American Journal of Pathology, Vol. 147, No. 5, November 1995

Cortical and Brainstem-Type Lewy Bodies Are Immunoreactive for the Cyclin-Dependent Kinase 5

Jean-Pierre Brion and Anne-Marie Couck

From the Laboratory of Pathology and Electron Microscopy, Free University of Brussels, Brussels, Belgium

The immunoreactivity of cortical and brainstem-type Lewy bodies has been investigated with antibodies to the cyclin-dependent kinase 5 (cdk5), to the extracellular regulated kinase 1 (ERK-1), and to the cdc2p34 kinase and with antibodies specific for phosphorylation epitopes typical of paired helical filament-tau (PHF-tau). Both cortical and brainstem-type Lewy bodies in diffuse Lewy body disease and brainstem-type Lewy bodies in Parkinson's disease were found to be immunoreactive for cdk5 but not for cdc2p34 or ERK-1 or with the PHF-tau antibodies. Double immunolabeling showed that cdk5-positive Lewy bodies were also ubiquitin immunoreactive and that cdk5 antibodies labeled as many Lewy bodies as ubiquitin antibodies in adequately fixed tissue. The cdk5 immunoreactivity of Lewy bodies was abolished by preabsorption of the antibody with a cdk5 peptide. The antibodies to cdk5 labeled a single 33-kd species on Western blots of human brain homogenates, with a similar intensity in control, diffuse Lewy body disease, and Alzheimer's disease, and this cdk5 species was found mainly in the particulate fraction of brain homogenates. This observation suggests that cdk5 might be a protein kinase involved in the phosphorylation of a molecular component of Lewy bodies, for example, neurofilament proteins known to be present in these inclusions. (Am J Pathol 1995, 147:1465–1476)

Lewy bodies are eosinophilic neuronal inclusions first described in pigmented neurons in idiopathic Parkinson's disease. Lewy bodies are now known to occur in several neurodegenerative diseases and in a variety of sites in the nervous system.[1–3] A wide distribution of Lewy bodies has been described in the diffuse Lewy body disease (DLBD), a dementing condition in which numerous Lewy bodies are found in cortical areas. Abundant cortical Lewy bodies can exist in the absence or in the presence of neurofibrillary tangles and/or senile plaques, the neuropathological lesions of Alzheimer's disease. There is not yet general agreement as to the relationship between these conditions. They might represent a spectrum of diseases with Lewy bodies, constitute separate entities, or be related to Alzheimer's disease.[1,4–7]

Brainstem-type Lewy bodies consist of a dense core composed of granular or vesicular material, surrounded by a less dense peripheral zone and an outer pale halo. Lewy bodies contain filaments, resembling intermediate filaments, randomly arranged throughout the whole structure.[8,9] The cortical Lewy bodies show some structural differences with typical Lewy bodies found in the substantia nigra and other brainstem nuclei; ie, they show a more uniform structure with a less marked demarcation between central and peripheral regions.

Lewy bodies have been repeatedly reported to be labeled by antibodies to neurofilament.[10–15] Extensive epitope mapping studies have indicated that the different domains of each neurofilament subunit are present in Lewy bodies and are the main building blocks of their filamentous component.[8,16] Lewy bodies are also systematically labeled by antibodies to ubiquitin.[14,15,17–20] These observations have suggested that Lewy bodies are composed of ubiquitinated neurofilaments. Lewy bodies have been reported to be immunoreactive with other cytoskeletal and noncytoskeletal molecules (for reviews, see

Supported by grants from the Belgian FRSM (3.4504.91 and 3.4507.95).

Accepted for publication July 13, 1995.

Address reprint requests to Dr. Jean-Pierre Brion, Laboratory of Pathology and Electron Microscopy, Free University of Brussels, School of Medicine, 808, route de Lennik, Bldg. C-10, 1070 Brussels, Belgium.

Refs. 2, 3, and 21), although these labelings are often inconsistent or concern only a proportion of Lewy bodies.

