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Dephosphorylation of tau protein and Alzheimer paired helical filaments by calcineurin and phosphatase-2A

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We have shown previously that brain tissue contains protein kinases which can phosphorylate tau protein to a state reminiscent of the pathological state of Alzheimer paired helical filaments (PHFs); these include proline-directed kinases which phosphorylate SP or TP motifs (such as MAP kinase and GSK-3) [Drewes et al. (1992); Mandelkow et al. (1992)], as well as a novel kinase which phosphorylates S262 of tau protein and thereby strongly reduces the binding of tau to microtubules [Biernat et al. (1993)]. Here we report on the corresponding phosphatases in brain which normally keep the 'pathological' sites free of phosphate. The major phosphatases acting on tau are calcineurin and PP-2A, but not PP-1. Both are present and active in brain extracts, they can dephosphorylate recombinant tau after prior phosphorylation with either MAP kinase, GSK-3, or brain extract, and the course of dephosphorylation can be monitored with antibodies diagnostic of the pathological state of tau. Both phosphatases also act directly on PHF tau isolated from Alzheimer brains.

Alzheimer's disease; Protein kinase; Protein phosphatase; Microtubule; Paired helical filament; Phosphorylation; Tau protein

1. INTRODUCTION

Tau from Alzheimer paired helical filaments differs from normal tau by abnormal aggregation and an abnormal state of phosphorylation ([16,38]; for reviews see [24,27,42]). In order to understand the origins of the disease it is of interest to clarify the causes of the abnormal phosphorylation, that is, the protein kinases and phosphatases acting on tau, and their regulation. We have previously identified two types of kinases which phosphorylate potentially pathological sites on tau, as well as their phosphorylation sites. The first type of kinases belongs to the family of proline-directed kinases (examples are MAP kinase and GSK-3); it induces the phosphorylation of SP and TP motifs in tau. This can be recognized by diagnostic antibodies which discriminate between normal tau and PHF-tau, by an M_r shift of the protein on SDS gels, and by phosphopeptide sequencing [4,10,30]. The second type of kinases phosphorylates S262 (e.g. a new 35 kDa/41 kDa kinase) and thereby strongly decreases the interaction between tau and microtubules [5]. Both types of kinase are active in brain extracts obtained from normal or Alzheimer brains [17]. However, we noted previously that the phosphorylation by the brain extract becomes significant only when phosphatase inhibitors were added to the extract. EGTA inhibits the Ca^{2+}-calmodulin dependent phosphatase calcineurin (alias PP-2B; [35]), while okadaic acid inhibits PP-1, PP-2A, and to a lesser extent calcineurin (IC_{50} = 10 nM, 0.1 nM, 5 μM, respectively; [3,7]). We have now studied the effects of these phosphatases in more detail and conclude that calcineurin and PP-2A are both capable of removing both types of pathological phosphates from tau. These results will be compared with other studies on the dephosphorylation of MAPs in vitro [13,14,31,39] and in situ [19].

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2. MATERIALS AND METHODS

Recombinant human isoform htau40 [12] was expressed in *E. coli* and purified as described [4]. Tau construct K18 was derived from htau40 and comprised the 4 microtubule binding repeats (residues Q244–E372; [5]. Tau from PHFs was prepared from brain tissue of Alzheimer patients following [15].

2.1. *Kinases*

pp42 MAP kinase was prepared from porcine brain by sequential chromatography on Q-Sepharose, Superdex G-75 and Phenylsuperose FPLC essentially as described [10].

2.2. *Phosphatases*

PP-2A was prepared from rabbit skeletal muscle as described [21]. The specific activities of the phosphatase preparations were 2800 U/mg for PP-$2A_{T55}$, 2300 U/mg for PP-$2A_{T72}$, 16,000 U/mg for PP-$2A_D$ and 18,000 U/ml for the catalytic subunit of PP-1 (1 unit dephosphorylates 1 nmol of ^{32}P-labeled phosphorylase/ min at 30°C). Calcineurin (PP-2B) was prepared from bovine brain as described [23] with a specific activity of 15 U/mg (1 unit dephosphorylates 1 nmol of ^{32}P-

labeled substrate peptide (1 μM) per min at 30°C). PP-1 catalytic subunit was prepared according to DeGuzman and Lee [9]), inhibitors I-1 and I-2 according to Waelkens et al. [37].

2.3. *Brain extracts*

In some experiments, phosphorylation and dephosphorylation were achieved by an extract of soluble proteins from brain. For phosphorylation experiments, fresh brain tissue cleaned from meninges and blood vessels was homogenized at 0.2 g tissue per ml buffer (25 mM Tris-HCl, pH 8.0, containing 5 mM EGTA, 2 mM DTT, 0.2 mM Na_3VO_4, 40 mM NaF, 10 μM okadaic acid, 1 mM PMSF, 10 μg/ml leupetin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 0.03% Brij-35) and desalted over a disposable column (PD10, Pharmacia) equilibrated in the same buffer. For dephosphorylation experiments, the extraction buffer was 25 mM Tris-HCl, 2 mM DTT, 0.03% Brij-35 and protease inhibitors as above.

