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Abnormal Alzheimer-like phosphorylation of tau-protein by cyclin-dependent kinases cdk2 and cdk5

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We have shown earlier that certain proline-directed kinases such as MAP kinase or GSK-3 can phosphorylate tau protein in an abnormal manner reminiscent of tau from Alzheimer paired helical filaments [Drewes et al. (1992); Mandelkow et al. (1992)]. Both kinases are abundant in brain tissue and associate physically with microtubules through several cycles of assembly and disassembly. In this report we show that cdk2/cyclinA incorporates ≈5 P_i into recombinant tau, and that it also induces the M_R shift and antibody reactivity typical of Alzheimer tau. However, since there is no cdk2 in brain [Meyerson et al. (1992)] we looked for other members of this family of kinases. Using an antibody against the conserved N-terminus we isolated a cdk-like kinase from brain which was capable of inducing the Alzheimer-like characteristics in tau by phosphorylation. Its size (31 kDa), target specificity (proline-directed), chromatographic behavior, and abundance in brain suggest that this kinase is similar or identical to the neuronal cdc2-like kinase nclk alias PSSARLE or cdk5 [Hellmich et al. (1992); Meyerson et al. (1992); Xiong et al. (1992); Tsai et al. (1993)]. This was confirmed by an antibody specific for cdk5. Like MAP kinase and GSK-3, this kinase is physically associated with microtubules and can be enriched by cycles of microtubule assembly and disassembly. Thus, cdk5 should be regarded as another kinase that could be held responsible for the changes in tau protein during Alzheimer disease progression.

Alzheimer's disease; Kinase; Microtubule; Paired helical filament; Phosphorylation; Tau protein

1. INTRODUCTION

Tau protein, a microtubule-associated protein, is the main component of the pathological paired helical filaments (PHFs) found in the brains of Alzheimer patients. PHF tau, in contrast to normal tau, is anomalously phosphorylated, suggesting that phosphorylation defects may be involved in Alzheimer's disease (for review, see [19,23,50]). Our previous studies on tau phosphorylation have yielded the following results. (a) A kinase activity can be prepared from normal or Alzheimer brains which phosphorylates mostly SP or TP motifs in the presence of the phosphatase inhibitors okadaic acid (for PP-2A) and EGTA (for calcineurin) [11]. (b) An 'Alzheimer-like' state of tau protein can be defined by antibodies which discriminate between normal and PHF tau and which react with SP motifs in a phosphorylation-dependent manner [2,25]. (c) Phosphorylation at SP and TP motifs of tau can be achieved by the proline-directed kinases MAP kinase or GSK-3 [8,26]; these kinases also induce the 'Alzheimer-like' antibody reactivity. These results were broadly in agreement with those of several other groups pointing to the importance of proline-directed kinases acting on tau (e.g. [12,15–17,21,33,35,45]).

These data suggested that MAPK or GSK-3 might be involved in the dysregulation of tau's phosphorylation, but they did not exclude the possiblity that other kinases might be equally important. In our experiments the cell cycle kinase cdc2/cyclin A or B (also a proline-directed kinase) phosphorylated tau only with low efficiency [8], but one could argue that the negative results on cdc2 might not be very meaningful since this kinase regulates the entry into mitosis which does not occur in differentiated neurons. In this report we describe the ability of other cyclin dependent kinases (cdk's) to phosphorylate tau. We notice that cdk2/cyclin A, a kinase regulating the G1 and S phases in vertebrate cells [41], was able to evoke the Alzheimer-like antibody reactivity. However, this kinase is present in many tissues but not in brain [28] and therefore seemed unlikely to affect tau in vivo. This prompted us to look for cdc2-like kinases in brain that would also phosphorylate tau in an abnormal fashion. This resulted in the isolation of a kinase of

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Abbreviations: CaMK, calcium-calmodulin dependent protein kinase; cdk, cyclin dependent kinase; EGTA, ethylene glycol bis(2-amino ethyl ether)-*N,N,N',N'*-tetraacetic acid; GSK-3, glycogen synthase kinase-3; FPLC, fast protein liquid chromatography; MAP(s), microtubule-associated protein(s); MAPK, mitogen activated protein kinase; PAGE, polyacrylamide gel electrophoresis; PHF, paired helical filament; PMSF, phenylmethylsulfonyl fluoride; PIPES, piperazine-*N,N'*-bis(2-ethane sulfonic acid); PKA, protein kinase A; PKC, proteinkinase C; SDS, sodium dodecyl sulphate; Tris, Tris-(hydroxymethyl)aminomethane.

$M_r \approx 31$ kDa which induces the Alzheimer-like state very efficiently when complexed to a 25 kDa cofactor, and in addition is bound to microtubules and Alzheimer PHFs, making it a particularly interesting kinase for studying the pathological phosphorylation of tau. Judging by biochemical properties this kinase is similar if not identical to nclk, alias PSSALRE or cdk5, a relative of cdc2 which is abundant in brain [14,24,28,37,42,49].

