Detection of a Cdc2-Related Kinase Associated with Alzheimer Paired Helical Filaments

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By immunocytochemical staining and Western blotting, we detected a Cdc2-related kinase in human brains. The kinase is recognized by antibodies against the carboxyl and the amino termini of p34<sub>Cdc2</sub> but is not recognized by antibodies against the PSTAIRE motif. It is slightly smaller than p34<sub>Cdc2</sub> in molecular mass (approximately 33 kd). This 33-kd Cdc2-related kinase is present in intracellular neurofibrillary tangles in neurons of elderly humans and in Alzheimer’s disease, and it is associated with paired helical filaments (PHF) from Alzheimer’s disease brains. Unlike the antibodies to the carboxyl and amino termini of p34<sub>Cdc2</sub>, antibodies to an abundant brain Cdc2-related kinase PSSLARE/Cdk5 did not immunolabel Alzheimer’s disease neurofibrillary lesions. PHF preparations were demonstrated to contain kinases capable of phosphorylating histone H1, PHF-Tau, and a synthetic peptide (YAVVRTPK-SPSSAK). By virtue of its physical association with PHF, the 33-kd Cdc2-related kinase may play a role in transforming normal Tau proteins to PHF-Tau characteristic of Alzheimer’s disease. (Am J Pathol 1995, 146:228–238)

Neurofibrillary tangles (NFT) and neuropil threads are histopathological hallmarks of brains afflicted with Alzheimer’s disease (AD).<sup>1–3</sup> These structures consist mainly of intraneuronal aggregates of paired helical filaments (PHF) and straight filaments of 15 to 18 nm in diameter; both exhibit immunoreactivity with the microtubule-associated protein Tau. The major protein component of PHF is a modified form of Tau (PHF-Tau). PHF-Tau differs from Tau in normal adult human brains (N-Tau) in a number of physiochemical properties. In comparison with N-Tau, PHF-Tau exhibits a higher apparent molecular mass, more phosphate (mol/mol), fewer isoforms, a more acidic isoelectric charge, and a lower content of lysine and higher content of glycine.<sup>4–5</sup>

By epitope mapping with phosphate-dependent anti-tau antibodies as well as direct protein sequencing, at least 10 phosphorylation sites have been located in the PHF-Tau molecule.<sup>7–15</sup> With the exception of three sites, all of the identified phosphorylated sites display amino acid sequences X-Ser/Thr-Pro-X, a structural motif for site-specific phosphorylation by proline-directed Ser/Thr protein kinases.<sup>14,16</sup> Mitogen-activated protein kinase (Mapk/Erk), glyco-gen synthase kinase-3 (GSK-3 or F<sub>A</sub> kinase) and cyclin-dependent kinases belong to the family of proline-directed kinases. Phosphorylation of N-Tau or recombinant Tau by Mapk/Erk<sup>18,19</sup> or GSK-3/F<sub>A</sub> kinase<sup>20,21</sup> has been shown to retard the gel electrophoretic mobility of these proteins, and also to alter N-Tau immunoreactivity to a state similar to PHF-Tau. It has recently been reported, however, that the distribution of GSK-3/F<sub>A</sub> kinase in AD and control brains is similar and that the observed GSK-3/F<sub>A</sub> immunoreactivity revealed a punctate instead of a filamentous pattern.<sup>21</sup> An antibody that recognized the mitogen-activated protein kinase Erk 2 was recently reported to recognize some intracellular and extracellular NFT.<sup>22</sup>

Recombinant Tau and purified Tau have also been shown to be substrates for certain cyclin/Cdc2 kinase complexes,<sup>23–25</sup> and Cdc2-related kinases such as Cdk2 and Cdk5.<sup>26,27</sup> The cyclin A/Cdc2 kinase complex, but not Mapk (Erk 2), is capable of phosphorylating a synthetic peptide containing the Tau-1 epitope (PKSGDRSGYSSPGSPGTG, #189–#207), an epitope that is fully phosphorylated in PHF-Tau but only partially phosphorylated in N-Tau or fetal Tau.<sup>28</sup>