2.4. *Tau from PHFs*

PHFs were prepared after Greenberg and Davies [15]. The PHFs were solubilized with 2 M guanidine thiocyanate for 2 h at 37°C, then centrifuged at 100,000 × *g*. The supernatant was dialyzed against water and lyophilized. For dephosphorylation assays the residual was dissolved in dephosphorylation buffer (see below) and incubated for 5 h with 5 U/ml calcineurin or 20 U/ml PP-$2A_{T55}$.

2.5. *Antibodies*

Monoclonal antibody AT8 directed against Alzheimer PHFs was a gift from Dr. A. Vandevoorde (Innogenetics S.A., Gent, Belgium), mAb SMI-31 and SMI-33 from Sternberger Monoclonals Inc. (Baltimore, MD, USA), and mAb Tau-1 was a gift from Dr. L. Binder (Univ. Alabama).

2.6. *Phosphorylation assays*

Recombinant htau40 (3 μM) was phosphorylated by 0.2–0.5 units of MAP kinase per ml (1 unit being the amount of kinase that catalyzes the phosphorylation of 1 nmol of htau40 per minute). The phosphorylation buffer contained 40 mM HEPES, pH 7.2, 0.2 mM PMSF, 1 mM DTT, 2 mM EGTA, 5 mM $MgCl_2$, and 2 mM [γ-^{32}P]ATP (specific activity adjusted to 40 Ci/mol). After 16 h of incubation at 37°C, the buffer was changed to 25 mM Tris-HCl, pH 7.4, 0.1 mM DTT, 0.1 mM PMSF (dephosphorylation buffer) by gel filtration on a G25-Superfine column (3.2 × 100 mm, Pharmacia). Protein concentration was determined by the BCA assay (Sigma) and incorporated radioactivity by Cerenkov counting of gel slices.

2.7. *Dephosphorylation assays*

^{32}P-labelled htau40 (2.5 μM in the standard assay) was incubated with different amounts of phosphatase at 30°C in dephosphorylation buffer (0.5 mg/ml ovalbumin or bovine serum albumin (Serva) was added to prevent non-specific interactions). The individual conditions for the dephosphorylation experiments are indicated in the figure legends. The reaction was stopped by addition of sample aliquots to boiling SDS sample buffer. Samples were electrophoresed on 7–15% gradient gels, the tau bands excised after Coomassie staining and counted. Calcineurin was preincubated with 10 mM $MgCl_2$ and 0.2 mM DTT at 30°C for 10 min. Incubations with calcineurin contained always 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 0.1 mg/ml calmodulin (Sigma).

2.8. *Nomenclature and abbreviations*

Phosphatase nomenclature: There are several names in use for the phosphatase PP-2A [6] alias PCS (for polycation stimulated phosphatase; [37]). The enzyme occurs as a heterodimer or heterotrimer. The dimer consists of a 36 kDa catalytic subunit (C-subunit, PP-$2A_c$) and a 65 kDa regulatory subunit (A-subunit). The trimer has an additional third subunit (B-subunit) which is variable (e.g. 55, 72 or 74 kDa). The enzymes used in this study are the two trimeric forms PP-$2A_{T55}$ (= PCS-H = PP-$2A_1$) and PP-$2A_{T72}$ (= PCS-M) and the dimeric form PP-$2A_D$ (= PCS-L = PP-$2A_2$).

PP-1 [6] alias ATP, Mg-dependent phosphatase (AMD phosphatase; [37]) consists of a 37 kDa catalytic subunit which may be complexed to regulatory subunits such as inhibitor-2 (whose phosphorylation by the kinase GSK-3 alias activating factor F_A leads to the activation of the phosphatase). It is usually bound to a tissue-specific targeting subunit. In this study we have used the purified catalytic subunit, PP-1_C.

PP-2B alias calcineurin (for review see [35]) is a Ca-calmodulin activated phosphatase which consists of a 60 kDa catalytic subunit (A-subunit, CN-A) which is activated by binding of calmodulin, and a 19 kDa regulatory calmodulin-like subunit (CN-B). In this study we have used dimeric calcineurin and activated it with Ca^{2+} and calmodulin.