2. MATERIALS AND METHODS

The methods used here have been described in recent publications so that only some key points will be repeated. Recombinant human tau proteins were derived from the cDNA clones of Goedert et al. [9] and expressed in *E. coli* [2], using derivatives of the pET expression vector [40]. Point mutants were made by PCR. Phosphorylation by the brain kinase activity was done following Gustke et al. [11]. Fig. 1A shows a diagram of the domain structure of htau40 and its main phosphorylation sites.

Antibodies against tau were obtained from Dr. L. Binder (TAU1, [4]), Innogenetics S.A., Ghent, Belgium; (AT8 [27]), and Sternberger Monoclonals Inc., Baltimore, MD (SMI 31, 33, 34; Sternberger et al. [39]). We had previously determined their epitopes [2,25]. TAU1 and SMI33 react with normal (or recombinant unphosphorylated) tau, AT8, SMI31 and SMI34 react with Alzheimer PHFs, or with tau phosphorylated by MAP kinase or the brain extract (Fig. 1A). An antibody against the conserved N-terminal region of cdc2 was obtained from UBI (Lake Placid, NY). The antibody against cdk5 was provided by Dr. L.-H. Tsai (MGH Cancer Center).

2.1. *Purification of 31 kDa kinase*

Fresh porcine brains obtained from the local slaughter house were cleaned from meninges and blood vessels. 500 g of tissue was homogenized at 4°C in 500 ml of buffer containing 25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 20 mM NaF, 2 mM EGTA, 2 mM DTT, 2 mM $MgCl_2$, 0.2 mM Na_3VO_4, 0.1% Nonidet P 40, 2 mM MgATP, 2 mM benzamidine, 0.5 mM PMSF, 2 μg/ml leupeptin, pepstatin and aprotinin and centrifuged at 100,000 × *g* for 60 min. The supernatant was pooled and clarified by another centrifugation step and then applied onto a Q-Sepharose column (200 ml; Pharmacia) equilibrated with buffer A (50 mM Tris-HCl, pH 8.0, containing 2 mM EGTA, 0.5 mM DTT, 3 mM $MgCl_2$, 0.2 mM Na_3VO_4, 0.1% Nonidet P40, 2 mM MgATP, 1 mM benzamidine,, 0.5 mM PMSF, 2 μg/ml leupeptin, pepstatin and aprotinin). The pH of the flow-through fraction was adjusted to 6.5 with 0.5 M MES and then applied onto a S-Sepharose column (50 ml, Pharmacia) equilibrated with buffer B (25 mM MES-NaOH, pH 6.0, containing 2 mM EGTA, 0.5 mM DTT, 3 mM $MgCl_2$, 0.2 mM Na_3VO_4, 0.1% Nonidet P40, 2 mM MgATP, 1 mM benzamidine, 0.5 mM PMSF, 2 μg/ml leupeptin, pepstatin and aprotinin). Bound protein was eluted with a gradient of 0–500 mM NaCl in buffer B at a flow rate of 5 ml/min in 100 min. The fractions (25 ml) were assayed by Western blotting with anti-cdc2 or anti-cdk5 antibody. Active fractions were pooled and dialysed against buffer B (pH 6.5) and then loaded on a SP-Sepharose HP column (10 ml; Pharmacia) equilibrated with the same buffer. Elution was performed stepwise with 0–500 mM NaCl (each step 25 mM for 2.5 min) at a flow rate of 4 ml/min. Anti-cdc2 (or anti-cdk5) positive fractions (10 ml) were pooled, dialysed against buffer A (pH 8.4) and then applied onto a Mono-Q HR 10/10 FPLC column (Pharmacia) equilibrated with the same buffer. Elution was carried out with a linear gradient from 0–500 mM NaCl at a flow rate of 2 ml/min over 80 min. Anti-cdc2 or -cdk5 positive fractions were pooled and dialysed against buffer A (pH 8.25). This pool was loaded on a Mono-Q HR 5/5 (Pharmacia) equilibrated with buffer A (pH 8.25) and bound protein was eluted with a stepwise gradient from 0–300 mM NaCl (each step 30 mM for 2 min) in the same buffer over 20 min at a flow rate of 0.5 ml/min.

Fractions (1 ml) positive on Western blots were assayed for their phosphorylation activity of tau protein or p34 substrate peptide (SV 40 T antigen peptide, Gibco BRL). Active fractions were submitted to gel filtration on a G200 Superdex HR 16/60 column (Pharmacia) equilibrated with buffer A (pH 7.4) containing 150 mM NaCl and eluted with the same buffer at a flow rate of 0.3 ml/min. Fractions (2 ml) were assayed for their phosphorylation activity of p34 substrate peptide and active fractions were pooled and dialysed against buffer A (pH 8.25) followed by ion exchange chromatography on a Mono-Q 1.6/6 Smart column (Pharmacia) equilibrated with the same buffer. Bound protein was eluted with gradient from 0–300 mM NaCl in the same buffer over 20 min at a flow rate of 0.1 ml/min. Active and anti-cdk5 positive fractions (0.1 ml) were pooled and quickly desalted on a PD 3.2/10 column (Pharmacia) equilibrated with buffer B (pH 6.5) and then applied onto a Mono-S 1.6/6 Smart column (Pharmacia). Elution was performed with a gradient of 50–250 mM NaCl in buffer B (pH 6.5) over 10 min and 0.1 ml fractions were collected. All steps were carried out at 4°C. The purified protein fractions were checked at each step with antibodies against MAP kinase and GSK-3 to ascertain that these kinases were no longer present. Phosphorylation assays were performed after each chromatography step.

