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Phosphorylation of microtubule-associated protein tau: identification of the site for Ca^{2+}-calmodulin dependent kinase and relationship with tau phosphorylation in Alzheimer tangles

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The microtubule array in neuronal cells undergoes extensive growth, dynamics and rearrangements during neurite outgrowth. While little is known about how these changes are regulated, microtuble-associated proteins (MAPs) including tau protein are likely to perform an important role. Tau is one of the MAPs in mammalian brain. When isolated it is usually a mixture of several isoforms containing between 341 and 441 residues that arise from alternative splicing. Tau can be phosphorylated by several protein kinases. Phosphorylation at certain sites results in major structural and functional changes, as seen by changes in electrophoretic mobility, interaction with microtubules, molecular length and elasticity. Here we show that the sites of phosphorylation by four kinases (PKA, PKC, CK and CaMK) all lie in the C-terminal microtubule-binding half of tau, but only the phosphorylation by CaM kinase shows the pronounced shift in electrophoretic mobility characteristic for tau from Alzheimer neurofibrillary tangles. By using a combination of limited proteolysis, protein sequencing and protein engineering we show that a single phosphorylation site is responsible for this shift, located at Ser 405 in the C-terminal tail of the protein outside the region of internal repeats. Phosphorylation at this site not only reduces the electrophoretic mobility of tau, it also makes the protein long and stiff, as shown earlier. The site is likely to be phosphorylated in tau from Alzheimer neurofibrillary tangles.

Key words: Alzheimer/Ca^{2+}-calmodulin dependant kinase/microtubule-associated protein tau/phosphorylation

Introduction

The C-terminal half of tau contains three or four internal repeats which are responsible for the binding to microtubules (Lee *et al.*, 1988; Himmler *et al.*, 1989). In this region tau is homologous to other MAPs such as MAP2 (Lewis *et al.*, 1988). One function of these proteins is to stabilize microtubules (Cleveland *et al.*, 1977), but phosphorylation by some kinases reduces the stabilizing effect (Jameson *et al.*, 1980; Burns *et al.*, 1984), suggesting a regulatory function of MAP phosphorylation. Certain types of phosphorylation can be distinguished by SDS–PAGE; in particular, CaMK (calcium calmodulin dependent protein kinase) is unusual in that it reduces the electrophoretic mobility of all tau isoforms (Baudier and Cole, 1987), and the underlying structural change is probably responsible for altering tau's elasticity (Lichtenberg *et al.*, 1988; Hagestedt *et al.*, 1989).

Much of the current interest derives from the fact that tau is part of the paired helical filaments, the major component of the neurofibrillary tangles of Alzheimer's disease (Kosik *et al.*, 1986; Goedert *et al.*, 1988). In mammalian brain, tau occurs in different states of phosphorylation (Binder *et al.*, 1985), but in Alzheimer tangles the phosphorylation is abnormal, as judged by phosphorylation-dependent antibody staining (Grundke-Iqbal *et al.*, 1986; Kosik *et al.*, 1986), and moreover it is of the same type as that induced by CaMK, i.e. Alzheimer tau shows a shift in SDS gels towards lower mobility (Baudier and Cole, 1987; Flament and Delacourte, 1989). Because of these observations current research is aimed at identifying the kinase(s) that act on tau and on its phosphorylation sites. Phosphorylation of tau has been demonstrated for the case of PKC and CaMK (Baudier and Cole, 1987). More generally, MAPs can be phosphorylated by PKA which is associated with the projection domain of MAP2 (Vallee, 1980), although there has been some debate on whether it acts on tau (Baudier and Cole, 1987). In the past the analysis of the phosphorylation sites has been hampered by the complexity of tau isoforms isolated from brain tissue. Because of this we have now turned to cloned and bacterially expressed tau isoforms (bovine Tau4, Himmler, 1989; Himmler *et al.*, 1989; or human Htau23, Goedert *et al.*, 1989) and several constructs derived from them.