Several of the neurofilament antibodies that label perikaryotic Lewy bodies recognize phosphorylated epitopes.[12,15,22] Neurofilaments in perikarya are poorly phosphorylated in most neurons of the central nervous system in normal conditions. On the contrary, an accumulation of phosphorylated neurofilaments in perikarya is observed in several neurodegenerative conditions in humans and animals.[23] This suggests that structural components of Lewy bodies might be subject to aberrant phosphorylation phenomena and that phosphorylation might play a role in their formation. Little information, however, is available on the nature of protein kinases involved in the generation of phosphorylated epitopes in Lewy bodies. In this study, we have investigated by immunocytochemistry the expression of selected protein kinases in neurons containing Lewy bodies.

Materials and Methods

Tissue Samples

Tissue blocks including the hippocampus and the adjacent parahippocampal gyrus were taken at autopsy from two normal subjects (71 and 75 years), from two patients with DLBD (60 and 70 years), two patients with idiopathic Parkinson's disease (65 and 70 years), and two patients with Alzheimer's disease (84 and 91 years). The tissue samples were fixed in 10% formalin for 2 weeks or in methacarn (methanol: acetic acid:chloroform, 6:1:1 volumes) for 48 hours, embedded in paraffin, and cut on a microtome in tissue sections with a thickness of 10 μm. The patients with DLBD had numerous cortical Lewy bodies in the fronto-temporal cortex, and brainstem-type Lewy bodies in the substantia nigra and the locus coeruleus. In one DLBD patient, rare neurofibrillary tangles and a few senile plaques were present in the cortical samples. The other DLBD patient had a moderate number of neurofibrillary tangles in the cerebral cortex, numerous neuropil threads, and a few senile plaques. The two patients with Alzheimer's disease had numerous and widespread neurofibrillary tangles in the cortex and fulfilled the criteria of stages V to VI according to the neuropathological stageing of Braak and Braak.[24] The patients with Parkinson's disease had numerous Lewy bodies in pigmented neurons in the substantia nigra and in the locus coeruleus and showed loss of pigmented neurons, extracellular pigments, and macrophages loaded with neuromelanin pigments.