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These findings raise the possibility that proline-directed Ser/Thr protein kinase(s) may play an important role in hyperphosphorylation of PHF-Tau. To further assess the potential role of Cdc2 kinase in PHF-Tau formation, we used immunocytochemical methods to investigate the distribution of this kinase in tissues and extracts from AD brains. In this paper, we provide evidence for the existence of a protein kinase that is closely related to p34Cdc2 and is physically associated with PHF. This PHF-associated kinase is smaller in molecular weight than several recently identified Cdc2-related kinases, which were referred to as PCTAIRE 1, PCTAIRE 2, PLSTIRE 3, and KKIALRE by Meyerson et al.29 These kinases were named according to the amino acid sequences of a region corresponding to the PSTAIRE region of p34Cdc2.

Materials and Methods

Antibodies

Anti-Cdc2 C, a polyclonal antibody against amino acid residues 290 to 297 (DNQIKKM)30,31 of human Cdc2, was used at 1:100 to 1:1000 dilution. One polyclonal antibody to an N-terminal region (amino acids 8 to 19) of Cdc2 (anti-Cdc2 N), and two antibodies to PSTAIRE (anti-Cdc2 (PSTAIRE), one polyclonal and one monoclonal) were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies to Cdc2-related kinases PSSALRE/Cdk5, PCTAIRE 1, PCTAIRE 2, PCTAIRE 3, PLSTIRE 3, and KKIALRE were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). These antibodies were raised against synthetic peptides with amino acid sequences unique to the carboxyl end of each kinase.29 Additional anti-PSSALRE/Cdk5 antibodies were obtained from Dr. Li-Huei Tsai29 (Massachusetts General Hospital, Boston, MA). These antibodies had no reactivity against p34Cdc2. Anti-Cdk2 kinase,32 anti-cyclin A,33 and anti-cyclin D that recognize cyclin D1 and D234 were prepared at Children's Hospital, Los Angeles. Tau 46, a monoclonal antibody to the carboxyl terminus of Tau (amino acids 404 to 441), was provided by Dr. V. Lee (University of Pennsylvania, Philadelphia, PA). Ab 39 is a monoclonal antibody capable of detecting both the extracellular and intracellular NFT.35 (McLean's fixative), 4% paraformaldehyde, or Bouin's fixative were used for immunoperoxidase staining. Overall, 10 AD and 2 normal brains were examined. In some studies, adjacent sections were stained with anti-NFT (Ab 39) and anti-Cdc2 antibodies, respectively. In other studies, the same sections were double stained with anti-NFT/Ab 39 and anti-Cdc2 antibodies, in which case the secondary antibodies were goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase and goat anti-mouse IgG1 conjugated to β-galactosidase (Southern Biotechnology, Birmingham, AL), respectively. The chromogens used for detecting the bound immunoglobulins were 3-3′-diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, MO) and X-gal (GIBCO BRL, Gaithersburg, MD). To confirm the specificity of the immunostaining, the anti-Cdc2 C antibody was absorbed with a synthetic peptide corresponding to the C-terminus of Cdc2 (DNQIKKM) or a peptide corresponding to the C-terminus (QDVTKPVPHRL) of Cdk2. Preimmune serum was used as negative control, and it gave no staining. For comparison, antibodies to PSSALRE/Cdk5 and other Cdc2-related kinases were also included in immunocytochemical studies.

In immunogold EM labeling studies, the S1 and T1 fractions (see below) were placed on EM grids and labeled according to a protocol reported before.36 The secondary antibodies were either goat antimouse or goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles (Amersham, Arlington Heights, IL). To reveal the structures exhibiting specific immunoactivities, EM grids were negatively stained with 2% uranyl acetate in distilled water.

For immunoblotting, samples were dissolved in sample buffer containing 2% sodium dodecyl sulfate (SDS), 2% β-mercaptoethanol, and 50 mmol/L Tris-HCl, pH 6.8. They were separated by SDS-polyacrylamide gel electrophoresis with 10% gels,37 and electrotransferred to nitrocellulose papers. The electroblots were blocked with 5% milk in Tris-buffered saline and incubated with antibodies. The bound immunoglobulins were detected with an alkaline phosphatase reaction,31 or a Vectastain ABC kit (Vector Laboratory, Burlingame, CA) or by an enhanced chemiluminescence detection system (Amersham).

