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<tr>
<th>Kinase</th>
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<tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>GSK-3β</td>
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</table>

Table 2. Generation of the AT100 epitope by simultaneous and sequential phosphorylation using different combinations of kinases.

<table>
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<tr>
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<th>Formation of AT100 epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK-3β + PKA</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>no</td>
</tr>
<tr>
<td>Cdk5 + MAP kinase</td>
<td>no</td>
</tr>
<tr>
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<td>Cdk5 + ISPK</td>
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</tr>
<tr>
<td>MAP kinase + ISPK</td>
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</table>

<table>
<thead>
<tr>
<th>Sequential phosphorylation</th>
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<tbody>
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<tr>
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<td>GSK-3β, PKA</td>
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<td>MAP kinase, PKA</td>
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<tr>
<td>MAP kinase + GSK-3β, PKA</td>
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<tr>
<td>Cdk5 + heparin, PKA</td>
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<tr>
<td>GSK-3β + heparin, PKA</td>
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<tr>
<td>MAP kinase + heparin, PKC</td>
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</tr>
<tr>
<td>GSK-3β + heparin, ISPK</td>
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</tr>
</tbody>
</table>

Phosphorylated by GSK-3β independently of whether heparin was present, and likewise the phosphorylation of Ser214 by PKA did not depend on heparin, indicating that the failure to induce the epitope was not due to a lack of activity of the kinases (data not shown).

Tables 1 and 2 summarize the experiments with the various kinase combinations. They show that all of the kinases tested were able to phosphorylate Tau (Table 1), yet almost none of the kinase combinations induced the AT100 epitope (Table 2). The key exception was to include polyanions [heparin, RNA and poly(Glu)], phosphorylate first with GSK-3β, then phosphorylate with PKA. By contrast, application of the kinases simultaneously or in the reverse order (PKA first, GSK3-β second) did not generate the AT100 epitope.

Fig. 5a illustrates the sequential phosphorylation by GSK-3β and PKA for the point mutants of Tau. As for the brain extract, the AT100 reaction was essentially absent for the mutants [Ala212]Tau and [Ala214]Tau (indicating the epitope at these residues), was unaltered for mutants at 198, 208 and 210 (indicating that these residues are neutral), and was weakened for mutants at residues 199, 202 and 205, suggesting that phosphorylation at these sites might enhance the AT100 reaction (Fig. 5a). Since phosphorylated Ser202 and Thr205 constitute the epitope of the antibody AT8, this antibody can be used as a sensor for phosphorylation around residue 200. The time dependence shown in the immunoblots (Fig. 5b) illustrates that the AT8 epitope appears before the AT100 epitope. (b) Time-course of sequential phosphorylation monitored by antibodies AT100 and A8. The epitope of AT8 (Ser202 and Thr205) precedes that of AT100. (c) Induction of the AT100 epitope on human Tau23 by different polyanions (heparin or tRNA). The protein was sequentially phosphorylated, first by GSK-3β in the presence of heparin (lane 2) or tRNA (lanes 3, 4), followed by PKA. Lane 1 shows the control without phosphorylation. Top, immunoblot with AT100, showing that heparin and tRNA induce the epitope. The same effect was observed with poly(Glu) (data not shown). Bottom, immunoblot with AT8, showing that this epitope is generated independently of AT100.
before PKA the epitopes of AT100 and AT8 (lanes 3 and 4) show that the reaction occurs only when Tau is phosphorylated initially with GSK-3β [with heparin (+ Hep); lanes 1 and 3]. The AT100 epitope is generated by subsequent phosphorylation with PKA (lane 1). Conversely, the initial phosphorylation with PKA (with or without heparin) prevents the formation of AT100 and AT8 epitopes (lanes 2 and 4). (b) Phosphopeptide map of Tau exposed to PKA (with heparin) for 15 min (left) shows that at this time Ser214 is phosphorylated almost exclusively, whereas extended phosphorylation (up to 15 h) leads to more than a dozen spots.

A further requirement for generating the AT100 epitope is the presence of polyanionic factors such as heparin, tRNA, or poly(Glu) (Fig. 5c). Such factors are known to modulate the activity of protein kinases so that phosphorylation reactions could be altered (Moreno et al., 1996). In our experiments the phosphopeptide maps suggest that roughly the same sites are phosphorylated with or without polyanions (data not shown). We therefore prefer an alternative interpretation. Polyanionic substances are known to change the state of Tau and induce it to self-assemble into PHF (Perez et al., 1997, 1996; Goedert et al., 1996; Kampers et al., 1996). These observations suggest that a PHF-like state is necessary to obtain the PHF-like epitope of AT100. The equivalence of different polyanions is illustrated in Fig. 5c, where heparin is compared with tRNA. The epitope of AT8 was formed even with low (or without) polyanions, but AT100 required a sufficient concentration of polyanions.