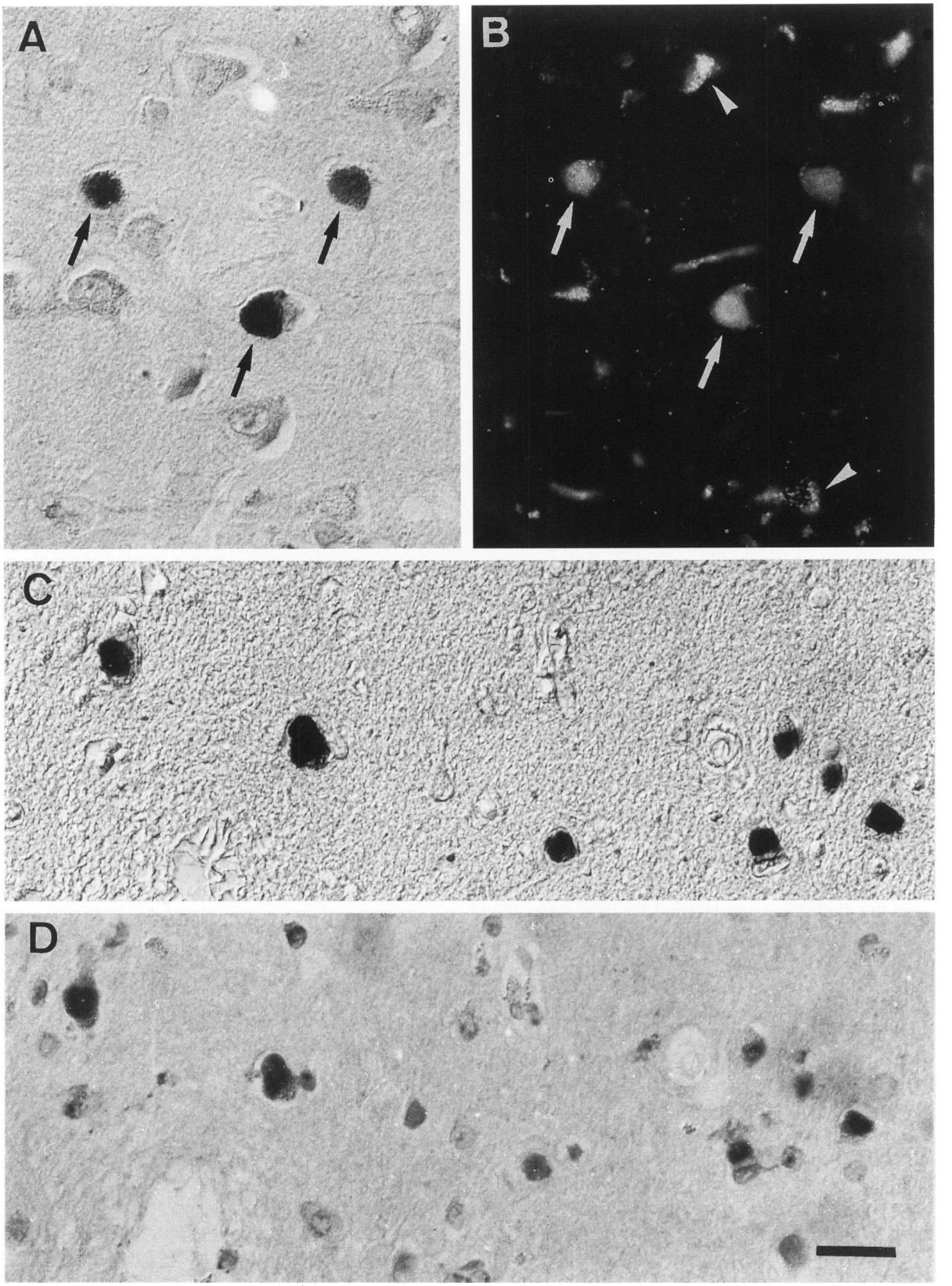
Antibodies

The two antibodies to cyclin-dependent kinase 5 (cdk5) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA (C-8 and H-291). These antibodies are affinity-purified rabbit immunoglobulins G (IgGs). The C-8 antibody was raised to a synthetic peptide (YFSDFCPP) corresponding to the last eight amino acid residues at the carboxy terminus of cdk5, and the H-291 antibody was raised to a fusion protein containing the full length human cdk5. The antibodies to extracellular-regulated kinase 1 (ERK-1) (Santa Cruz, C-16) are affinity-purified rabbit polyclonal antibodies raised to a synthetic peptide corresponding to an amino acid sequence in the carboxy terminus of ERK-1. The antibody to cdc2p34 (Santa Cruz, clone 17) is a mouse monoclonal antibody raised to recombinant cdc2p34. This antibody has previously been shown to label immature neuronal cells in proliferative layers of the developing brain.[25] The mouse monoclonal anti-ubiquitin antibody (MAb 1510, raised to ubiquitin covalently coupled to keyhole limpet hemocyanin) was purchased from Chemicon (Temecula, CA); this antibody has previously been shown to label Lewy bodies.[8] The mouse monoclonal antibodies AT8, AT180, and AT270 (kindly supplied by Innogenetics, Ghent, Belgium) were raised to paired helical filaments-tau (PHF-tau) and recognize specific sites on the tau molecule in a phosphorylation-dependent manner; ie, their reactivity with tau proteins is dependent on the phosphorylation of Ser202 (AT8), Thr231 (AT180), and Thr181 (AT270).[26,27]

Immunocytochemistry

For single immunolabeling, tissue sections were immunolabeled with the peroxidase-antiperoxidase method with diaminobenzidine for chromogen, as previously reported.[28] The antibodies were used at the following concentrations: anti-cdk5, 2 μg/ml; anti-ERK-1, 0.5 μg/ml; cdc2p34, 10 μg/ml; AT8, 0.5 μg/ml; AT270, 0.5 μg/ml; or at the following dilutions: AT180, 1/1000; anti-ubiquitin, 1/1000. The immunolabeling of tissue sections with these antibodies was