Purification of cdk2/cyclin A (GST fusion protein) from baculovirus transfected Sf9 insect cells was done by precipitation with glutathione-agarose beads, as described elsewhere [31].

2.2. *Phosphorylation of tau*

0.4 μl of kinase fractions were added to aliquots of a solution of htau34 or other tau constructs (0.2 mg/ml) in 40 mM HEPES, pH 7.4, containing 2 mM [γ-^{32}P]ATP (25–50 Ci/mol), 3 mM $MgCl_2$, 5 mM EGTA, 2 mM DTT, 0.5 mM PMSF, to a final folume of 4 μl. After incubation at 37°C for different time intervals, the reaction was stoppen by addition of SDS-PAGE sample buffer. After electrophoresis on 7–15% gradient gels and staining with Coomassie blue, tau bands were excised and the incorporated radioactivity was determined by Cerenkov counting.

2.3. *Phosphorylation of p34 substrate peptide*

0.5 μl of kinase fractions were added to aliquots of a solution of the substrate peptide (0.5 mM) in 50 mM Tris-HCl, pH 7.4 containing 0.1 mM [γ-^{32}P]ATP (1 Ci/mmol), 2 mM $MgCl_2$, 1 mM DTT, 5 mM EGTA, 0.5 mM PMSF to a final volume of 5 μl. After incubation at 37°C for 30 min, the reaction was stopped by addition of an equal volume of 20% TCA. Samples were centrifuged for 5 min at 14,000 × *g* to remove protein. 8 μl of the supernatant was spotted on P81 cellulose paper disks (Gibco BRL) and washed four times with 0.1 M phosphoric acid. The disks were dried and radioactivity was determined by Cerenkov counting.

3. RESULTS

3.1. *cdk2 transforms tau into an Alzheimer-like state*

The criteria which we have used for judging the abnormal phosphorylation state of tau were developed in earlier studies (see [11,25,50]). They include a shift in the SDS gel in several stages, quantification of incorporated phosphate, sequencing of phosphopeptides, and reaction with diagnostic antibodies whose epitopes and phosphorylation dependence are known, and which discriminate between normal tau and PHF-tau. These antibodies recognize SP motifs, either in the phosphorylated or unphosphorylated state (Fig. 1A). In addition, we synthesized several tau constructs, derived from the isoform htau23, where critical SP and TP motifs were mutated into AP as shown in Fig. 1B. They include the constructs AP11 (all 6 SP motifs of htau23 mutated into