PHF Preparation

PHF were prepared by a method described previously26 with minor modifications. Brain samples were obtained from histopathologically confirmed AD subjects no more than 8 hours postmortem. Samples of
frontal or temporal cortex were homogenized in three volumes of 0.25 mmol/L Tris-HCl buffer, pH 7.4, containing 0.15 mol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride (referred to as TBS). After centrifugation at 27,000 × g for 20 minutes, the pellet was collected, and the supernatant was recentrifuged at 100,000 × g for 1 hour. The pellet (TBS/P) contained PHF as judged by immunoblotting with antibodies Tau 46 and PHF 1 and was further treated with either 1% Triton X-100 or Sarkosyl (Sigma) and recentrifuged at 100,000 × g to obtain post-detergent pellets. The Triton X-100 and the Sarkosyl pellets are referred to as TBS/TP and TBS/SP. The initial pellet fraction was rehomogenized in five volumes of sucrose extraction buffer, which contains 10 mmol/L Tris-HCl, pH 7.4, 0.8 mol/L NaCl, 10% sucrose, 2 mmol/L EGTA, and 1 mmol/L phenylmethylsulfonyl fluoride, and centrifuged again at 27,000 × g for 20 minutes. The resulting supernatant, which contained PHF, was collected, mixed either with Sarkosyl or Triton X-100 to a final concentration of 1%, and then recentrifuged at 100,000 × g for 2 hours. The resulting pellet was then suspended in 3 ml of TBS, homogenized with a Dounce homogenizer, and layered over a discontinuous sucrose gradient consisting of 3 ml each of 1.0 mol/L, 1.5 mol/L, and 2.0 mol/L sucrose in TBS. After centrifugation for 2 hours at 100,000 × g, the 1.0 mol/L sucrose layer, which is highly enriched in PHF, was collected. This fraction was designated as S1 or T1 depending on whether Sarkosyl or Triton X-100 was used during the extraction.

The TBS/P fraction was used for affinity interactions with p9\textsuperscript{Ckhs}1, Affigel-10 (a Cdc2 kinase-binding subunit, human sequence) according to a protocol described previously\textsuperscript{32} with a concentration of 5 mg of p9\textsuperscript{Ckhs}1 ligand per ml of Affigel-10 (Bio-Rad Laboratories, Richmond, CA). The TBS/P fraction was incubated with p9\textsuperscript{Ckhs}1-Affigel-10 for 30 minutes at 4°C followed by centrifugation at 1000 × g for 10 minutes. The pellet was recovered, washed, and extracted with 2% SDS. The extracted proteins were subjected to gel electrophoresis and immunoblotting.

**Kinase Assay**

In vitro kinase assays were carried out in a reaction buffer containing 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 25 mmol/L NaF, 25 mmol/L β-glycerophosphate, 0.1 mmol/L sodium vanadate, 0.1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1% Triton X-100, 10 mmol/L MgCl\textsubscript{2} and 0.1 mmol/L [γ-\textsuperscript{32}P]ATP (specific activity 1500–2000 cpm/pmol). Histone (5 μl of type III S, 1.0 mg/ml) was added as substrate to 45 μl of reaction buffer containing various brain fractions. The phosphotransferase reaction was initiated by the addition of ATP at 37°C for 1 hour. For samples containing histone and/or brain proteins, the reaction was terminated by an addition of 6× SDS-sample buffer and boiling for 5 minutes. The reaction mixture was resolved on a 10% SDS-polyacrylamide gel, electrophoresed to nitrocellulose paper, and evaluated by autoradiography and/or immunoblotting. For samples containing synthetic peptide (VAVVTRPPKSPSSAK), the phosphotransferase reactions were stopped by the addition of perchloric acid, followed by centrifugation to separate peptides and spotting of supernatant onto phosphocellulose paper.\textsuperscript{10} The papers were washed with 75 mmol/L phosphoric acid and distilled water, dehydrated in acetone, air-dried, and placed in scintillation fluid. The incorporation of \textsuperscript{32}P was quantified by liquid scintillation counting.