The reason why sequential phosphorylation is necessary for the AT100 epitope is illustrated in Fig. 6. If GSK-3β is applied before PKA the epitopes of AT100 and AT8 are generated (Fig. 6a), but if PKA is applied first there is no antibody reaction (Fig. 4b), not with the purified kinases (Fig. 5a). This can be explained by assuming that Thr(P)217 is stimulatory for the phosphorylation of the AT8 epitope, but if it is absent (as in the [Ala217]Tau mutant) the stimulation can be achieved via other kinases in the brain extract, acting on other unknown sites.

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**Fig. 6. Phosphorylation of Ser214 by PKA (with or without heparin) prevents the phosphorylation at (Ser or Thr)-Pro residues around the AT8 and AT100 epitopes.** (a) Blots with AT100 (lanes 1 and 2) and AT8 (lanes 3 and 4) show that the reaction occurs only when Tau is phosphorylated initially with GSK-3β [with heparin (+ Hep); lanes 1 and 3]. The AT100 epitope is generated by subsequent phosphorylation with PKA (lane 1). Conversely, the initial phosphorylation with PKA (with or without heparin) prevents the formation of AT100 and AT8 epitopes (lanes 2 and 4). (b) Phosphopeptide map of Tau exposed to PKA (with heparin) for 15 min (left) shows that at this time Ser214 is phosphorylated almost exclusively, whereas extended phosphorylation (up to 15 h) leads to more than a dozen spots.

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**Fig. 7. Mapping of AT100 epitope by proteolysis and antibody labeling.** The human isoform Tau23 was phosphorylated as in Fig. 5 (first GSK-3β, then PKA) to generate the AT100 epitope, then cleaved with AspN protease and stained with antibodies AT100 (lanes 1 and 2), AT8 (lanes 3 and 4) and 12E8 (lane 5). Cleavage for up to 20 h leaves one major fragment (A; 21.5 kDa). Sequencing of the peptide by Edman degradation after blotting onto a poly(vinyl difluoride) membrane showed that the sequence was D146GKTQIA AT. The molecular mass and the positions of Asp residues suggests that the peptide extends at least to Pro251 (preceding the potential cleavage site at Asp252) and thus covers domains P1 and P2. This includes the epitope of AT8 (lanes 3 and 4). There is no significant cleavage at Asp193 after 20 h. 12E8 detects two main fragments (B and C), both starting with D252LKNVKSK in the first repeat. Fragment B probably extends to Lys344, fragment C to Val313, neither of which includes the AT8 or AT100 epitopes. The conditions were chosen such that PKA phosphorylates essentially only one residue, as seen from the phosphopeptide maps (Fig. 6b). The single spot seen after 15 min contains Ser(P)214 (as shown by isolation of the spot, sequencing, and comparison with defined marker peptides). In other words, phosphorylation of Ser214 by PKA blocks the subsequent phosphorylation of Thr212 and around residue 200 by GSK-3β so that the AT100 and AT8 reactions can no longer take place. Thus, there is an antagonistic relationship between Ser214 and the preceding (Ser or Thr)-Pro motifs. Phosphorylation of the (Ser or Thr)-Pro motifs (by GSK-3β) is stimulatory for phosphorylation of Ser214 (by PKA), resulting in the AT100 epitope, but phosphorylation of Ser214 protects the neighboring sites against proline-directed phosphorylation. This effect occurs independently of polyanions.

To confirm the location of the AT100 epitope we combined immunoblotting with proteolytic cleavage. One convenient cleavage site is behind Tyr197, generating essentially two fragments (projection and assembly fragments; Steiner et al., 1990; Fig. 2). However, this cleavage could not be used because the phosphorylation around residues 200 by GSK-3β made Tau highly resistant to chymotryptic cleavage (analogous to observations with the protease calpain; Litersky and Johnson, 1992; Mercken et al., 1995). We therefore chose AspN protease, which generated only a few interpretable fragments of Tau (Fig. 7). Extended cleavage generates one major fragment (21.5 kDa, Fig. 7) starting at Asp146 as determined by sequencing from the blot. The molecular mass, the positions of Asp residues in Tau, and the absence of the 12E8 reaction suggests that the peptide extends to Pro251 and thus covers domains P1 and P2, including
the epitopes of AT8 and AT100. The AT100 epitope, once it is formed after phosphorylation, is retained in the P1–P2 domain, even though its generation on these domains de novo is very inefficient because it requires P2 and flanking regions on both sides. Thus the underlying conformation appears to remain intact after proteolysis. Labeling of the degradation products of Tau with another antibody, 12E8 (sensitive to phosphorylated Ser262 and Ser356, which are generated by PKA; Seubert et al., 1995) reveals a different set of fragments (Fig. 7b), both of which begin at Asp252 in the first repeat as determined by sequencing. Neither of these is recognized by AT8 and AT100, confirming that these epitopes lie in the P domain and do not require the repeat domain, once they are formed.