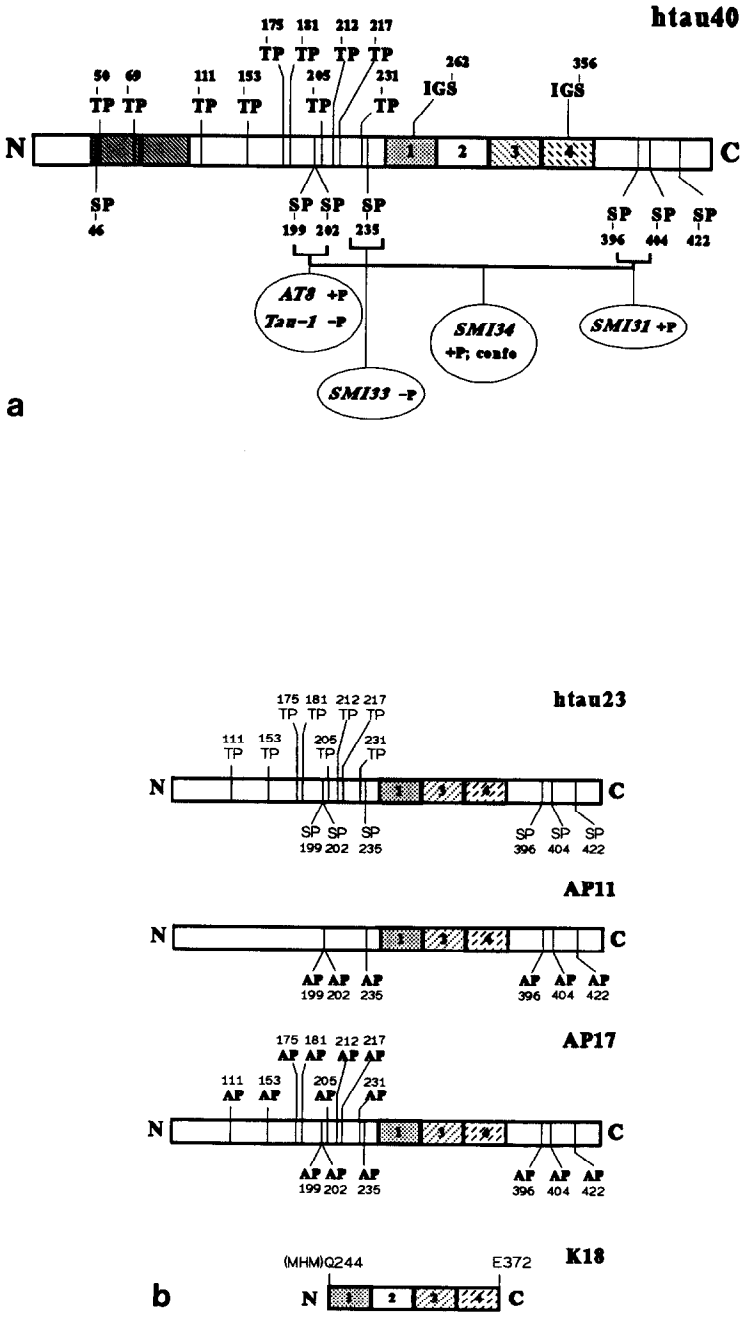


Fig. 1 (A) Bar diagram of htau40, the largest isoform of human tau in central nervous tissue, showing the location of all Ser-Pro and Thr-Pro motifs and the epitopes of several diagnostic antibodies. The numbering follows that of Goedert et al. [9]. The two hatched boxes near the N-terminus (29 residues each) are inserts that may be absent in smaller tau isoforms. The four boxes in the C-terminal half indicate the microtubule-binding repeats; repeat 2 may be absent. MAP kinase can phosphorylate all Ser-Pro or Thr-Pro motifs [8]. The main sites phosphorylated by the brain extract after 20 h are Ser^{46}, Ser^{199}, Ser^{202}, Ser^{235}, Ser^{396}, Ser^{404}, Ser^{422}; Thr^{181}, Thr^{212} (all within Ser-Pro or Thr-Pro motifs), and Ser^{262} and Ser^{356} (in Ile-Gly-Ser motifs; see [11]). GSK-3 phosphorylates preferentially the Ser-Pro motifs [26]. The diagnostic antibodies shown recognize Ser-Pro motifs in phosphorylated or unphosphorylated form (+P or −P [2,25]). (B) Diagram of htau23 and tau constructs used in this study. From top to bottom: htau23 (the smallest tau isoform, lacking repeat no. 2 and the two N-terminal inserts), AP11 (all six SP before and after the repeats changed into AP), AP17 (all 14 SP or TP motifs of htau23 changed to AP), K18 (the four repeats, Q244-E372, which contain no SP or TP motifs).

AP) and AP17 (all 6 SP and 8 TP motifs mutated into AP, see [3]).

The phosphorylation of tau by cdk2 is illustrated in Fig. 2. The M_r shifts up with time in several stages, very similar to our earlier observations with the brain kinase activity, MAP kinase and GSK3 (Fig. 2_1). Autoradiography (Fig. 2_2) shows the incorporation of phosphate (typically 5–6 P_i for htau23). The diagnostic antibodies AT8, TAU-1, SMI31, SMI34, and SMI33 react to phosphorylation similarly as with MAPK and GSK-3, indi-

cating that SP motifs before the repeat region (S199 and/or S202, S235) and after the repeats (S396, S404) become phosphorylated (Fig. 2_3–2_7); note that AT8, SMI31, and SMI34 react with PHFs where the epitopes containing SP motifs are phosphorylated, while TAU-1 and SMI33 react with normal tau where the epitopes are not phosphorylated. This illustrates the characteristic transformation of tau into the 'Alzheimer-like' state.

In spite of these similarities between MAPK, GSK-3 and cdk2, differences become apparent when one compares the tau mutants. As described earlier, MAPK phosphorylates essentially all SP and TP motifs while GSK-3 affects mainly the SP motifs. This has little effect on the antibody reactions (since these depend mainly on SP motifs; Fig. 1A), but it can be seen from the total P_i incorporated. In the case of cdk2, we find ≈ 5.5 P_i in htau23 (which contains 6 SP and 8 TP motifs), 4.5 P_i in AP11 (no SP, 8 TP), but only 0.6 P_i in AP17 (no SP, no TP), and 0.3 P_i in the repeat domain K18 (no SP, no TP). The comparison of total phosphate incorporated with the available SP or TP motifs shows that cdk2 targets preferentially the TP motifs (i.e. the opposite of GSK-3), but SP motifs are also affected as shown by the antibody reactions. In the case of cdk5, we find ≈ 4.2 P_i in htau23, otherwise its effect on SP or TP sites tends to be similar to that of cdk2.