Figure 1. *Double immunolabeling (temporal cortex) in DLBD with the C-8 antibody to cdk5* (**A** *and* **D**) *and the anti-ubiquitin antibody* (**B** *and* **C**). *The same tissue sections were double labeled by the two antibodies either simultaneously* (**A** *and* **B**) *or sequentially* (**C** *and* **D**). *The same cortical Lewy bodies (eg,* **arrows** *in* **A** *and* **B**) *are labeled by both antibodies.* **Arrowheads** *in* **B** *point on autofluorescent lipofuscin granules. Scale bar, 25 μm.*


A
B
C
D

also tested after pretreatment for 10 minutes at 37°C with trypsin (400 μg/ml in 0.05 mol/L Tris-HCl, pH 7.6, 0.3 mol/L NaCl, 0.02 mol/L $CaCl_2$).

Double Immunohistochemical Staining

Some sections were double immunolabeled with the C-8 antibody to cdk5 in association with the mouse monoclonal antibody to ubiquitin. Sections were first incubated with the monoclonal antibody to ubiquitin and were then sequentially incubated with a donkey anti-rabbit antibody conjugated to biotin (Amersham, Arlington Heights, IL; diluted 1/100) and streptavidin conjugated to fluoroscein isothiocyanate (Amersham; diluted 1/50). Sections were mounted in gelvatol and viewed under epi-illumination in a Zeiss Axioplan microscope, photographed, unmounted, and then immunolabeled with the antibody to cdk5 by the peroxidase-anti-peroxidase method. In another set of experiments, tissue sections were first labeled with the anti-ubiquitin antibody (peroxidase-anti-peroxidase method), revealed with α-chloronaphthol, photographed, and unmounted. The α-choronaphthol polymerization product was then dissolved in alcohol and antibodies were subsequently eluted from the tissue sections by treatment for 1 hour with 0.1 mol/L glycine, pH 2.2. The tissue sections were labeled with the anti-cdk5 antibody by the same peroxidase-anti-peroxidase method.

Molecular Specificity of the cdk5 Antibodies

The specificity of the cdk5 immunolabeling was controlled by preabsorbing the C-8 antibody to cdk5 with the synthetic peptide used for generating this antibody; 2 μg of antibodies were absorbed overnight at 4°C with 10 μg of the peptide and the absorbed antibodies were used for immunolabeling of tissue sections. To test for a potential cross-reactivity with ubiquitin, 2 μg of antibodies to cdk5 were absorbed with up to 200 μg of ubiquitin from bovine red blood cells (Sigma Chemical Co., St. Louis, MO). As a control, the diluted anti-ubiquitin antibody was absorbed with the same quantity of ubiquitin. Control sections were also immunolabeled with the IgG fraction (2 μg/ml) of a nonimmune rabbit serum instead of antibodies to cdk5. These IgGs were purified from serum by chromatography on diethylaminoethyl (DEAE Affi-Gel blue, Bio-Rad Laboratories, Richmond, CA). Fractions collected from the column were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and pooled.

A search for potential sequence homology between the cdk5 peptide and other molecules was performed using the data bases PDB, SwissProt, PIR, SPUpdate, GPUpdate, and GenePept. A complete homology was observed between this peptide and an identical sequence present in human, bovine, and rat cdk5, in the 33-kd subunit of tau protein kinase II, and in a gene product of *Caenorhabditis elegans*; no other homologies were identified.