Results

Four different kinases phosphorylate the C-terminal half of tau

In attempting to define the Alzheimer-like phosphorylation site of tau we initially started with a preparation from bovine or porcine brain. However, this protein is a mixture of several isoforms in a heterogeneous state of phosphorylation which makes a clear-cut analysis difficult. We therefore turned to genetically engineered and bacterially expressed tau and several derivatives of it. The largest protein was identical to bovine brain Tau4 (Himmler *et al.*, 1989). It consists of 430 amino acid residues. We broadly distinguish three domains, the N-terminal domain, residues 1–231, a repeat domain, residues 232–356, and a C-terminal domain,

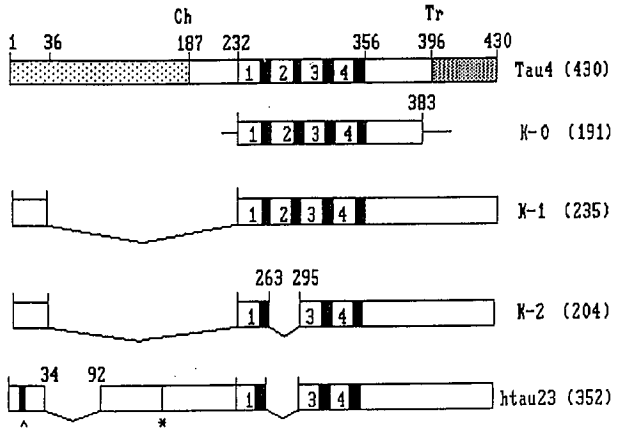

Fig. 1. Diagram of the amino acid sequences of tau and its derivatives. The top bar represents the sequence of isoform 4 of bovine brain tau (430 residues, Himmler *et al.*, 1989), the other three bars show the constructs K0, K1 and K2. The four internal repeats in the microtubule-binding domain are labeled 1–4 (residues 232–356), the homologous 18-residue stretches are black (residues 245–262, 276–293, 307–324, 339–356). Construct K0 contains the tau sequence 232–383 (mainly the four repeats), plus leading and trailing sequences from the vector that are not homologous with tau (positions 216–231, 384–406, shown only as lines). K1 contains residues 1–36 and 232–430 so that most of the N-terminal domain up to the repeat region is missing. K2 is similar to K1, except that the second repeat is missing (264–294). K2 was derived from Htau23 (bottom bar, see Goedert *et al.*, 1989; note the different numbering used here: residues 186–352 of Htau23 correspond to 233–430 of Tau4 without the second repeat, residues 1–185 of Htau23 correspond to 1–232 of Tau4, with several deletions and insertions around the positions indicated by the asterisk and the small arrow). Limited chymotryptic digestion (Ch) of Tau4 generates the peptides Met1–Tyr186 (termed 'projection fragment', dotted) and Ser187–Leu430 ('assembly fragment'). The C-terminal tail generated by tryptic digestion comprises residues 396–430 (hatched).

residues 357–430 (Figure 1). The repeat domain contains four internal repeats of 31 or 32 residues; it represents the microtubule-binding region (Lee *et al.*, 1988; Himmler *et al.*, 1989; and see below). Construct K0 contains essentially the repeat domain, part of the C-terminal domain (357–383), and short leading and trailing sequences from the vector which are not homologous to tau (corresponding to residue positions 216–231 and 384–406). Construct K1 is identical to the full length Tau4 in the repeat and C-terminal domains (232–430) but has only the first 36 residues of the N-terminal domain. Construct K2 is similar to K1, except that the second repeat is missing (residues 264–294).

Tau4 can be phosphorylated by four protein kinases occurring in brain tissue, PKA, PKC, CK and CaMK (Figure 2). The case of CaMK is illustrated in Figure 2c, showing that phosphorylation induces the upward shift in the SDS gel. This is typical of CaMK phosphorylation *in vitro* and of tau in Alzheimer tangles.

Since phosphorylation is thought to affect the affinity of tau to microtubules, our next step was to separate the microtubule binding and non-binding parts. Taxol-stabilized microtubules were incubated with tau and briefly digested with chymotrypsin. As shown in Figure 2a and b this cleaves tau in two parts. One of them, the projection fragment, stays in the supernatant when the microtubules are pelleted. The other microtubule binding fragment can be released from microtubules in high salt. N-terminal sequencing shows that the cleavage occurs at Tyr186. Thus the projection fragment contains residues 1–186, the assembly fragment contains residues 187–430 (244 residues, Figure 1, top). The latter includes the repeat domain, consistent with the earlier studies showing that this is responsible for microtubule binding. Although the projection fragment is smaller than the