3.2. *Preparation of a 31 kDa tau-kinase from brain and identification as cdk5*

The above results demonstrated that cdk2 phosphorylated tau protein at all SP motifs sensitive to the diagnostic antibodies, and many TP motifs as well. Since cdk2 is present in many tissues but not in brain we asked whether a similar kinase was present in brain cells and would qualify as a cause for abnormal phosphorylation. We developed a purification scheme which was based on two criteria. One was the kinase activity towards tau protein, as judged by P_i incorporation, gel shift, and reaction with the diagnostic antibodies. The second was the reaction with the cdc2 antibody (UBI) directed against the N-terminal region of cdc2 which is conserved in all cdk's known so far.

As shown in Fig. 3, several ion exchange steps followed by gel permeation chromatography resulted in the purification of a single band at 31 kDa (Fig. 3A) which reacted with the cdc2 antibody. The kinase activities eluting from the ion exchange columns were high (up to 6 P_i incorporated into tau in 16 h). Surprisingly, however, the kinase activity almost disappeared during gel chromatography so that the highly purified kinase (Fig. 3A, lane 7) incorporated only < 1 P_i into tau. This suggested that a crucial factor was lost in the last step.

Since most cdk's are active only in complexes with their regulatory subunits (cyclins) we modifed the purification to find the putative cofactor, using an additional antibody specific for the cdk variant most prominent in brain (cdk5). The first five steps of the modified

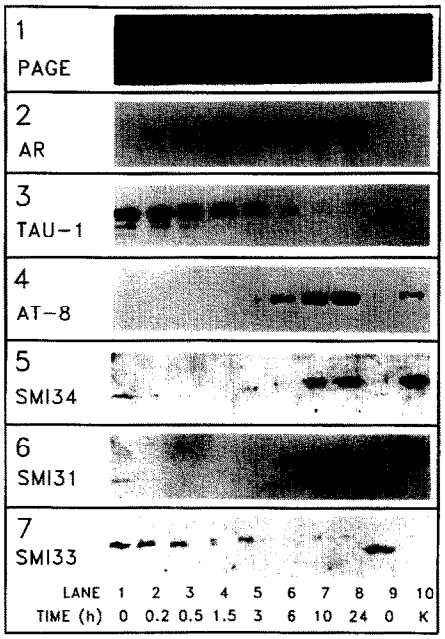


Fig. 2. Time course of phosphorylation of htau40 by cdk2 and immunoreactivity. (1) SDS-PAGE of htau40 after incubation with the kinase between 0 and 24 h at 37°C. The minor lower band in lane 1 is a fragment. Note the progressive shift to higher M_r values, similar to the effects of brain extract and MAP kinase. (2) Autoradiography. (3) Immunoblot with the antibody TAU1 whose reactivity is lost after ≈ 2 h (following the phosphorylation of S199 and/or S202). (4) Immunoblot with the PHF-specific antibody AT8 whose reactivity requires the phosphorylation of S199 and/or S202. (5) Immunoblot with antibody SMI34 (conformation senstive and against phosphorylated SP motifs before or after the repeats). (6) Blot with SMI31 (epitope includes phosphorylated S396 and S404). (7) Blot with antibody SMI33 which requires a dephosphorylated S235.

procedure were the same as above, and showed that the 31 kDa band reacted not only with the cdc2 antibody but also with the cdk5 antibody (Fig. 3B). After ion exchange chromatography on Mono-Q HR 5/5 we observed two major peaks (fraction 7–9 and 10–12, according to elution with ≈150 mM and 210 mM NaCl) as determined by the anti-cdk5 antibody (Fig. 3B). Although both fractions were capable of phosphorylating tau (not shown), the activity towards the p34 substrate peptide was mainly detected in the second peak (Fig. 3B). Applying fraction 7 of the first peak to gel filtration G200 Superdex yielded a fraction eluting from the column at ≈ 30 kDa containing a single band on SDS-PAGE at ≈ 31 kDa (not shown, similar to Fig. 3A, lane 7). However, this fraction showed only little activity in phosphorylating tau even after several-fold concentration (< 1 mol P_i/mol tau) and p34 substrate peptide (< 300 cpm).

On the other hand, fraction 10 of the second peak of the Mono-Q HR 5/5 when submitted to the same gel filtration procedure yielded several fractions that showed immune reactivity reactivity against cdk5 but not against MAP kinase or GSK-2 (Fig. 3A, lane 8) which eluted in the range of 50–70 kDa and showed

phosphorylating activity towards the p34 substrate peptide (Fig. 3C). These fractions (13–18) were pooled and applied onto a Mono-Q 1.6/6 Smart column (Fig. 3D, lane 1). The positive and active fractions eluted mainly in the range of 160–200 mM NaCl and were further purified by ion exchange chromatography on a Mono-S 1.6/6 Smart column (Fig. 3D, lanes 2–5). This step yielded anti-cdk5 positive fractions eluting in the range of 200–220 mM NaCl. These fractions (7–9) contained several protein bands, including two with $M_r \approx 25$ and 31 kDa (Fig. 3D, lanes 3–5) and were capable of phosphorylating htau40, incorporating 3.2, 4.2 and 2.9 mol P_i per mol htau40, respectively, and inducing an M_r shift of htau40 in SDS-PAGE. Correspondingly, these fractions show high phosphorylation activity towards the p34 substrate peptide.