PP-$2A_1$ = PCS-H = PP-$2A_{T55}$ = trimeric form with 55 kDa B-subunit (A + C + B, 35 + 65 + 55 kDa)
PCS-M = PP-$2A_{T72}$ = trimeric from with 72 kDa B″-subunit (A + C + B″, 36 + 65 + 72 kDa)

PP-$2A_2$ = PCS-L = PP-2AD = dimeric form (A + C, 36 + 65 kDa)

PP-$2A_c$ = 36 kDa catalytic subunit of PP-2A

PP-2B = calcineurin (active in the form CN-A + CN – B + calmodulin)

Other abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol-*O*,*O*′-bis(2-aminoethyl ether)-*N*,*N*,*N*′-tetraacetic acid; GSK-3, glycogen synthase kinase-3; MAP kinase, p42 mitogen activated protein kinase (alias ERK2); MAP, microtubule associated protein; PHF, paired helical filament; PMSF, phenylmethylsulfonyl fluoride; OA, okadaic acid.

3. RESULTS

3.1. *Tau phosphatase activity in the brain extract*

The findings reported here were initially triggered by the observation that an extract prepared from mammalian brain possesses a kinase activity towards tau that induces the Alzheimer-like antibody reactivity (by phosphorylating mostly SP and TP motifs; [4,29]) and reduces microtubule interactions by phosphorylating Ser^{262} [5]. But it was also clear that phosphatases played an important role since efficient phosphorylation was achieved only in the presence of phosphatase inhibitors [17]. The extract by itself allowed only minimal phosphorylation (< 0.5 P_i incorporated into tau). With 5 mM EGTA (to block the Ca^{2+} dependent phosphatase calcineurin) the phosphorylation increased to ≈ 4 P_i. 10 nM OA (to block PP-2A) had no effect, nor did 10 μM OA (to block both PP-2A and PP-1). However, with 5 mM EGTA and 10 μM OA combined a level of ≈ 5 P_i was achieved, about 1 P_i more than with 5 mM EGTA alone. These data suggested that endogenous phosphatases, particularly calcineurin, were more potent than the endogenous kinases so that the Alzheimer-like phosphorylation of tau was prevented in normal brain extract.

These earlier experiments were designed to explore phosphorylation but were not optimized for studying dephosphorylation. We therefore modified the proto-

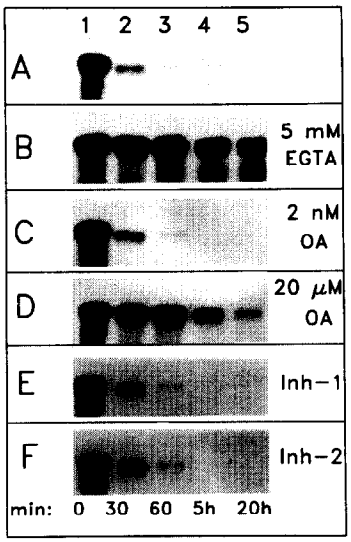


Fig. 1. Dephosphorylation of htau40 with extract from porcine brain under various conditions as indicated below. Extract from porcine brain (5 μl, total protein concentration adjusted to 1 mg/ml) was incubated at 30°C with 50 μl of htau40 (2.5 μM, phosphorylated with MAP kinase, 9.3 mol of ^{32}P incorporated per mol of tau). Aliquots were withdrawn at the indicated time intervals (O min, 30 min, 60 min, 5 h, 20 h), mixed with boiling SDS sample buffer, electrophoresed on 7–15% polyacrylamide gels and processed for autoradiography. (A) Control: dephosphorylation of tau by the brain extract without any phosphatase inhibitors added. The dephosphorylation is rapid and complete in less than an hour. (B) Addition of 5 mM EGTA to inhibit calcineurin. Phosphate remains bound to tau for 20 h. (C) and (D) Addition of okadaic acid (an inhibitor of PP-1, PP-2A, and PP-2B with IC_{50} values of 10 nM, 0.1 nM, and 5 μM, respectively, see [7]). At 2 nM OA there is no inhibition of phosphatase activity, at 20 μM it is substantial. (E) and (F) Addition of the inhibitors I-1 and I-2 of PP-1 at 0.2 μM. There is no inhibition of phosphatase activity, showing that PP-1 is not responsible for dephosphorylating tau in the extract.

col: we omitted ATP (to disable the kinases), and instead of using unphosphorylated recombinant tau as substrate we first phosphorylated it with purified MAP kinase (or GSK-3) and then observed its dephosphorylation by the brain extract. In the absence of inhibitors, dephosphorylation is nearly complete within 30 min (Fig. 1A), illustrating the presence of endogenous phosphatases. 5 mM EGTA inhibits the dephosphorylation completely (Fig. 1B), indicating that calcineurin is a major phosphatase of tau in the extract. Okadaic acid fails to stop the dephosphorylation at a concentration of 2 nM (Fig. 1C), but is an efficient inhibitor at 20 μM (Fig. 1D). Considering that the IC_{50} values are 10 nM and 0.1 nM for PP-1 and PP-2A, this would argue superficially that PP-1 is the dominant phosphatase; however, this conclusion is not stringent since PP-2A concentrations are often too high so that nM OA does not suffice to block all the enzyme (see [7,40]). In fact the experiments of Fig. 1E and F with the inhibitors I-1 and I-2 of PP-1 show that blocking this phosphatase makes no difference to the dephosphorylation of tau. These data leave calcineurin and PP-2A as two possible candidates.