**Results**

**Immunocytochemistry**

Alzheimer brain sections were specifically immunolabeled by antibodies directed against the extreme C-terminus of p34\textsuperscript{Cdc2}. The results are shown in Figure 1. Immunoperoxidase reaction products were localized to NFT and abnormal neurites in the neuropil (so-called neuropil threads) and senile plaques (Figure 1a, b). Similar immunostaining was also demonstrated by the anti-Cdc2 N antibody (Figure 1c-e), indicating an association of Cdc2 and/or closely related kinases with AD neurofibrillary pathology. NFT and abnormal neurites were more intensely stained by anti-Cdc2 C than anti-Cdc2 N antibodies, and more abnormal neurites were positive with anti-Cdc2 C antibody. The difference could be due to differences in the accessibility of epitope to antibody binding, as both anti-Cdc2 C and anti-Cdc2 N antibodies appear to have a comparable titer by immunoblotting.

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**Figure 1.** Immunocytochemistry of paraformaldehyde-fixed Vibratome sections. A section of cerebral cortex from AD (a) shows numerous fibrillar immunoreactive neuritic processes (neuritic threads) and cell bodies (NFT), and (b) dystrophic neurites in a senile plaque with a polyclonal antibody to the C-terminus of p34\textsuperscript{Cdc2} (anti-Cdc2 C). Antibodies to the amino terminus of p34\textsuperscript{Cdc2} (anti-Cdc2 N) also stained (c) NFT and (d and e) dystrophic neurites in senile plaques. Antibodies to the PSTARE domains of p34\textsuperscript{Cdc2} failed to stain any structures in either normal or AD brain sections (f). No specific staining was detected when the anti-Cdc2 C antibody was preincubated with C-terminal p34\textsuperscript{Cdc2} synthetic peptide (g). In normal brains (h) some pyramidal neurons, such as those in this figure, had fine granular cytoplasmic immunoreactivity in tissue that had been briefly fixed in Bouin’s solution.
Both anti-Cdc2 (PSTAIRE) antibodies exhibited little if any immunoreactivity with brain sections (Figure 1f) or brain extracts (see below). The results suggest that the kinase(s) associated with AD neurofibrillary lesions may be a Cdc2-related kinase rather than the p34
\textsuperscript{Cdc2}
 kinase per se. Antibodies to Cdk2, PSSALRE/Cdk5, PCTAIRE 1, PCTAIRE 2, or PLSTIRE did not stain AD NFT and abnormal neurites (data not shown).

The number of NFT detected by anti-Cdc2 C or anti-Cdc2 N antibody was fewer than that detected by Ab 39. The difference is unlikely due to differences in antibody titer, as comparable results were obtained when more concentrated anti-Cdc2 antibodies were used. The absence of Cdc2 immunoreactivity in some NFT may be due to proteolytic degradation. Alternatively, it may reflect either the state of NFT development or the physiology of the enzyme system.

The immunoreactivity of anti-Cdc2 C antibody to NFT was completely absorbed by synthetic Cdc2 C-terminal peptide (Figure 1g) but not by Cdk2-specific peptide (data not shown), demonstrating that the observed immunoreactivity was specific. NFT were not labeled in the sections incubated with normal rabbit serum or cyclin A and cyclin D (data not shown). The anti-cyclin A antibodies detected a small subset of astrocytes, although no specific immunoreactivity was detected in neuronal cells. Immunostaining was optimal on tissue fixed briefly in 4% paraformaldehyde, PLP, or Bouin’s solution. In the latter fixative, weak staining was also detected in the cell bodies of pyramidal neurons, although some pyramidal neurons without NFT were immunolabeled by anti-Cdc2 N and Cdc2 C antibodies. The staining of neurite threads by anti-Cdc2 antibodies was greater in Bouin’s fixative than in paraformaldehyde. NFT staining could be detected in acetone- or methanol-fixed frozen sections, although higher antibody concentrations were required (1:100) and the reactivity was weaker than in fixed tissue cut with a Vibratome.

In normal brain tissues, a subset of neurons (Figure 1h) were labeled by anti-Cdc2 antibodies. In the hippocampus of one of the normal elderly subjects studied, a few NFT were detected by thioflavin stain and they were recognized by Ab 39. Virtually all of these NFT were immunolabeled by anti-Cdc2 antibodies. Collectively, the results are consistent with those obtained from AD brains, indicating the specific association of Cdc2-like immunoreactivity with NFT.