GSK-3β and PKA are the primary kinases responsible for the AT100 epitope in the brain extract. The experiments described above indicate that the AT100 epitope can be generated by the mixture of kinases present in the brain extract, or by the sequential application of purified kinases GSK-3β and PKA. But whether these two kinases were the active ingredients in the brain extract remained an open question. To narrow down the possibilities, such as Alz-50 and MC-2, whether these two kinases were the active ingredients in the AT100 reaction in cells formed after phosphorylation, is retained in the P1–P2 domain, even though its generation on these domains de novo is very inefficient because it requires P2 and flanking regions on both sides. Thus the underlying conformation appears to remain intact after proteolysis. Labeling of the degradation products of Tau with another antibody, 12E8 (sensitive to phosphorylated Ser262 and Ser356, which are generated by PKA; Seubert et al., 1995) reveals a different set of fragments (Fig. 7b), both of which begin at Asp252 in the first repeat as determined by sequencing. Neither of these is recognized by AT8 and AT100, confirming that these epitopes lie in the P domain and do not require the repeat domain, once they are formed.

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**Fig. 8. Kinase inhibitors suggest that GSK-3β and PKA are responsible for generating the AT100 epitope in the brain extract.** GSK-3β is specifically inhibited by LiCl (lanes 1–4), and PKA is inhibited by its regulatory subunit RII (lanes 5–7). (a) In the control (lane 1), the epitope of AT100 is induced by the brain extract (as in Figs 1 and 3), but it disappears with increasing concentrations of the GSK-3β inhibitor LiCl (lanes 2–4). Similarly, the RII subunit of PKA suppresses the AT100 epitope (lanes 6 and 7). (b) By comparison, the epitope of AT8 is not significantly influenced by LiCl or the RII subunit, suggesting that this epitope can be phosphorylated by other proline-directed kinases in the brain extract, such as MAP kinase (Drewes et al., 1992).

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**Fig. 9. Induction of the AT100 epitope in Sf9 insect cells transfected with human Tau23 and human Tau40 isoforms using the baculovirus vector.** (A) SDS/PAGE illustrates the phosphorylation and molecular mass shift of Tau 23 [hTau23(BV)] and Tau40 [hTau40(BV)] (lanes 2 and 4) compared with the unphosphorylated protein expressed in E. coli (lanes 1 and 3). (B–D), immunoblots with antibodies Tau-1, AT8 and AT100. All samples show a reaction with Tau-1, indicating that part of the protein is unphosphorylated around residue 200. The AT8 and AT100 reactions are seen only with Tau from the Sf9 cells.

**DISCUSSION**

In Alzheimer’s disease Tau occurs in a hyperphosphorylated form and in a particular state leading to the aggregation of PHF (Grundke-Iqbal et al., 1986). This has led to a strong interest in antibodies against Tau that are sensitive to its conformation or to its state of phosphorylation. Such antibodies are potentially useful as diagnostic tools, both for Alzheimer brain tissue and for the development of cellular or animal models. Some antibodies, such as Alz-50 and MC-1 (Carmel et al., 1996; Jicha et al., 1997), are sensitive to a folded conformation of Tau, while phosphorylation dependent antibodies include the AT series (Mercken et al., 1992), the SMi series (Sternberger et al., 1985), PHF-1 (Greenberg et al., 1992), Tau-1 (Binder et al., 1985) and others (Lee et al., 1991; Brion et al., 1993; Hasegawa et al., 1993; Ishiguro et al., 1995). Most of these antibodies have contiguous epitopes which are located on phosphorylatable sequence motifs and can be deleted by point mutations. Some of them depend on additional structural features, such as a folded conformation (e.g. SMi34; Lichtenberg-Kraag et al., 1992) or a sequential phosphorylation, such as AT100. This antibody is of particular interest because it appears to be highly specific for the Alzheimer state of Tau, in contrast to other antibodies with phospho-epitopes that also recognize a fraction of Tau in normal
Phosphorylate several sites on Tau with varying efficiencies, and investigation.