3.2. *Dephosphorylation of tau by calcineurin and PP-2A*

The nest step was to test the individual phosphatases, and to see whether they freed up the abnormal phosphorylation sites. Tau was pre-phosphorylated by MAP kinase to a level of ≈10 P_i/molecule. Phosphorylation by this kinase can be recognized by four features: (i) the amount of phosphate as measured by autoradiography, (ii) the nature of the phosphorylation sites (SP or TP motifs), (iii) the shift towards higher M_r in the SDS gel, similar to the Alzheimer tau, (iv) the reaction with certain antibodies whose epitopes are sensitive to phosphorylation. Calcineurin reversed all of these changes (Fig. 2). The M_r shifted down again (Fig. 2A) and the incorporated phosphate disappeared (Fig. 2B). Significantly, the reaction with the PHF-specific antibodies disappeared (e.g. AT8, sensitive to phosphorylated S199 and/or S202, or SMI31 reacting with phosphorylated S396/S404; [29]). Conversely the reactivity with antibodies recognizing unphosphorylated SP motifs reappeared (e.g. SMI33 for unphosphorylated S235, or Tau-1 for unphosphorylated S199/S202, Fig. 2C–E). This response is complementary to what one observes when tau is phosphorylated with proline-directed kinases such as MAP kinase or GSK-3. We conclude that calcineurin can dephosphorylate all SP and TP motifs of human tau which are targets of MAP kinase.

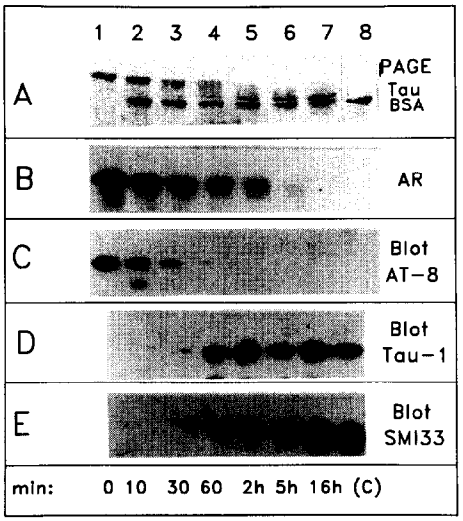


Fig. 2. (A) Time course of dephosphorylation of MAP kinase-phosphorylated htau40 with calcineurin monitored by gel shift and antibody response. ^{32}P-labeled hTau 40 (2.5 μM) containing 7.4 mol of phosphate per mole of tau was incubated with PP-2B (final concentration 130 μg/ml, corresponding to 2 U/ml). Aliquots were withdrawn after the indicated time intervals and mixed with SDS sample buffer. (A) Coomassie stained SDS gradient gel (7–15%). Note the downward shift in the gel upon dephosphorylation. The weak and constant band below tau is BSA present in the calcineurin sample. (B) Autoradiograph, showing the loss of phosphate. (C) Immunoblots of the same samples with PHF-specific mAb AT-8, showing the loss of phosphate at S199/S202. (D) Immunoblot with mAB Tau-1, showing the appearance of the dephoshorylated epitope at S199/S202. (E) Immunoblot with mAb SM133, showing the appearance of dephosphorylated S235.

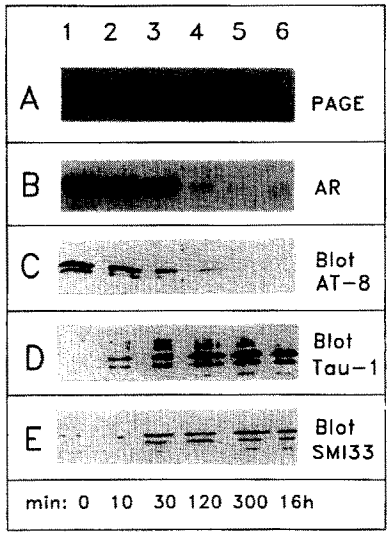
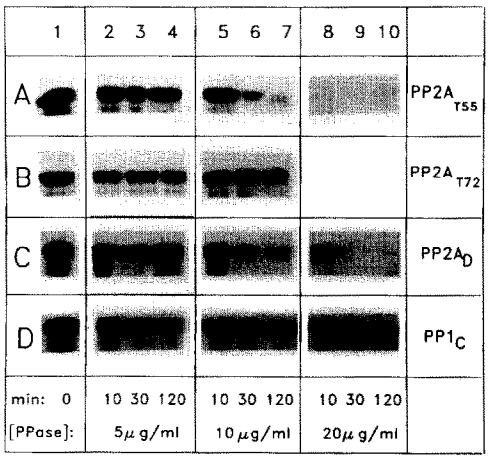
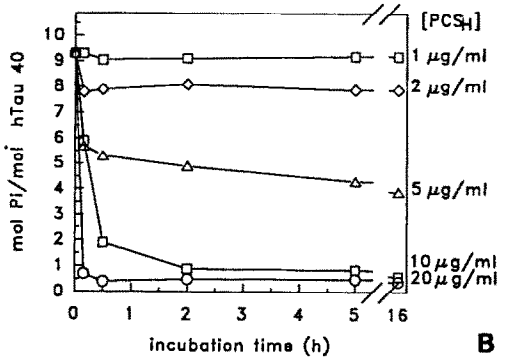