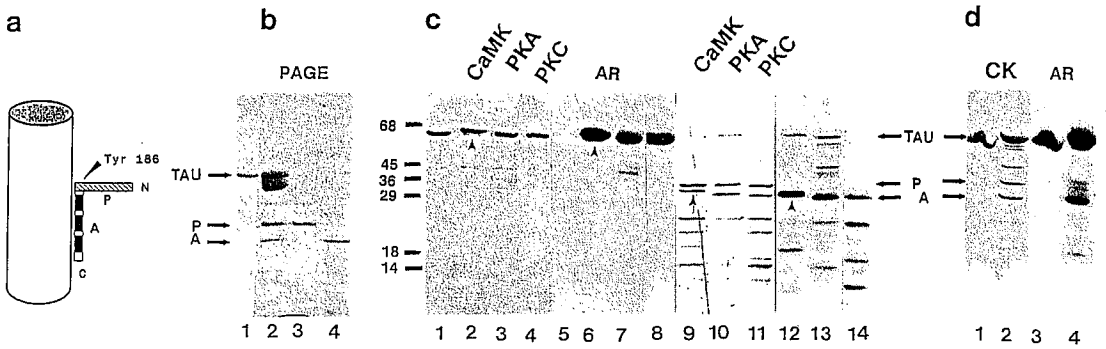

Fig. 2. Phosphorylation of Tau4 and its fragments by different kinases. (**a**) Diagram of a microtubule with a bound tau molecule, showing the relationship of the projection domain P (N-terminal, hatched) and the assembly domain (A) containing the internal repeats. (**b**) Limited chymotryptic digestion of Tau4. Lane 1, SDS–PAGE of bacterially expressed Tau4; lane 2, SDS–PAGE of Tau4 bound to microtubules and then briefly digested with chymotrypsin. The two top bands are α and β tubulin which are not cleaved in these conditions. The two cleavage products labeled P and A (M_r ~36 and 31 kd) are the N-terminal projection domain (residues 1–186) and the C-terminal assembly domain (residues 187–430). The fragments were identified by N-terminal sequencing. Note that the projection fragment runs anomalously slowly, i.e. the high M_r value of tau, compared to its actual mass, is due to this fragment. Lane 3, projection fragment remaining in the supernatant after pelleting the microtubules with the attached assembly fragment. Lane 4, assembly fragment of Tau4 after dissociating it from the microtubules with high salt. The lower bands are smaller fragments of the assembly fragment. (**c**) Phosphorylation of Tau4 with three different kinases. Lane 1, SDS–PAGE of Tau4, lanes 2–4, Tau4 after phosphorylation with CaMK, PKA and PKC. Note that only CaMK causes a shift in the gel. Lanes 5–8, autoradiograms of lanes 1–4 showing that each kinase phosphorylates the protein. Lanes 9–11, SDS–PAGE of fragments of Tau4 produced by limited chymotryptic digestion after phosphorylation with CaMK (lane 9), PKA (lane 10) and PKC (lane 11); lanes 12–14, corresponding autoradiograms. The pair of bands at the top (small arrow) are the projection and assembly domains (P and A). Only the assembly domain (and subfragments of it) contain phosphate, but not the projection domain. Only the assembly domain is shifted after phosphorylation with CaMK (lane 9). (**d**) Phosphorylation of Tau4 and its subfragments by casein kinase. Lane 1, SDS–PAGE of phosphorylated Tau4; lane 2, limited chymotryptic cleavage of phosphorylated Tau4, lanes 3 and 4, autoradiograms. Note that the phoshorylation is mainly in the assembly domain with minor labeling in the projection domain (lane 4).

assembly fragment it runs more slowly in the gel, i.e. the anomalously high M_r value of tau is mainly due to the projection fragment. However, the M_r shift induced by phosphorylation is seen only in the assembly fragment.

The phosphorylation of Tau4 and its subfragments by PKA, PKC and CK is shown in Figure 2c and d. In each case the sites are located in the assembly fragment (187–430), as in the case of CaMK. However, neither of the other three kinases induces an M_r shift.