**Immunogold EM**

The S1 and T1 fractions of PHF preparations (see Materials and Methods) were used for immunogold labeling. Both fractions contained comparable amounts of PHF-Tau proteins based upon Western analysis, although by examination of uranyl-acetate-stained samples, the S1 fraction appeared to contain fewer contaminants than the T1 fraction (data not shown). PHF in the S1 (Figure 2) or T1 fraction were uniformly labeled by Tau 46. The results are consistent with previous studies indicating that Tau is a prominent component of PHF.

PHF in S1 and T1 fractions were recognized by both anti-Cdc2 C and anti-Cdc2 N antibodies (Figure 2). Unlike Tau 46, which reacted with nearly all PHF detected on EM grids, only a fraction of these filaments exhibited Cdc2 immunoreactivity (compare in Figure 2 S1, Tau 46 with S1, Cdc2 C or Cdc2 N). In comparison with anti-Cdc2 C antibody, somewhat fewer immunogold particles were detected on PHF with the anti-Cdc2 N antibody. Anti-Cdc2 (PSTAIRE) antibodies did not label PHF (Figure 2). Anti-PSSALRE/Cdk5 also failed to stain PHF (Figure 2). These observations are consistent with the results of the immunocytochemical studies, which demonstrated an association of Cdc2 C and Cdc2 N immunoreactivity with some neurofibrillary lesions in AD and the absence of PSTAIRE and PSSALRE/Cdk5 immunoreactivity. Moreover, the results also demonstrated the strong association between PHF and the Cdc2-related protein, as some (if not all) of the Cdc2 immunoreactivity was detectable in PHF treated with Triton X-100 or Sarkosyl.

The anti-cyclin D antibody did not label any filamentous structure but instead labeled a very small number of aggregates of unknown nature (data not shown). The significance of the cyclin immunoreactivity is uncertain at this time.

**Western Blot Analysis**

Five different fractions, TBS/P, TBS/TP, TBS/SP, T1, and S1, were used for immunoblotting. To estimate the relative amount of PHF in different preparations, each fraction was probed concomitantly with Tau 46 (Figure 3). Fractions with comparable amounts of PHF-Tau were compared for immunoreactivity with a battery of anti-Cdc2 antibodies. The Cdc2 immunoreactivity was detected mainly in TBS/TP and T1 fractions (Figure 4). When the loading of samples on gel was increased five-fold or more (not shown), the Cdc2 C and Cdc2 N immunoreactivities, but not the PSTAIRE immunoreactivity, could also be detected in the TBS/SP or S1 fraction, which contained Sarkosyl-insoluble materials. The results, in agreement with
Association of a Cdc2-Related Kinase with PHF

Figure 2. Immunogold labeling of PHF preparations. All filaments in the S1 or T1 fractions (not shown) were stained by anti-Tau antibody Tau 46. Only a fraction of the filaments in these fractions was recognized by the anti-Cdc2 antibody and anti-Cdc2 N antibody. Filaments in these fractions were not recognized by anti-PSTAIRE/Cdk5.

those obtained in immunogold labeling studies, suggest that Sarkosyl is capable of extracting some but not all of the Cdc2-immunoreactive proteins. Moreover, the detection of Cdc2-related proteins in PHF preparations extracted with Triton X-100 indicates that these proteins are tightly associated with PHF. In this regard, it is interesting to note that neurofilament-enriched preparations from bovine brains have been reported to contain p34\textsuperscript{Cdc2}\textsubscript{8},-related kinase, which is not extractable by Triton X-100.\textsuperscript{41}

Of the four anti-Cdc2 antibodies tested, only anti-Cdc2 C and anti-Cdc2 N reacted with a protein in the AD brain fractions with a molecular mass similar to p34\textsuperscript{Cdc2} (Figure 4A, B). Anti-Cdc2 (PSTAIRE) antibodies did not cross-react with this protein in any fraction (Figure 4C), although the PSTAIRE immunoreactivity was readily detected in recombinant p34\textsuperscript{Cdc2} (Figure 4C, first lane). Incubation of electrophoretic with alkaline phosphatase did not improve the reactivity of anti-Cdc2 (PSTAIRE) antibodies with the protein in brain fractions that was reactive to anti-Cdc2 C and anti-Cdc2 N (data not shown), suggesting that the absence of Cdc2 (PSTAIRE) immunoreactivity may not be due to phosphorylation of the epitope.