Fig. 3. (A) Time course of dephosphorylation of MAP kinase-phosphorylated Tau with PP-$2A_1$, as monitored by gel shift and antibody response. ^{32}P-labeled hTau 40 (2.5 μM) was incubated with PP-$2A_1$ (final concentration 10 μg/ml) and aliquots were withdrawn after the indicated time intervals and mixed with SDS sample buffer. (A) Coonmassie stained SDS gradient gel (7–15%). (B) Autoradiograph of the same gel. (C) Immunoblots of the same samples with PHF-specific mAb AT-8. (D) Immunoblot with mAb Tau-1. (E) Immunoblot with mAb SMI33. The phosphate content of the protein was as follows: 0 min, 9.3 mol/mol (100%); 10 min, 7.5 mol/mol (81%); 30 min, 5.6 mol/mol (60%); 120 min, 1.9 mol/mol (20%); 300 min, 1.4 mol/mol (15%); 16 h, 1.0 mol/mol (11%).

A similar picture emerges when tau protein is prephosphorylated with MAP kinase and then dephosphorylated with PP-2A (Fig. 3). While the AT8 reactivity disappears, that of Tau-1 or SMI33 reappears upon dephosphorylation (Fig. 3C–E). The other phosphorylation-dependent antibodies tested earlier with MAP kinase react in an analogous fashion (data not shown, compare Lichtenberg et al. [29] with Mandelkow et al. [30]).

PP-2A can exist in a dimeric form, PP-$2A_D$, containing the catalytic 36 kDa subunit C and the regulatory 65 kDa subunit A. This complex can take up one of several other regulatory subunits, B, B′, B″, with molecular weights between 54 and 72 kDa, to form a heterotrimer PP-$2A_T$ (for review, see [6]). We wanted to test the efficiency of these alternative complexes and studied the dimeric form PP-$2A_D$ and two different trimeric forms, PP-$2A_{T55}$ and PP-$2A_{T72}$. As shown in Fig. 4A, PP-$2A_{T55}$ was most efficient in removing the phosphates at SP or TP motifs; the time course of dephosphorylation is illustrated for several enzyme concentrations in Fig. 4b. The





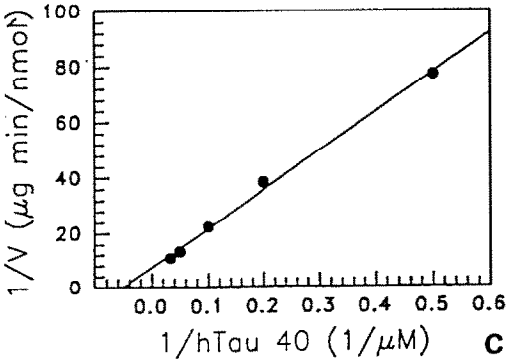


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Fig. 4. Kinetics of dephosphorylation of htau40 by PP-$2A_1$ and PP-$2A_2$ after prior phosphorylation by MAP-kinase (phosphate content 9.3 moles per mol). Methods were as in Figs. 1 and 2. (A) Tau was dephosphorylated with varying amounts of phosphatase and different time intervals as indicated, electrophoresed on 7–15% gradient gels and submitted to autoradiography. The first lane shows the phosphorylated tau before dephosporylation. The dephosphorylation times (10, 30, 120 min) and phosphatase concentrations (5, 10, 20 μg /ml) are as indicated. (A) PP-$2A_{T55}$ (PCS-H), (B) PP-$2A_{T72}$ (PCS-M), (C) PP-$2A_D$ (PCS-L). (D) PP-1, catalytic subunit. Lanes 2–4: 5 μg/ml. Lanes 5–7: 10 μg/ml. Lanes 8–10: 20 μg/ml. Time course of the dephosphorylation with different amounts of phosphatase as indicated.(B) Time course of dephosphorylation of htau40 by PP-$2A_{T55}$ as shown by phosphate content of samples withdrawn at the indicated time intervals. (C) Double reciprocal plot of the rate of the dephosphorylation of htau40 by PP-$2A_{T55}$ yields $K_m = 50$ μM, $V_{max} = 0.6$ nmol/μg/min.