We were most interested in the CaMK phosphorylation sites since this was the only kinase tested to produce the gel shift in tau. We narrowed down the phosphorylation sites of the CaMK by using several engineered constructs of tau. Both K1 and K2 are good substrates for the CaMK, and they also show the shift in the SDS gel (Figure 3a, lanes 2, 4 and 6). These constructs contain most of the assembly fragment and a short stretch from the N terminus which is not phosphorylated, as shown in Figure 2 above. By contrast, construct K0 is a very poor substrate and does not show the shift in the gel. It differs from the others by the lack of the N-terminal 36 residues (which are unimportant for phosphorylation) and by the lack of the C-terminal tail (residues 384–430). This already suggests that the phosphorylation site of CaMK must be near the C terminus, outside the region of internal repeats (see Figure 1), and that this region is responsible for the phosphorylation-dependent shift in mobility. The other three kinases efficiently phosphorylate all three constructs, but neither one induces the M_r shift, as with intact Tau4. This means that at least part of the phosphorylation sites are in the region of internal repeats. It is consistent with the observation that phosphorylation reduces the interaction between tau and microtubules (details elsewhere).

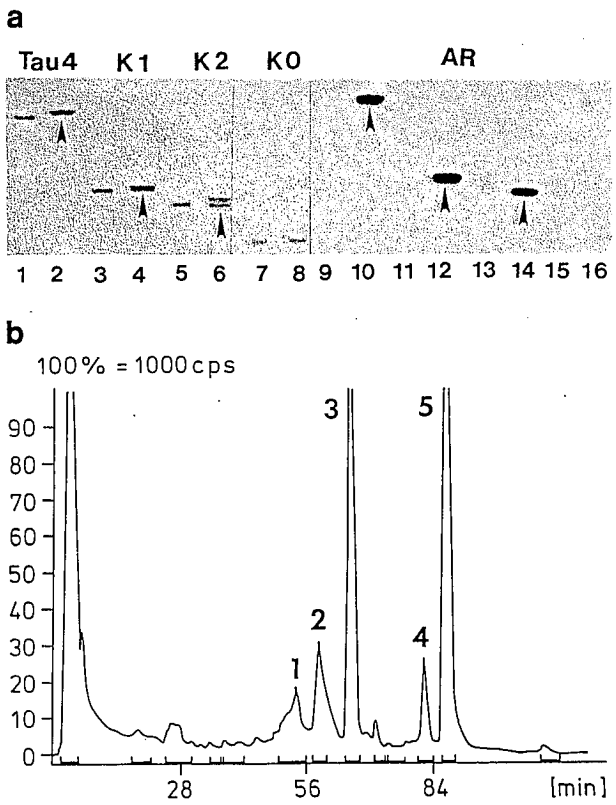

Fig. 3. (a) Phosphorylation of Tau4, its constructs and peptides by CaMK. Lanes 1 and 2, unphosphorylated and phosphorylated Tau4. Lanes 3 and 4, unphosphorylated and phosphorylated K1. Lanes 5 and 6, unphosphorylated and phosphorylated K2. Lanes 7 and 8, unphosphorylated and phosphorylated K0. Lanes 9–16, autoradiograms of lanes 1–8. Note that all constructs show an upward shift in the gel upon phosphorylation, except K0 (indicating that the phosphorylation site is beyond residue 383). Note that Tau4, K1 and K2 are highly phosphorylated, whereas K0 shows only marginal phosphorylation. (b) HPLC elution profile of ^{32}P phosphorylated tryptic peptides of Tau4. The sequences of all five peptide peaks (labeled 1–5) start at the same residue, His 396. (The multiplicity of the peaks may arise from incomplete digestion, e.g. at Lys 427, or oxidation of Met, or from structural isomers due to aggregation.)

CaM kinase phosphorylates Ser 405 in the C-terminal tail of tau

To identify the CaMK site(s) we made a total tryptic digest of phosphorylated Tau4, K1 and K2, and separated the peptides by reverse phase HPLC. Figure 3b shows the radioactive peptides for the case of Tau4. There are two major and three minor peaks. After rechromatography of these fractions on a C_{18} column they were analyzed by gas phase sequencing, with the surprising result that they all started at His 396, consistent with tryptic cleavage after Arg 395. Similar results were obtained with constructs K1 and K2 (not shown).