The band labeled with anti-Cdc2 C and anti-Cdc2 N antibodies co-migrated with recombinant p34\textsuperscript{Cdc2} on 10% gels (Figure 4) and with p34\textsuperscript{Cdc2} proteins purified from MG-63 osteosarcoma or EW-1 Ewings sarcoma cell lysates purified by p\textsuperscript{G\textsubscript{20}BRK\textsubscript{1}} affinity Affigel-10 beads (data not shown). The apparent mo-
lecular mass of the Cdc2-related AD protein appeared to be slightly smaller (approximately 1 kd) than that of authentic p34\(^{\text{CDC2}}\), inasmuch as the results obtained with 12% gels suggested that the Cdc2-related protein migrated slightly ahead of p34\(^{\text{CDC2}}\) (Figure 5A). The small difference in molecular weight is unlikely due to proteolysis, because the amino and the carboxyl termini of p34\(^{\text{CDC2}}\) were preserved in our Cdc2-related AD protein.

To further characterize the properties of the Cdc2-related protein in PHF preparations, we performed p9\(^{\text{Cskhs1}}\)-Affigel-10 affinity precipitations on TBS/P as described by Hall et al.\(^{34}\) Although the recovery of the anti-Cdc2 cross-reactive protein was determined to be nonquantitative, the purification of the protein was substantial enabling comparative Western analysis of the precipitated protein. Proteins migrating at the 33- to 34-kd region were clearly detected with anti-Cdc2 C antibodies (Figure 5A) but had virtually no cross-reactivity with anti-Cdc2 (PSTAIRE) antibodies that readily detect authentic p34\(^{\text{CDC2}}\) (Figure 5B). Thus, the immunoreactivity and the molecular mass of the p9\(^{\text{Cskhs1}}\)-Affigel-10-associated protein detected in TBS/P appear to be the same as that in PHF preparations.

In Vitro Kinase Assays

T1 and TBS/TP fractions from AD brains were tested for phosphotransferase activity toward exogenous histone H1. Incubation of the respective fractions with kinase reaction mixtures containing histone H1 and [\(\gamma\)\(^{32}\)P]ATP led to the phosphorylation of histone (Figure 6). Comparable results were obtained with samples prepared from fresh and frozen brains. Phosphorylation of histone H1 was not detected in control reaction mixtures lacking the T1 or the TBS/TP fraction. Besides histone H1, three additional bands were phosphorylated in samples containing T1. These phosphorylated proteins were identified as PHF-Tau by immunoblotting with anti-Tau antibodies (data not shown). In reaction mixtures containing T1, but lacking histone, the PHF-Tau proteins were also phosphorylated (Figure 6). The results indicate that PHF-enriched preparations contain kinases capable of phosphorylating PHF-Tau. These kinases are also capable of phosphorylating a synthetic peptide containing the VAVRTPPKSPSSAK motif, which corre-
well as with PHF, Cdc2-related The was to, and plaques

Figure 5. Immunoblotting of samples obtained from p34 2,3-phospho-1.4,5-pyridine diphosphate (PSTAIRE) affinity precipitation of TBS/TP fraction with anti-Cdc2 antibodies. The samples were separated on 12% gel. A: Anti-Cdc2 C antibody recognized proteins of molecular mass 33 to 34 kD in samples (lanes a and b) from two AD brains respectively. These proteins were not recognized by (B) anti-PSTARE antibody. The 33- to 34-kD proteins migrated slightly ahead of p34 2,3-phospho-1 proteins (lane c) that were purified from M63 osteosarcoma lysates by p34 2,3-phospho-1 affinity beads.

sponds to amino acid residues 226 to 240 of the longest human Tau isoform. In samples containing TBS/TP, numerous proteins were phosphorylated; however, the identity of these endogenous proteins has not yet been determined.