In conclusion, this cdc2-like kinase activity towards tau occurs in brain tissue and has an M_r of 31 kDa. These features are suggestive of other cdc2-like kinases cloned recently from brain, termed nclk [14], PSSARLE [28], or cdk5 [42,49]. Indeed, the 31 kDa protein reacted with an antibody specific for cdk5. Thus, the kinase isolated here is very similar if not identical to cdk5. We find that this kinase shows good activity towards tau only when complexed with another protein of M_r 25 kDa, presumably a regulatory subunit analogous to one of the cyclins. The nature of this protein is not known at present, but its molecular weight is distinctly smaller than the known cyclins (as noted by Lew et al. [24]).

3.3. *cdk5 Induces the Alzheimer-like immunoreactivity of tau and is bound to microtubules and PHFs*

Given the above results it remains to be shown that the 31 kDa kinase indeed transforms tau into the Alzheimer-like state and is associated with brain microtubules. These points are illustrated in Figs. 4 and 5. The reaction with the diagnostic antibodies (Fig. 4) is similar to the examples shown previously for cdk2 (Fig. 2), MAP kinase [8], or GSK-3 [26], indicating the phosphorylation of the SP motifs for which these antibodies are sensitive (serines 199, 202, 235, 396, 404; see Fig. 1).

The association with microtubules can be shown by cycles of temperature-dependent microtubule assembly and disassembly. This is the classical method for copurifying microtubule-associated proteins, including the structural MAPs and microtubule-bound enzymes [46]. Fig. 5 shows that the 31 kDa kinase is included in the MAP fraction after three or more cycles of microtubule assembly (lanes 1–6), and is also associated with PHFs (lane 7). In this regard, cdk5 is similar to PKA [30], MAP kinase, and GSK-3 [12,17,26], but distinct from PKC or CaMK which do not copurify with microtubules [34]. Thus, we now know three kinases, cdk5, MAP kinase, and GSK-3, all of which are present in neurons, which are capable of transforming tau into the Alzheimer-like state, and therefore could account for the pathological phosphorylation of tau in Alzheimer brains.

4. DISCUSSION

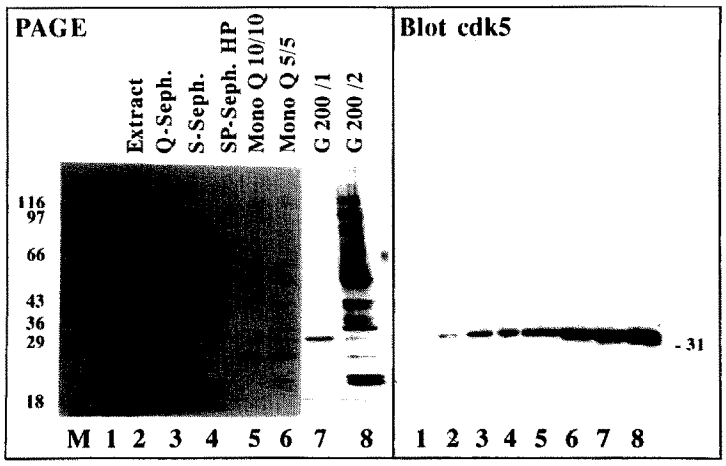
Tau protein is the major component of the paired helical filaments of Alzheimer's disease. It is therefore of considerable interest to find out in what way the PHF- tau differs from normal tau. There are several criteria for assessing the difference. (a) PHF tau is aggregated in an abnormal fashion; this effect is probably based on the repeat domain of tau [47,48]. (b) PHF tau has an abnormally high M_r due to phosphorylation [6,10]. An M_r shift can be induced by kinases affecting S/T-motifs (e.g. MAP kinase, GSK-3, [8,12,17,26]), and in this sense the criterium of M_r shift coincides with the criterium based on PHF-specific antibodies. However, an M_r shift can also be induced at other sites whose relationship to the Alzheimer-like state is not clear (e.g. CaMK [1,38]; PKA, [3,36]). (c) PHFs contain full length tau molecules [18], but also tau abnormally cleaved after E391 [29]. (d) PHF tau is obviously no longer bound to microtubules, and it is generally thought that Alzheimer tau binds less tightly to microtubules (e.g. [5]). It has been difficult to verify this point rigorously. In our experience, most of the well-known kinases have only a weak effect on tau's affinity for microtubules in vitro. Exceptions are the brain extract kinase activity and a 35 kDa/41 kDa kinase, both of which phosphorylate S262 and thereby strongly reduce the stoichiometry [3]. This kinase has no influence on the reaction with PHF-specific antibodies tested so far, nor on the M_r shift; however, it is interesting because S262 is also phosphorylated in PHFs [13]. (e) Finally, abnormal phosphorylation of tau can be detected by antibodies which distinguish between PHF-tau and normal tau in a phosphorylation-dependent manner (as in Fig. 2). The antibodies we have tested so far recognize SP motifs either in a phosphorylated or unphosphorylated state [2,25]. (Note that there are also PHF-specific antibodies that do not depend on phosphorylation but rather on some conformation or abnormal proteolysis, such as mAB Alz50 [20] or mAb 423 [29]).