The site of phosphorylation was determined by three independent methods, (i) solid phase sequencing of the radioactive peptides, (ii) gas phase sequencing of the peptides after conversion of phosphoserines into S-ethylcysteines, or (iii) sequencing of the phosphopeptides and detection of the

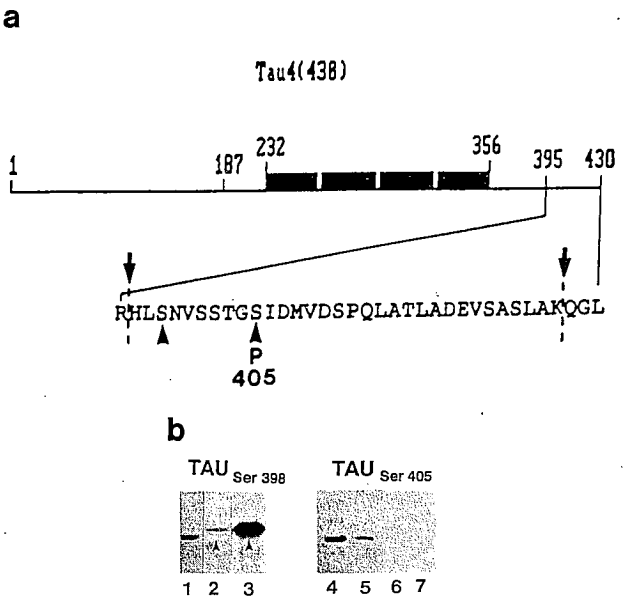

Fig. 4. Phosphorylation sites and site-directed mutagenesis of tau. (a) Diagram of Tau4, with the internal repeats shown as black bars, and the C-terminal tryptic peptide (396–430). The residue phosphorylated by CaM kinase and responsible for the shift in the gel is Ser 405 (labeled P). Tryptic cleavage sites defining the peptide are Arg 395 and Lys 427 (arrows). (b) Site-directed mutagenesis of Htau23. Lanes 1–3, Ser 398 mutated into Gly, lane 1 without, lane 2 with phosphorylation by CaMK, lane 3 autoradiogram of lane 2. Note that phosphorylation still takes place, as seen by the shift in the gel and by the autoradiogram of lane 3. This shows that Ser 398 is not the site of phosphorylation causing the shift. Lanes 4–7, Ser 405 mutated into Ala, lane 4 without, lane 5 with phosphorylation by CaMK, lanes 6 and 7 are autoradiograms of lanes 4 and 5. Note that there is no shift in the gel and only some faint unspecific phosphorylation. Thus the phosphorylation of Ser 405 is responsible for the change in electrophoretic mobility.

DTT adduct of dehydroalanine (Meyer *et al.*, 1990). In each case, Ser 405 was labeled with phosphate (see Figure 4a). Protein that showed a complete shift to the higher M_r value contained nearly one P_i per molecule, suggesting already that Ser 405 is the only phosphorylatable residue by CaMK. This is remarkable in view of the unusually large number of Ser and Thr in tau. Even the C-terminal tryptic peptide contains 9 Ser + Thr. The sequencing signal could be identified for 11 cycles in solid phase sequencing and 21 cycles in gas phase sequencing, showing that Ser 398, 401, 402, 411 and Thr 403 and 416 are not phosphorylated. This leaves only two other potential sites, Ser 422 and 424, which can be excluded by site-directed mutagenesis (see below).

It is noteworthy that CaMK phosphorylates Tau4 and K1 more efficiently than K2. K2 is special in that it is the only phosphorylatable construct that has only three repeats. As discussed below, this suggests that the second repeat can induce a conformation of tau which facilitates phosphorylation by CaMK at the C-terminal tail.

Site-directed mutagenesis of potential phosphorylation sites

After realizing that only the tryptic peptide 396–430 was phosphorylated we initially expected that Ser 398 was the phosphorylated residue because it is part of a reported target sequence R-X-X-S of CaM kinase (Edelman *et al.*, 1987). We therefore used Htau23 to change Ser 398 into Gly by site-directed mutagenesis, but the protein could still be phosphorylated and showed the shift in the gel (Figure 4b, lanes 1–3). This prompted us to determine the phosphorylation site by the S-ethylcysteine method, as described above.

To check if Ser 405 was indeed the crucial residue for the M_r shift we converted it into Ala. The modified Htau23 could no longer be phosphorylated by the CaMK, and there was no shift either. This excludes all other potential sites in the C-terminal peptide. It confirms that Ser 405 is essential not only for the phosphorylation, but also for the conformational change that underlies the altered mobility of the protein. In fact there is a 1:1 correspondence between phosphorylation and the M_r shift: every phosphorylated tau molecule will migrate more slowly so that the slow fraction is nearly 100% phosphorylated and the fast fraction is not. Thus in the case of CaMK the average degree of phosphorylation can be read off directly from the gel. These results are illustrated in Figure 4b, lanes 4–7.