Discussion

By immunocytochemical methods, AD brain sections were found to be immunolabeled by antibodies to the carboxyl and amino termini of the authentic p34 Cdc2, but not by antibodies to the PSTAIRE motif of kinase subdomain III. The negative result with anti-Cdc2 (PSTAIRE) antibodies suggests that the Cdc2-immunoreactive AD protein is related to, but not identical to, the authentic p34 Cdc2. The immunoreactivity was localized mainly to abnormal neurites in senile plaques and neuritil threads and a fraction of NFT. The Cdc2-related protein is physically associated with PHF, as revealed by immunogold EM labeling as well as biochemical extraction and immunoblotting studies. Therefore, this protein kinase may play a role in the covalent modification of normal Tau to PHF-Tau. The absence of Cdc2 C and Cdc2 N immunoreactivities in some NFT may be due to dissociation of kinase from NFT when the phosphorylation of Tau is completed or due to proteolytic degradation. Alternatively, the enzyme may be inaccessible to antibody binding in some NFT.

By SDS-polyacrylamide gel electrophoresis and immunoblotting, the Cdc2-related AD brain protein was determined to be slightly smaller than p34 Cdc2 in molecular mass. This Cdc2-related protein reacted with anti-Cdc2 N and anti-Cdc2 C antibodies but not with anti-Cdc2 (PSTAIRE) and anti-Cdk2 antibodies. Moreover, this protein was detected in fractions enriched in PHF. The results are consistent with those obtained with immunocytochemical and immunogold labeling methods, indicating the physical association of this Cdc2-related kinase with PHF. The absence of PSTAIRE immunoreactivity is unlikely due to a modification of the PSTAIRE region or adjacent amino acid residues by phosphorylation, as treatment of electoblots with alkaline phosphatase had no effect on the reactivity of Cdc2-related and PHF-associated kinase with anti-PSTAIRE antibodies. The lack of immunoreactivity of the PHF-associated kinase with anti-Cdc2 (PSTAIRE) antibodies may indicate the divergence of the PSTAIRE motif in Cdc2-related PHF.

Figure 6. Autoradiographs show phosphorylation of histone and PHF-Tau proteins by kinases in PHF-enriched fractions. TBS/TP fraction from two AD brains and a T1 fraction were incubated with [γ-32P]ATP alone or with [γ-32P]ATP and histone. Histone phosphorylation (marked by star) was detected only in samples containing ATP. In the TBS/TP fraction, proteins other than histone were also phosphorylated. Phosphorylation of PHF-Tau proteins (marked by bars) were detected in samples containing T1 fraction.
kinase. Alternatively, it may be due to biochemical modifications other than phosphorylation.

The association between PHF kinase and PHF appeared to be strong, inasmuch as Cdc2-related immunoreactivity was detected in proteins from PHF fractions treated with Triton X-100 (immunoblotting) as well as in PHF washed in Triton (immunogold EM). Extraction with Sarkosyl was effective in removing some but not all of the PHF kinase from PHF. Additional studies are needed to determine the efficiency of Sarkosyl in extraction of PHF kinase. It is interesting to note that p9\textsuperscript{Ckhsa\textsubscript{1}} is capable of binding to at least a subset of Cdc2-related PHF kinases. This property is being used for further purifications of PHF kinase.

Many isoforms of Cdc2-related enzymes have been identified recently.\textsuperscript{29,46} Among them only the authentic Cdc2, Cdk2, and a Cdc2-related bovine kinase\textsuperscript{41} have been reported to exhibit a high affinity for p9\textsuperscript{Ckhsa\textsubscript{1}} or related ligands.\textsuperscript{29,46} Inasmuch as neither cyclin A nor D-type cyclins were detected in brain homogenates or in neurons by immunocytochemistry (tissue sections), and as the PSTAIRE region has been shown to be involved in cyclin binding, our results suggest that Cdc2-related PHF kinase may associate with a regulatory subunit other than a classical cyclin.