dimer PP-2A$_D$ is somewhat less active (Fig. 4A, row C), and the other trimeric form PP-2A$_{T72}$ showed a much lower level of phosphatase activity (Fig. 4A, row B). This suggests that the third subunit (B) has a strong influence on what type of substrate is recognized by PP-2A. Similar observations have been made with other substrates dephosphorylated at SP or TP motifs by PP-2A isoforms (e.g. Agostinis et al. [2]). Fig. 4C shows a double reciprocal plot for PP-2A$_{T55}$ with K_m values for PP-2A$_{T55}$ and PP-2A$_D$ are in the range 25–50 μM, and V_{max} around 0.2–0.6 nmol/μg/min. By comparison, purified PP-1 has almost no phosphatase activity towards tau (Fig. 4A, row D).

An interesting detail observed with both calcineurin and PP-2A is that a large fraction of the phosphate (about 50%) is liberated quickly within about 10 min, before one observes the downward shift in the gel and the transition from Alzheimer-like to normal antibody reactivity (around 30 min in Figs. 2 and 3). Since the shift and the antibodies are mainly sensitive to the SP motifs this means that the initial dephosphorylation is mainly due to the TP motifs. Thus it appears that the phosphates on TP motifs are more labile than those on SP motifs (similar to the preference of T over S in model peptides; [1]). Note that this sequence is the reverse of the phosphorylation reaction by MAP kinase or GSK-3 where SP motifs tend to be phosphorylated before the TP motifs [17]. The experiments illustrated here were done with tau pre-phosphorylated with MAP kinase, but we note that very similar results are obtained with GSK-3. As shown previously [30], GSK-3 phosphorylates tau less efficiently ($\approx$ 4 P$_i$ incorporated) and prefers SP motifs over TP motifs, but otherwise the dephosphorylation reactions by the different phosphatases are comparable (data not shown).

3.3. *Dephosphorylation of tau's microtubule binding domain*

Thus far we dealt mainly with the dephosphorylation of phosphorylated SP and TP motifs, the ones responsible for the Alzheimer-like antibody reactivity. However, these sites have only a minor influence on tau's binding to microtubules. Instead, this depends mainly on the repeat region and in particular on S262 which is phosphorylated by a different kinase in brain [5] and is found to be phosphorylated in Alzheimer PHF tau [20]. We wished to know if the phosphatases affected that site and hence the microtubule binding. Since there are no diagnostic antibodies reacting with S262 we made use of tau construct, K18, comprising only the four repeats (equivalent to residues Q244–E372 in the htau40 sequence). In this construct, the brain kinase activity phosphorylates S262 and S356 in the two IGS motifs in the first and fourth repeat ([5]; note that the phosphorylation of S356 has no influence on microtubule binding). Fig. 5 (left) shows how the construct is phosphorylated by the brain extract and then dephosphorylated completely by calcineurin and PP-2A. For comparison, the