Discussion

Our studies were prompted by two observations. One is that tau occurs in Alzheimer neurofibrillary tangles in a specific state of phosphorylation whose effect is to shift the M_r to a higher apparent value; a similar shift is induced *in vitro* by phosphorylation with CaM kinase, but not by other kinases (Baudier and Cole, 1987). Second, tau is an unusually elastic molecule, and phosphorylation by CaM kinase has a dramatic effect on this property by making the structure long and stiff (Hagestedt *et al.*, 1989). We therefore set out to determine the residues phosphorylated by CaMK, hoping that this will eventually lead to an understanding of the structural states of tau *in vitro* and in the Alzheimer tangles. At the same time we compared the effects of CaM kinase with other kinases occurring in brain tissue. Since tau prepared from brain is a mixture of isotypes in ill-defined states of phosphorylation we chose to work with pure bacterially expressed tau constructs in order to arrive at interpretable results.

The results differed in several ways from our initial expectations. Firstly tau can be phosphorylated by all four kinases tested, including PKA (this point was a matter of debate, see Baudier and Cole 1987). Good substrates for PKA are peptides of the type R-R-X-S (for a review see Edelman *et al.*, 1987) which are not found in tau. However, the kinase has a broad specificity and the less stringent motif R-X-X-S occurs several times. The same motif can also be a target for PKC and CaMK. By contrast, CK phosphorylates serines near acidic residues; several such motifs can also be found in tau. However, the sequence alone is of limited predictive value, as illustrated by the example of CaMK, and we will show elsewhere that the same reservation holds for other kinases.

Considering the potential CaMK sites near the C terminus in more detail, we note that Ser 398 is not phosphorylated although it is in a R-X-X-S sequence, whereas the phosphorylated Ser 405 is not part of such a motif. Thus the comparison with R-X-X-S is not very meaningful; however, a more appropriate comparison can be made with the sites phosphorylated by MLCK, another calmodulin dependent kinase. The phosphorylatable myosin light chains contain a motif K/R-X_n-S, where n is of the order of 6–9; moreover, MLCK is autophosphorylated at Ser 160 which is also in a R-X_9-S motif (Meyer and Mayr, 1987). The same motif is found in tau, R395-X_9-Ser405, suggesting a relationship with MLCK substrates.

Another notable observation was that all kinases phosphorylated exclusively the C-terminal microtubule assembly domain and not the N-terminal projection domain (defined by chymotryptic cleavage at Tyr 186). The reason for this is not evident from the sequence whose general character is similar in both domains, and putative target sequences exist in both domains. However, the observation agrees well with several studies (e.g. Jameson *et al.*, 1980; Burns *et al.*, 1984) showing that phosphorylation reduces the affinity between tau and microtubules. More specifically, the microtubule binding site(s) are believed to lie in the region of the internal repeats corresponding to residues 232–356 (Lee *et al.*, 1988; Himmler *et al.*, 1989). PKA, PKC and CK indeed phosphorylate residues in this region (Figure 2b, c). Only CaMK is special in that it phosphorylates the C-terminal tail outside the internal repeats.

Perhaps the most surprising result is that the phosphorylation of a single site by CaMK is sufficient to induce the conformational change that is responsible for the shift in M_r, equivalent to an increase of 4 kd. Among the sites phosphorylated in tau *in vitro* only the modification of Ser 405 has the pronounced effect on the protein, and when this residue is point mutated to Ala both the phosphorylation by CaMK and the M_r shift disappear. The same Ser 405 is presumably responsible for controlling the length and the elasticity of tau, as seen by electron microscopy, since the experimental conditions were similar in the two types of experiment (Hagestedt *et al.*, 1989). None of the other kinases produced the M_r shift although they phosphorylated the protein quite efficiently. Since tau from Alzheimer tangles shows the shift (Baudier and Cole, 1987; Flament and Delacourte, 1989) these data strongly indicate that Ser 405

is among the residues phosphorylated in the Alzheimer tangles.

The phosphorylation of Alzheimer brain tau has been termed 'abnormal' since it is prominent in diseased but not in normal brains, as judged by labeling with the monoclonal antibody TAU1 specific for dephosphorylated tau and by the M_r shift (Binder *et al.*, 1985; Grundke-Iqbal *et al.*, 1986; Kosik *et al.*, 1986; Flament and Delacourte, 1989). The epitope for TAU1 is around residues 178–197, i.e. on the N-terminal side of the internal repeats (Kosik *et al.*, 1988). This suggests that in brain tissue the TAU1 antibody is sensitive to phosphorylation sites other than Ser 405. The Alzheimer-like phosphorylation of tau is therefore abnormal in two respects, around the TAU1 binding site (as detected by the antibody's affinity) and at Ser 405 (as seen by the M_r shift).