By in vitro kinase assays, PHF-enriched preparations were demonstrated to contain phosphotransferase capable of phosphorylating PHF-Tau proteins. Our results support the study of Vincent and Davies\textsuperscript{43} in which PHF purified by Alz50 immunoaffinity column chromatography was found to have phosphotransferase activities capable of phosphorylating PHF-Tau. In addition to PHF-Tau, we demonstrated the phosphorylation of exogenous histone H1 and synthetic peptide VAVRTPPKSPPSAK, which exhibits the X-Ser/Thr-Pro-X motif that can be phosphorylated by different proline-directed kinases. This type of phosphorylation is unlikely due to Mapk/Erk, because Mapk/Erk cannot efficiently utilize histone H1 as a substrate.\textsuperscript{43–45} Our data, however, do not rule out the possibility of Mapk/Erk in PHF preparations.

The Cdc2-related PHF kinase is different from the following characterized Cdc2-related kinases identified recently in human and bovine brain tissues: PCTAIRE\textsuperscript{1}, PCTAIRE\textsuperscript{2}, PCTAIRE\textsuperscript{3}, PSSALRE/Cdk5, PLSTIRE, and KKIALRE.\textsuperscript{29,46} PSSALRE kinase/Cdk5 was reported to phosphorylate neurofilament proteins and Tau in vitro.\textsuperscript{26,27,47,48} Unlike Cdc2-related PHF kinase, the above mentioned kinases do not contain the amino acid sequences (DNQIKKK) of authentic p34\textsuperscript{Cdc2} kinase. Furthermore, with the exception of PSSALRE and PLSTIRE kinases, which contain 291 and 326 amino acid residues, respectively, the molecular mass of the other four kinases is significantly larger than that of p34\textsuperscript{Cdc2} and Cdc2-related PHF kinase. Antibodies specific for these six Cdc2-related kinases were found in our studies to have no reactivities against PHF, although they readily immunolabeled cultured neuroblastoma cells (unpublished observations).

At this time we are uncertain about the relationship between Cdc2-related PHF kinase and the Cdc2-related kinases isolated from rat spinal cord\textsuperscript{49} and from bovine brain cytoskeleton-enriched fractions.\textsuperscript{41} The structure of these nonhuman Cdc2-related kinases has yet to be determined; however, the rat Cdc2-related kinase was recognized by antibodies to the carboxyl terminal of p34\textsuperscript{Cdc2} but not by an anti-Cdc2 (PSTAIRE) antibody, which is similar to Cdc2-related PHF kinase. The rat spinal cord kinase has been reported to be proline directed and to phosphorylate neurofilament proteins and synthetic peptides containing the KSPXK motif. The KSPXK motif is not present in Tau proteins. Similar to Cdc2-related PHF kinase, the bovine Cdc2-related kinase isolated by Mawal-Dewan et al.\textsuperscript{41} was found in cytoskeleton-enriched fractions extracted with 1% Triton X-100. This bovine kinase binds to p13\textsuperscript{Suc\textsubscript{1}} beads and is capable of phosphorylating Tau proteins and high molecular weight neurofilament proteins.

A number of enzymes, including Mapk/Erk and GSK-3/F\textsubscript{A}, have been demonstrated to phosphorylate Tau protein in vitro. GSK-3/F\textsubscript{A} kinase does not appear to have a close association with PHF, as judged from the results of immunocytochemical studies of others.\textsuperscript{41} The GSK-3/F\textsubscript{A} kinase is known for its selectivity for phosphate residues closely spaced near the target phosphorylation sites, exhibiting a tendency toward secondary or hierarchical phosphorylation, but is unlikely to perform the initiating event.\textsuperscript{50,51} Mapk/Erk immunoreactivity has been localized to AD neurofibrillary lesions by immunocytochemical methods; however, it remains to be determined whether Mapk/Erk are also associated with PHF. In contrast, the PHF-associated kinase identified here is a strong candidate for involvement in the hyperphosphorylation of PHF-Tau in AD brains. The striking differences in Cdc2 immunoreactivity observed between NFT- and non-NFT-bearing neurons in AD brains suggest that Cdc2-related PHF kinase is abnormally accumulated in neurons with neurofibrillary lesions. It is also conceivable that this PHF kinase is dysregulated in AD. Additional molecular and biochemical characterizations of this enzyme may provide additional insights into the pathogenesis of AD.
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References