Western Blotting

Human brain extracts from a control subject, a patient with DLBD, and a patient with Alzheimer's disease were prepared by homogenizing tissue samples from the frontal cortex (0.5 g/ml) on ice in 50 mmol/L Tris-HCl, pH 7.4, 0.1 mol/L NaCl, 10 mmol/L EDTA, 1 mmol/L phenymethylsulfonylfluoride, 25 μg/ml leupeptin, and 25 μg/ml pepstatin. The homogenates were centrifuged for 30 minutes at 27,000 × *g* (4°C) and the supernatants (S1) and pellets (P1) were retained and kept at −20°C. Protein concentrations were determined by the method of Bradford. The samples were analyzed by electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate. After the electrophoresis, proteins were electrophoretically transferred on nitrocellulose sheets with a semi-dry electroblotter (Ancos, Denmark). For Western blotting, the nitrocellulose sheets were blocked in semi-fat dry milk (10% w/v) and incubated successively with the antibodies to cdk5, a donkey anti-rabbit antibody conjugated to biotin, and streptavidin conjugated to alkaline phosphatase. The alkaline phosphatase activity was detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Results

Lewy Bodies Are Labeled with Anti-cdk5 but Not with Anti-ERK-1 or Anti-cdc2p34 Antibodies

In DLBD, numerous cortical Lewy bodies were observed to be intensely labeled by the C-8 antibody to cdk5 (Figure 1, A and D, and Figure 2A). This antibody to cdk5 labeled cortical Lewy bodies in a homogeneous manner, ie, without a distinction between a core and a periphery. Although many of these cdk5-positive Lewy bodies had a spherical shape, a significant number of them had more irregular shapes. These Lewy bodies were mainly found in deep cortical layers. The cdk5 immunoreactivity of