1. Known to phosphorylate Tau, antibodies and two-dimensional called into question (Skurat and Roach, (Ser-Pro or Thr-Pro for GSK3, (Arg or Lys)-Xaa-Xaa-(Ser or sidered the classical example of sequential phosphorylation,

1. GSK-3 (Hanger et al., 1994). The third step (Thr212 phosphorylation) occurs only when the first two conditions are met, and it is blocked if the fourth step (Ser214 phosphorylation) occurs prematurely. This pathway explains several observations. The AT100 epitope always appears after the AT8 epitope; inverting the order of kinases to PKA first, GSK-3β second does not generate the AT100 epitope; omitting the polyanions does not generate it either, etc. In other words, Ser(PT)214 can protect its neighborhood against proline-directed phosphorylation. The same phosphorylation site in Tau also affects microtubule dynamics (Brant et al., 1994; Illenberger et al., 1998). Our results obtained with the sequential phosphorylation of Tau correlate well with a recent study employing synthetic phosphopeptides derived from Tau. This study showed that the reaction of AT100 can be induced on peptides containing the equivalent of phosphorylated Thr212 and S214 (Hoffmann et al., 1997).

Given this complicated scenario it might seem surprising that the brain extract can generate the AT100 epitope, even though it contains a cocktail of kinases and phosphatases. However, this becomes understandable when we consider that the two crucial kinases are PKA and GSK-3, as seen from the effect of the specific kinase inhibitors Rp and LiCl (Stambolic et al., 1996; Hong et al., 1997). Apparently GSK-3 is more active than PKA in the extract and thus can set the scene for the AT100 epitope.

Since the extract contains RNA and extracellular matrix material the requirement for polyanions is met as well.

An open question is why MAP kinase, which is present in the extract and can phosphorylate Tau efficiently (Drewes et al., 1992), generates the AT8 epitope but not the AT100 epitope, even though residues Thr212→Pro213 would constitute a suitable target motif. Two factors may contribute to this observation. First, MAP kinase has a lower activity in cells than GSK-3β, presumably because it is more tightly regulated (Lovestone et al., 1994). Second, although purified MAP kinase is capable of phosphorylating more sites on Tau, some of these could be inhibitory for the AT100 epitope. This issue is currently under investigation.

The phosphorylation of glycogen synthase by GSK-3 is considered the classical example of sequential phosphorylation, started by the priming phosphorylation of glycogen synthase by casein kinase II and continuing in an N-terminal direction by GSK-3 (Woodgett, 1995). Aspects of this model have been called into question (Skurat and Roach, 1996), but it has been a useful guide to probe possible sequential phosphorylation mech-
anisms for Tau (e.g. Singh et al., 1995). It would therefore be tempting to relate the model of glycogen synthase phosphorylation to our current results; however, we believe that a direct comparison is not appropriate. In our case a ‘priming’ phosphorylation of Ser214 by PKA) is inhibitory, not stimulatory. Stimulatory phosphorylation (around the AT8 epitope) is achieved by GSK-3β itself (and not by another kinase), and this phosphorylation appears to occur independently of the AT100 epitope. The difference between the two targets of GSK-3, Tau and glycogen synthase, are due to their different structures. Tau largely lacks a compact folded structure and behaves like a highly soluble but natively denatured protein (Schweers et al., 1994). The effect of polyanions could be explained by a change in conformation, which may be locally restricted but leads to the formation of insoluble PHF (Perez et al., 1996; Goedert et al., 1996; Kampers et al., 1996). Similarly, the accumulation of phosphorylation sites (which also have an anionic character) might induce a local change in structure, which could interfere positively or negatively with neighboring regions of Tau and with protein kinases.

This is a simple view of the observed chain of reactions, and structural details must be obtained to provide further information. In the absence of structural information we can only speculate on the basis of other examples. Proline-rich sequences occur in a number of proteins and are often involved in protein-protein recognition. Two recently solved structures, that of the actin regulator protein profilin, with a poly(Pro) peptide, and that of a tyrosine kinase Src homology 3-domain combined with a Pro-rich peptide from a Src homology 2 domain, show that the Pro-rich sequences are in extended conformations, similar to that of a poly(Pro) helix (Archer et al., 1994; Musacchio et al., 1994). A similar conformation occurs in collagen, and the sequences around the AT8 and AT100 epitopes are reminiscent of collagen-like triplets. Phosphorylation at Ser or Thr before the Pro residues would tend to stiffen the chain and keep it in an extended form. The poly(Pro)-like conformation would put successive phosphorylated side chains roughly on one side of the coil; this would help to explain why antibodies such as AT8 or AT100 recognize pairs of phosphorylation sites. Furthermore the AT100 epitope lies just upstream of the heptapeptide K224KVAVVR, which is particularly effective in binding the P2 domain to microtubules. This supports a model where a phosphorylation-dependent conformational change in the P2 domain regulates Tau’s interactions with microtubules (Goode et al., 1997).

The most significant conclusion from these studies is that the epitope of an antibody that is highly characteristic of Alzheimer Tau has been mapped, the reaction sequence and kinases bulle-associated tau-proteins is due to differences in phosphorylation, which would help to explain why antibodies such as AT8 or AT100 (rich sequences are in extended conformations, similar to that of ping and phosphoamino acid analysis by two-dimensional separation

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