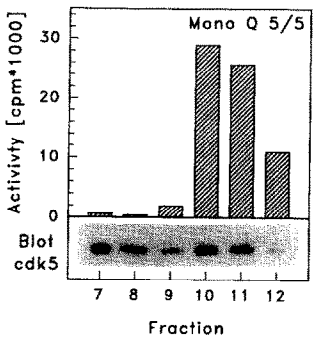
This prompted us to look for proline-directed kinases, leading the way to MAP kinase [8] and GSK-3 [26]. MAP kinase incorporates up to 15 P_i into tau and phosphorylates all SP and TP motifs (17 in htau40, 14 in htau23, see Fig. 1). GSK-3 is more restricted; both isoforms (α and β) incorporated up to 3–4 P_i, mainly at SP motifs. Since the diagnostic antibodies react with SP-containing epitopes, MAP kinase and GSK-3 generate very similar immunoreactivities in blots. By comparison, the major phosphorylation sites we found for the brain extract include all SP motifs, two TP motifs, and the non-SP sites S262 and S356 (targets of the 35 kDa/41 kDa kinase).

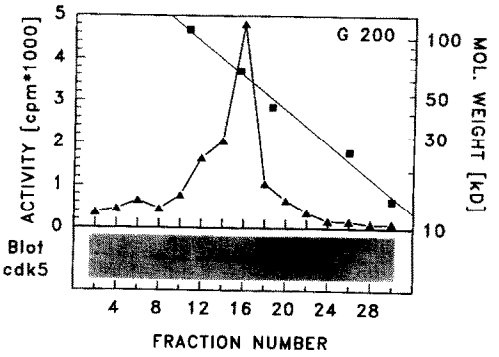
One of the best known proline-directed kinase is cdc2

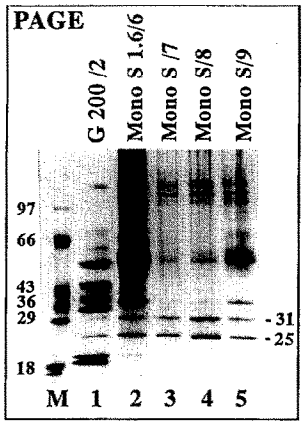
which controls the cell cycle at the G2-M transition. This kinase can also affect the dynamic instability of microtubules, probably by phosphorylating one of the MAPs [43]. Although this kinase phosphorylates tau to some extent it does not induce the full PHF-like antibody reaction or gel shift [8,35,45]. It seemed therefore that this kinase was not involved in pathological phosphorylation, an assumption made plausible by the fact that neurons do not undergo mitosis anyway. However, cdc2 has relatives, the cdk family of kinases (for review see [28]). When we tested cdk2 it became clear that this kinase was capable of phosphorylating tau in an abnormal manner at the SP motifs 199, 202, 235, 396 and 404, judging from the Alzheimer-like antibody reaction, as well as many of the TP motifs.

Nevertheless, even cdk2 seemed an unlikely candidate for a pathologically active kinase since it occurs in most tissues except in brain. We therefore looked for cdk-like









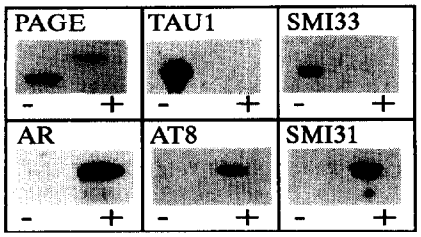


Fig. 4. Phosphorylation of tau by cdk5 purified from brain. This experiment is analogous to Fig. 2 for cdk2, but only the time points 0 (no phosphorylation −) and 16 h (phosphorylation, +) are shown. PAGE, SDS-PAGE gel; AR, autoradiography, TAU1, AT-8, SMI33, SMi31, immunoblots with antibodies.

kinases from brain (using initially the commercial cdc2 antibody) and tested their effect on tau. This resulted in the purification of the 31 kDa kinase shown in Fig. 3. It incorporated ≈ 4 P_i into tau, the targets were SP and TP motifs, and evoked the reaction with the diagnostic antibodies. The properties of this kinase were very similar or identical to the neuronal cdc2-like kinase nclk, also known as PSSARLE or cdk5 [14,28,42,49]. In particular the kinase required a 25 kDa protein as cofactor for full activity, as described by others (e.g. [24]). The similarity of the kinase was confirmed by the reaction with an antibody specific for cdk5.