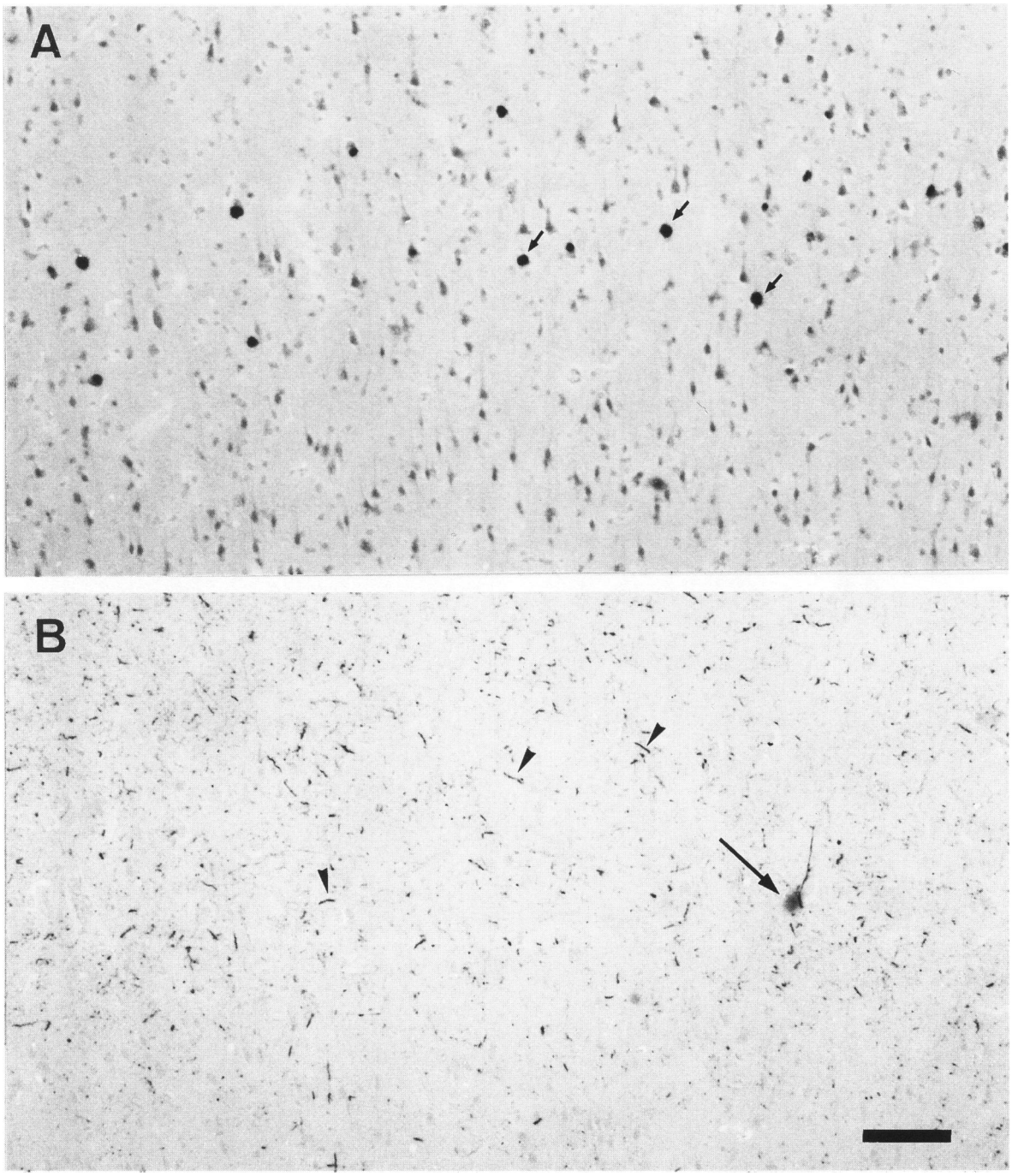