In any case the present results argue that the abnormal behavior (in terms of reduced mobility) lies not in a particularly high degree of phosphorylation, but rather in a particular residue. How a single phosphate could elicit these dramatic responses on the structure of the protein is at present a mystery. Analogous examples could be cited, for example the phosphorylation-dependent hinge motion in glycogen phosphorylase (Barford and Johnson, 1989). However, the structural changes in tau are much larger, with length changes in excess of 10 nm. It is possible that the magnitude of the effect depends on the interaction of several tau molecules, or on interactions between the CaMK site and sites labeled by other kinases, but it would be premature to speculate on its origin. At any rate it is important to point out that the conformational change induced by CaMK action at Ser 405 *in vitro* does not exclude similar conformational changes induced by the action of other kinases *in vivo* at Ser 405 or other sites

Some additional points are worth mentioning in this context: Kosik *et al.* (1989) pointed out that the four internal repeats contain two conserved serines and suggested that Ser 345 (in our numbering) is phosphorylated by CaMK, resulting in a reduced affinity for microtubules. We have not found any CaMK sites in the repeat region *in vitro* so that the explanation of the phosphorylation effect on microtubule binding is less obvious. It is conceivable that the C-terminal tail containing Ser 405 contributes to microtubule binding, or that it affects the structure of the microtubule-binding region in an indirect manner. *In vivo* there could be influence of sites phosphorylated by different kinases on each other. Moroever, we find that construct K2, lacking the second internal repeat, is not as readily phosphorylated as intact Tau4 or K1; this is another indication that the C-terminal tail and the repeat region might influence one another.

Finally we note that tau belongs to a class of MAPs which are homologous in their C-terminal microtubule binding domains but differ in their N-terminal projection domains; another prominent member of this class is MAP2 (Lewis *et al.*, 1988). The homology is found not only in the region of internal repeats, but also in the C-terminal tail, and in particular MAP2 also contains the RX_9S sequence. We found that the assembly fragment of MAP2 can be phosphorylated by CaMK, resulting in a similar M_r shift as in tau (H.Wille, unpublished). This suggests that the phosphorylation site, kinase specificity and structural reorganization are common to proteins of the MAP2/tau class and may be of more general regulatory importance, independent of the pathology of Alzheimer's disease.

Materials and methods

Cloning and expression of tau will be described in detail elsewhere. Briefly, we used the plasmid $pET_{Nde}43$-12 (Himmler *et al.*, 1989) containing the bovine Tau4 gene between the *NdeI* and *Bam*HI restriction sites of the expression vector pET-3b (Rosenberg *et al.*, 1987). Construct K0 is a fragment of Tau4 encoded between the *PstI* and *RsaI* sites; it contains in addition a non-homologous 16 residue leader sequence and a 23 residue tail from the bacteriophage T7 sequence encoded in the pET-3a expression vector (Figure 1). K1 is derived from Tau4 by deleting 195 amino acid residues encoded between two *PstI* sites. K2 is a chimera consisting of 36 residues from the amino terminus of bovine Tau4 (from the plasmid $pET_{Nde}43$-12) and 168 residues from the carboxy part of human Tau23 (from the plasmid pUC18/htau23, see Goedert *et al.*, 1989). The carboxy terminal part is completely homologous to the corresponding part of bovine Tau4, except that the second internal repeat is absent (residues 264–294, see Figure 1). The tau constructs were isolated by making use of the heat stability of the protein and by FPLC Mono S (Pharmacia) chromatography (for details see Hagestedt *et al.*, 1989).

Protein kinases

CaM kinase from rabbit muscle was prepared according to Woodgett *et al.* (1983). PKC from rat brain was prepared according to Walton *et al.* (1987). CK II from human placenta was a generous gift of Dr W.Pyerin (DKFZ Heidelberg). PKA from rabbit muscle was obtained from Sigma.