Altogether, we have now defined four classes of kinases that are present in neurons, phosphorylate tau in an abnormal manner, and could thus play a role in the genesis of Alzheimer's diseases. Three of these classes, represented by MAPK [8], GSK-3 [12,26], and cdk5 (this report) are proline-directed kinases which phosphorylate SP and/or TP motifs and induce the PHF-like conformation recognized by the diagnostic antibodies. The kinases are regulated by different phosphorylation cascades and respond to different extracellular signals, but are probably linked at common intermediate points (for review, see [7]). The fourth kinase (35/41 kDa) is not proline-directed, it phosphorylates S262 and is the only one that has a major effect on tau's binding to microtubules in our hands. The interplay between the kinases

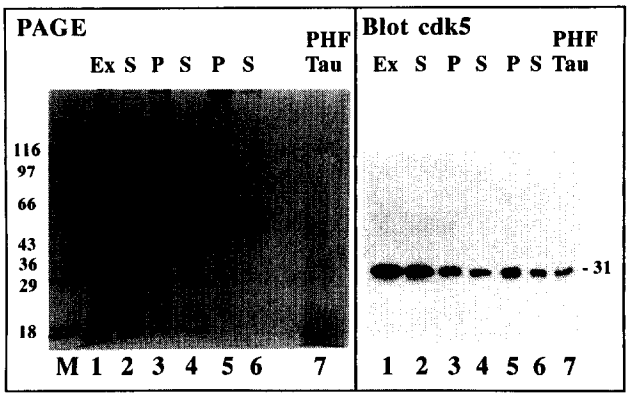


Fig. 5. Copolymerization of cdk5 with porcine brain microtubules. (A) SDS gel of microtubule purification stages. Lane 1, Ex = brain extract = supernatant after first cold spin at 4°C. Lane 2, S = supernatant of first hot spin = tubulin and MAPs not assembled into microtubules after warning to 37°C; Lane 3, P = pellet of redissolved microtubules. The other lanes (4–6, S, P, S) show two further cycles of assembly and dissassembly by temperature shifts. Lane 7, PHF-tau prepared prepared according to Wischik et al. [48]. (B) Blot with anti-cdk5 antibody, showing the 31 kDa kinase. It shows that the kinase copurifies with microtubules and is associated with PHFs.

and the corresponding phosphatases in the genesis of AD remains to be established. As shown elsewhere [51], both calcineurin and phosphatase-2A can clear the sites affected by the kinases mentioned above. It may be significant that the three proline-directed kinases can be purified as microtubule-associated proteins and are present in neurofibrillary tangles, suggesting that MAPs act as anchors for the kinases and thus are potential targets for them.

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Fig. 3. Purification of cdk5 from brain. (A) Left panel: SDS-PAGE after Coomassie blue staining (lane 1–6) or silver staining (lanes 7,8). Lane M, molecular weight standards as indicated, (lane 1) crude brain extract; (lane 2) flow-through fraction of Q-Sepharose ion exchange chromatography; (lane 3) pool of fractions of S-Sepharose ion exchange chromatography; (lane 4) pool of fractions of SP-Sepharose HP ion exchange chromatography; (lane 5) pool of fractions of Mono-Q HR 10/10 ion exchange chromatography; (lane 6) pool of fractions after Mono-Q HR 5/5 ion exchange chromatography; (lane 7) fraction 24 of G-200 gel filtration, showing a single band at 31 kDa; (lane 8) pool of fractions 13–18 of G-200 Superdex gel filtration. Right panel: immunoblot with anti-cdk5 antibody, lanes 1–8 show the same samples as seen on the left panel. (B) Anion exchange chromatography by Mono Q HR 5/5. Upper panel, fractions 7–12 were screened for phosphorylation of p34 substrate peptide. Lower panel, immunoblot with anti-cdk5 antibody of the same fractions as seen above. Note that the cdk5 elutes in two maxima as determined by the specific antibody, but only the right peak (fraction 10–12) has high activity for phosphorylating the peptide substrate, whereas the left peak (fraction 7–8) has low activity. (C) Gel filtration (Superdex G-200) of fraction 10 obtained after Mono-Q HR 5/5 ion exchange chromatography. Upper panel, plot of phosphorylation activity for p34 substrate peptide of every second fraction (triangles). Lower panel, immunoblot of the same fractions as seen above. Note that the highest phosphorylation activity coincides with the presence of cdk5 as indicated by immunoblot and corresponds to a molecular weight of ca. 50–70 kDa (see calibration line with marker proteins, squares). (D) Final purification of active brain cdk5. SDS-PAGE after silver staining. Lane 1, pool of fractions 13–18 obtained after G-200 Superdex gel filtration; lane 2, pool of fractions 5–7 of Mono-Q 1.6/6 Smart ion exchange chromatography; lanes 3–5, fractions 7, 8 and 9 of Mono-S 1.6/6 Smart ion exchange chromatography. Lane M, molecular weight standards as indicated.

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