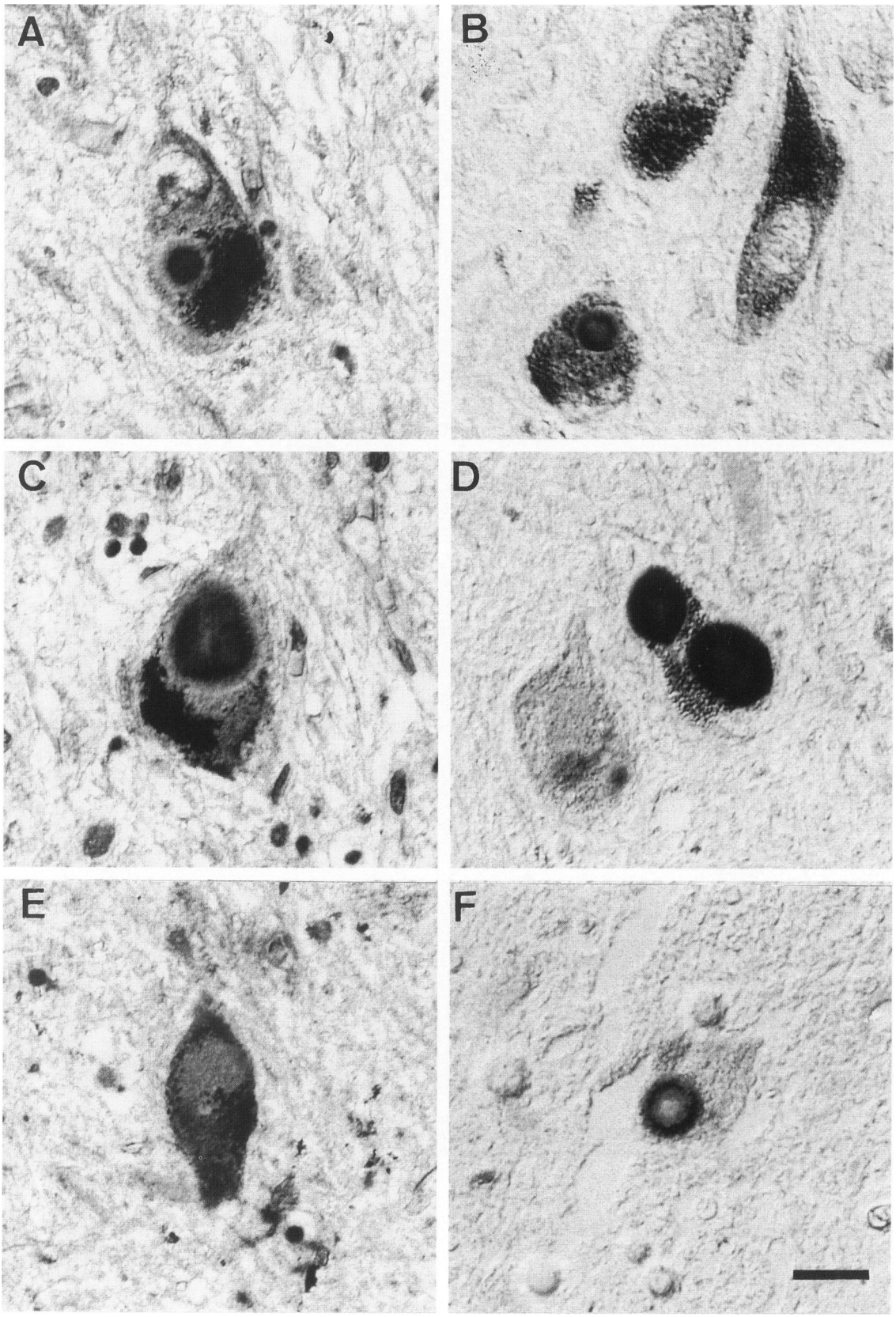