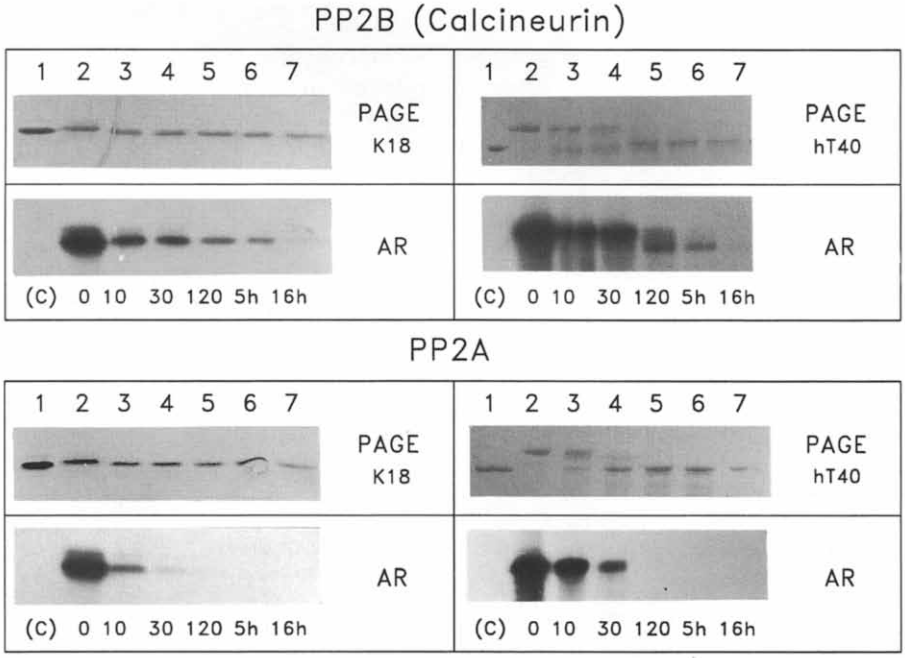

Fig. 5. Dephosphorylation of Tau with PP-2A$_{T55}$ and PP-2B after prior phosphorylation with brain extract. hT40 and K18, a tau construct containing only the repeat region, were phosphorylated with extract from fresh mouse brain and brain proteins removed by boiling. The resulting ^{32}P-labeled htau40 (2.5 μM, containing 9.0 mol of phosphate per mole) or ^{32}P-labeled K18 (8 μM, containing 2.5 mol of phosphate per mol) were incubated with PP-2A$_1$ (20 U/ml) and PP-2B (2 U/ml). Aliquots were withdrawn at the indicated time intervals, mixed with boiling SDS sample buffer and electrophoresed on 7–15% gels. Lane 1: protein before phosphorylation; lane 2: after phosphorylation with brain extract; lane 3: 10 min incubation; lane 4: 30 min; lane 5: 120 min; lane 6: 5 h; lane 7: 16 h.

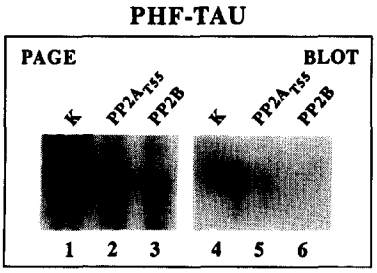

Fig. 6. Dephosphorylation of PHFs from Alzheimer brain tissue by PP-A_{T55} and calcineurin. Lanes 1–3: SDS-PAGE (silver stain); lanes 4–6: immunoblot with antibody AT-8. Lanes 1 and 4: PHF-tau reacts with AT-8; lanes 2 and 5: dephosphorylation with PP-$2A_{T55}$ cases a shift towards lower M_r and abrogates the reaction with AT-8; lanes 3 and 6: Calcineurin has the same effects as PP-2A.

same experiment is shown for htau40 (Fig. 5, right). PP-2A is more efficient than calcineurin with regard to the IGS motifs in the repeat domain, contrary to the SP or TP motifs where calcineurin is more efficient (Figs. 1 and 2). The results show that both phosphatases act on all potentially 'pathological' phosphorylation sites of tau, both by proline-directed and non-proline-directed kinase described previously.

3.4. *Dephosphorylation of PHF-tau from Alzheimer brains*

In the experiments described so far we showed that the PHF-like phosphorylation of tau induced by MAP kinase or the brain extract could be removed by calcineurin or PP-2A. It remains to be shown that PHFs from Alzheimer brains can be dephosphorylated by the same phosphatases. This is demonstrated in Fig. 6. Dephosphorylation by calcineurin and PP-$2A_{T55}$ leads to a shift of the tau isoforms towards a lower M_r (lanes 2, 3), and the reactivity with PHF-specific antibody AT-8 disappears (lanes 5, 6).

4. DISCUSSION

The phosphorylation and dephosphorylation of MAPs has been studied by a number of authors since this appeared to regulate microtubule assembly. Several protein kinases were able to phosphorylate tau, and in studying the reverse process it was reported that calcineurin and PP-2A were particularly active in dephosphorylating MAPs such as MAP2 and tau ([14,31,39]; for review, see [23]). A question not resolved by these earlier studies was the nature of the phosphorylation sites, and which sites were targeted by particular kinases and phosphatases. These issues became relevant for Alzheimer's disease research when it was discovered that tau was the main protein of Alzheimer PHFs, and that this protein was abnormally phosphorylated (for review, see [27]).

After tau was cloned and sequenced [11,12,26] it became possible to identify phosphorylation sites and epitopes of antibodies which were sensitive to phosphorylation. This led to the determination of the sites several kinases (PKA [5,33]; PKC [8]; CaMK [34]; cdc2 [25,36]). Of particular interest was the finding that certain kinases transformed tau into an 'Alzheimer-like' state, as judged by the reaction with diagnostic antibodies [4,29]. These kinases were present in normal brains, phosphorylated SP and TP motifs and thus belonged to the family of proline-directed kinases [17]. Kinases of this type were MAP kinase [10], GSK-3 [18,22,30] cdk2 and cdk5 [41]. Another type of 'pathological' phosphorylation was that of S262 which was observed in PHFs [20] and strongly reduced the interaction with microtubules, in contrast to the proline-directed kinases [5].