Protein phosphorylation

All phosphorylation reactions were done in 50 mM Tris–HCl, pH 7.2, 2 mM DTT and 0.2 mM ATP at 37°C for 2 h. Radioactive labeling was done with [γ-^{32}P]ATP (NEN Du Pont) at 10 mCi/ml, 3000 mCi/mmol, diluted to 200–300 Ci/mol ATP for peptide sequencing or 50–70 Ci/mol ATP for autoradiography. Reaction conditions were as follows. (i) CaM kinase, 5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 10 μg/ml calmodulin, 1 unit CaM kinase/mg substrate (1 unit = nmol P_i/min, determined with synapsin as substrate). (ii) PKA, 10 mM $MgCl_2$, 2 μM cAMP, 0.03 units kinase/mg substrate (activity determined with casein). (iii) PKC, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 50 μg/ml phosphatidylserine, 1 μg/ml diacylglycerol, 3 units kinase/mg substrate (activity determined with histone IIIS). (iv) CK II, 15 mM Mg^{2+} acetate, 100 mM NaCl, 1 mM spermidine, 0.2 units kinase/mg substrate (activity determined with casein).

Limited proteolysis

The proteins were phosphorylated as described above and immediately incubated with chymotrypsin (Sigma, TLCK treated, final concentration 1 μg/ml) at room temperature for 30–40 min. The reaction was stopped by adding an equal volume of SDS sample buffer and boiling for 2 min.

Preparation of assembly and projection fragments of Tau4

PC-tubulin (8 mg/ml) was polymerized in the presence of Tau4 (2 mg/ml) in reassembly buffer (0.1 M PIPES, pH 6.9, 1 mM each of GTP, $MgSO_4$, EGTA and 20 μM taxol, 37°C, 20 min). Chymotrypsin was added to a final concentration of 1 μg/ml (digestion for 1 h at room temperature, stopped with 2 mM phenylmethylsulfonylfluoride). The microtubules were pelleted at 100 000 *g* for 10 min, the supernatant containing the unbound projection fragment of Tau4 was removed, and the pellet was resuspended in cold reassembly buffer. The protein was polymerized once more, microtubules were stabilized by taxol, and the assembly fragment of Tau4 was detached from microtubules by adding 0.8 M NaCl. The solution was pelleted, and the supernatant containing the assembly fragment was removed.

Sequencing of peptides

The protein was phosphorylated as described above, unbound nucleotide was removed by passage over a C_{18} cartridge (Waters). The protein was lyophilized and then resuspended in 10 mM NH_4HCO_3, pH 8.0, and digested for 24 h by adding 1/50 (w/w) of trypsin (Sigma, TPCK treated) four times at 6 h intervals (this procedure was used to optimize digestion conditions). The digest was lyophilized once more and resuspended in buffer A (10 mM ammonium acetate, pH 6.0) for the first HPLC gradient. Separation was done on Millipore-Waters or Beckman HPLC systems with a C_{18} reverse phase column (Vydac, 4.6 × 250mm, flow rate 1 ml/min at room temperature). Crude fractions were obtained by a linear gradient of 0–40% acetonitrile in 10 mM ammonium acetate. Radioactivity was

determined by a scintillation counter (Searle M5). The radioactive peaks were reapplied to the same column and eluted with a linear gradient of 0–40% acetonitrile in 0.1% trifluoroacetic acid. The sequence analysis of the peptides was performed using a 477A pulsed liquid phase protein/peptide sequencer and a 120A on-line PTH amino acid analyzer (Applied Biosystems). Phosphoserines were identified by several methods: gas phase sequencing after conversion of phosphoserines into S-ethylcyteines; by the formation of the dithiothreitol adduct of dehydroalanine from serine phosphate prior to sequencing; or by solid phase sequencing of radioactive peptides (Meyer *et al.*, 1990).

Abbreviations used

CaM kinase, calcium-calmodulin dependent protein kinase; CK, casein kinase II; EGTA, ethylene glycol-O,O′-bis(2-amino ethyl ether)-N,N,N′,N′-tetraacetic acid; HPLC or FPLC, high performance or fast protein liquid chromatography; MAP(s), microtubule-associated protein(s); MAP_2, microtubule-associated protein 2; MLCK, myosin light chain kinase; PAGE, polyacrylamide gel electrophoresis; PIPES, piperazine-N,N′-bis(2-ethane sulfonic acid); PKA, protein kinase A (cAMP dependent kinase); PKC, protein kinase C (Ca^{2+} phospholipid dependent kinase); SDS, sodium dodecyl sulphate; Tris, Tris(hydroxymethyl)aminomethane.

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