In this report we have used our previous knowledge about phosphorylation sites, antibody epitopes, and kinases to ask which of the 'abnormal' sites are kept unphosphorylated in a normal brain. In other words, since phosphorylation results from a competition between kinases and phosphatases, and since the kinases affecting the 'abnormal' sites were present in normal brain, the corresponding phosphatases have to be present as well. We therefore studied the phosphatase activity in brain extract by selective inhibitors, and compared this with purified phosphatases. SP or TP motifs were first phosphorylated with MAP kinase (or GSK-3), and their dephosphorylation monitored by autoradiography and phosphorylation dependent antibodies. In the case of S262 the state of phosphorylation was monitored by comparing constructs that had no SP or TP motifs (such as K18), since antibodies specific for that site were not available.

We conclude that both types of abnormal phosphorylation sites can be dephosphorylated by calcineurin and PP-2A, with the trimeric form PP-$2A_{T55}$ (alias PP-$2A_1$, PCS-H) being the more active than other trimeric or dimeric forms (PP-$2A_{T72}$ = PCS-M, PP-$2A_D$ = PP-$2A_2$ = PCS-L, Fig. 4). This suggests a strong regulatory function of the B subunit of PP-2A. By contrast, PP-1 is inactive. These results are based on the experiments with purified phosphatases. However, in the brain extract, calcineurin appears to be the more active phosphatase.

This can be illustrated in two ways. We had shown

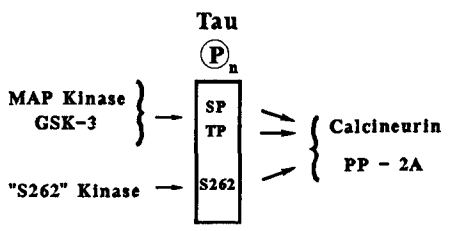

Fig. 7. Diagram showing the two types of abnormal phosphorylation sites on tau protein (S/T-P or S262), the kinases phosphorylating these sites (MAP kinase/GSK-3 or S262 kinase), and the two phosphatases calcineurin and PP-2A which counteract the phosphorylation of these sites.

previously [17] that phosphorylation of tau by the brain extract (containing the endogenous MAP kinase, GSK-3, and other kinases) was possible only by inhibiting the phosphatases with the Ca^{2+} chelator EGTA and with okadaic acid. EGTA was more important than OA; EGTA alone allowed phosphorylation of tau up to 4 P_i, OA alone had no effect (no phosphorylation since the phosphatase activity was too high), EGTA plus OA allowed a somewhat higher phosphorylation than EGTA alone (5 P_i). These results argue that calcineurin is the main antagonist of MAP kinase or GSK-3 in the brain extract.

In this paper we started from defined initial conditions since we phosphorylated tau first by purified MAP kinase and then observed the activity of the brain extract (Fig. 1). With no inhibitors added, the phosphates were removed quickly; EGTA was completely inhibitory, while OA even at μM concentrations was only partially inhibitory. Our interpretation is again that calcineurin is the predominant phosphatase of the sites targeted by MAP kinase. When calcineurin is inhibited by EGTA, neither PP-1 (which is inactive anyway towards tau, see above) nor PP-2A can dephosphorylate tau. When PP-2A is inhibited by OA at sub-μM levels, there is still full phosphatase activity. It takes OA levels around 10 μM to observe partial inhibition. We suspected earlier [17] that this would point to a role of PP-1 (since PP-1 requires higher concentrations of OA for inhibition than PP-2A), but we note now that this may well be due to the partial inhibition of calcineurin by OA since the IC_{50} concentration is about 5 μM [3].

The question of phosphatases that remove the MAP kinase target sites from tau has been addressed by Goedert et al. [13]. They concluded that PP-1 was inactive and PP-2A was active, in agreement with our observations. However, they found calcineurin to be ineffective as a phosphatase, in contrast to our conclusions. Other authors had studied more generally the dephosphorylation of MAPs such as MAP2 or tau which share a similar protein character and microtubule binding region [28]. For example, Goto et al. [14] reported on the MAP-phosphatase activity of calcineurin, and Yamamoto et al. [39] described similar results for PP-2A. Since the protein sequences and phosphorylation sites were not known at the time the data could not be interpreted in detail. Now we know that these two phosphatases act as scavengers to keep sites clean that might otherwise appear as phatological sites in ageing neurons. There are, however, differences in efficiency: PP-2A acts more rapidly than calcineurin on the phosphorylated IGS motifs in the repeat region, while the opposite is true for the SP or TP motifs.

Finally, we note that calcineurin and PP-2A also act on PHF-tau isolated from Alzheimer brains. The phosphorylation sites of PHF-tau have been partially mapped, they include Ser^{262} and several SP or TP motifs [20,32]. Thus the phosphorylation state resembles that achieved by the experiments with brain extract [17] and explains why the same phosphatases can dephosphorylate PHFs. A similar state of phosphorylation can be achieved in situ by incubating brain slices with OA; here, too, the effect can be reversed primarily by calcineurin [19].

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