Figure 2. *Immunolabeling on adjacent tissue sections (temporal cortex) in DLBD with the C-8 antibody to cdk5* (A) *and the AT180 antibody to phosphorylated tau* (B). *The Lewy bodies* (small arrows *in* A) *are labeled by the cdk5 antibody but not by the AT180 antibody, whereas neurofibrillary tangles* (arrow *in* B) *and neuropil threads* (arrowheads *in* B) *are labeled by the AT180 antibody but not by the cdk5 antibody. Scale bar, 100 μm.*

Lewy bodies was observed only after proteolytic treatment of tissue sections and was strong in methacarn-fixed tissue but much weaker in formalin-fixed tissue. The H-291 antibody to cdk5 also labeled Lewy bodies although more weakly than the C-8 anti-cdk5 antibody (not shown).

Brainstem-type Lewy bodies in the substantia nigra and the locus coeruleus were also labeled by the antibodies to cdk5 in DLBD (Figure 3, A–E) and in Parkinson's disease (Figure 3F). The peripheral zone of brainstem-type Lewy bodies was often more strongly labeled than the inner core, the halo being weakly or


A
B
C
D
E
F

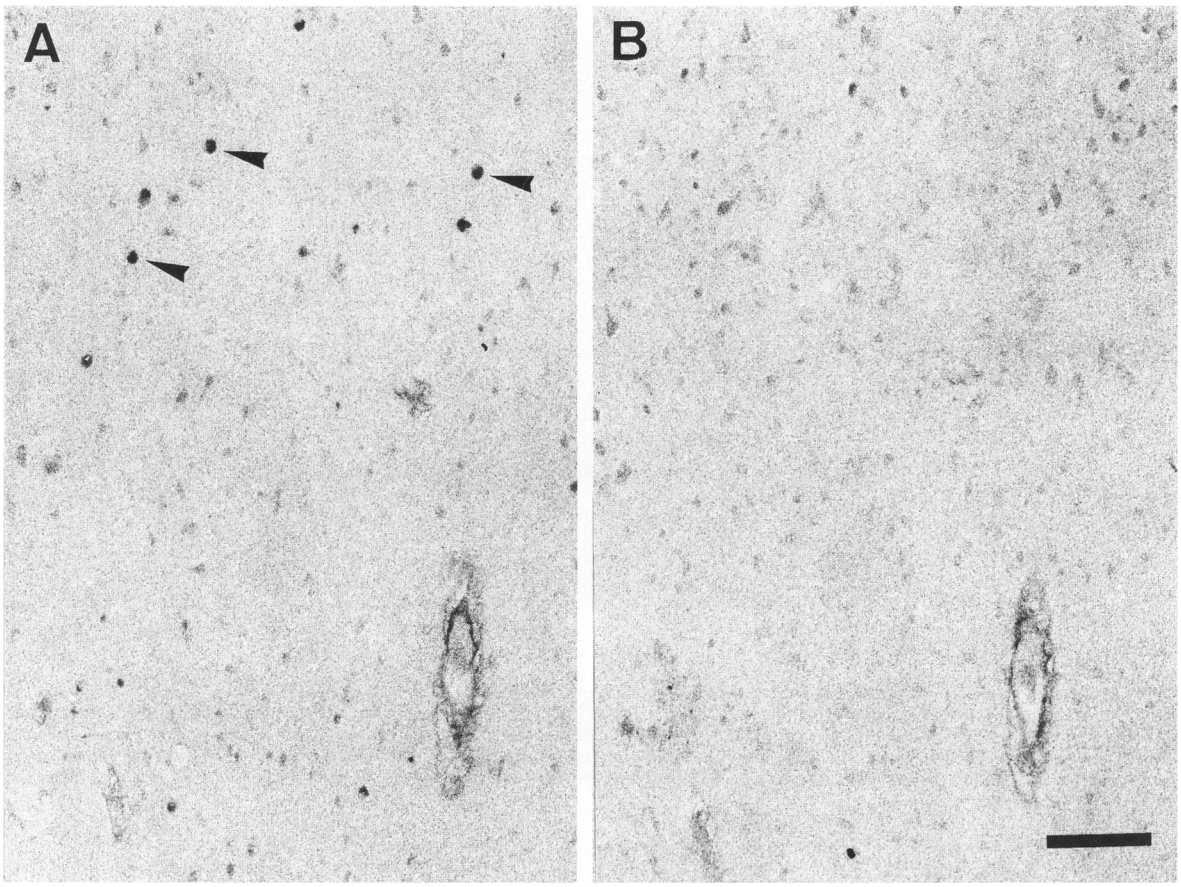


Figure 4. *Immunolabeling on adjacent tissue sections (temporal cortex) in DLBD with the C-8 antibody to cdk5* (A) *or with the same antibody preabsorbed with the cdk5 peptide* (B). *The labeling of Lewy bodies* (arrowheads *in* A) *is abolished after absorption of the cdk5 antibody. Scale bar, 100 μm.*

not labeled. Some brainstem-type Lewy bodies showed a homogeneous labeling. Pale bodies were weakly labeled by the cdk5 antibodies (Figure 3E).

The anti-ERK-1 and the anti-cdc2p34 antibodies did not label Lewy bodies. The anti-ERK-1 antibody labeled the perikarya of many small pyramidal neurons, mainly in cortical layers II and III, as reported in a previous study.[29] After proteolytic treatment, the anti-cdc2p34 antibody weakly labeled nuclei of neuronal and non-neuronal cells in a speckled manner; an immunoreactivity for cdc2p34 or a cdc2p34 homologue in nuclei of neurons and glial cells in adult mice has been recently observed,[30] although this immunoreactivity was located in the nucleoli of these cells.

Immunocytochemical Controls

The preabsorption of the C-8 antibody to cdk5 with the cdk5 peptide abolished the labeling of Lewy bodies (Figure 4). The preabsorption of cdk5 antibodies with ubiquitin did not decrease this staining whereas the labeling with the anti-ubiquitin antiserum was dramatically decreased by preabsorption with ubiquitin. No staining was observed when the IgGs purified from a nonimmune serum instead of cdk5 antibodies were used in the immunolabeling procedure.

Comparison of cdk5 and Ubiquitin Imunoreactivities of Lewy Bodies

In DLBD cases, the comparison of adjacent sections of temporal cortex labeled with ubiquitin or cdk5 antibodies showed that ubiquitin- and cdk5-immunoreactive Lewy bodies were found in the same cortical layers. The double immunolabeling demonstrated that most if not all ubiquitin-positive Lewy bodies

Figure 3. *Immunolabeling with the C-8 antibody to cdk5 in substantia nigra in DLBD* (A–E) *and in Parkinson's disease* (F). *cdk5-positive Lewy bodies show a homogeneously and strongly labeled core* (A *and* D), *sometimes with a weakly labeled halo* (A), *or a strongly labeled peripheral rim with a less intensely labeled core* (B, C, *and* F). *Pale bodies* (E) *in pigmented neurons are weakly labeled. Scale bar, 20 μm.*

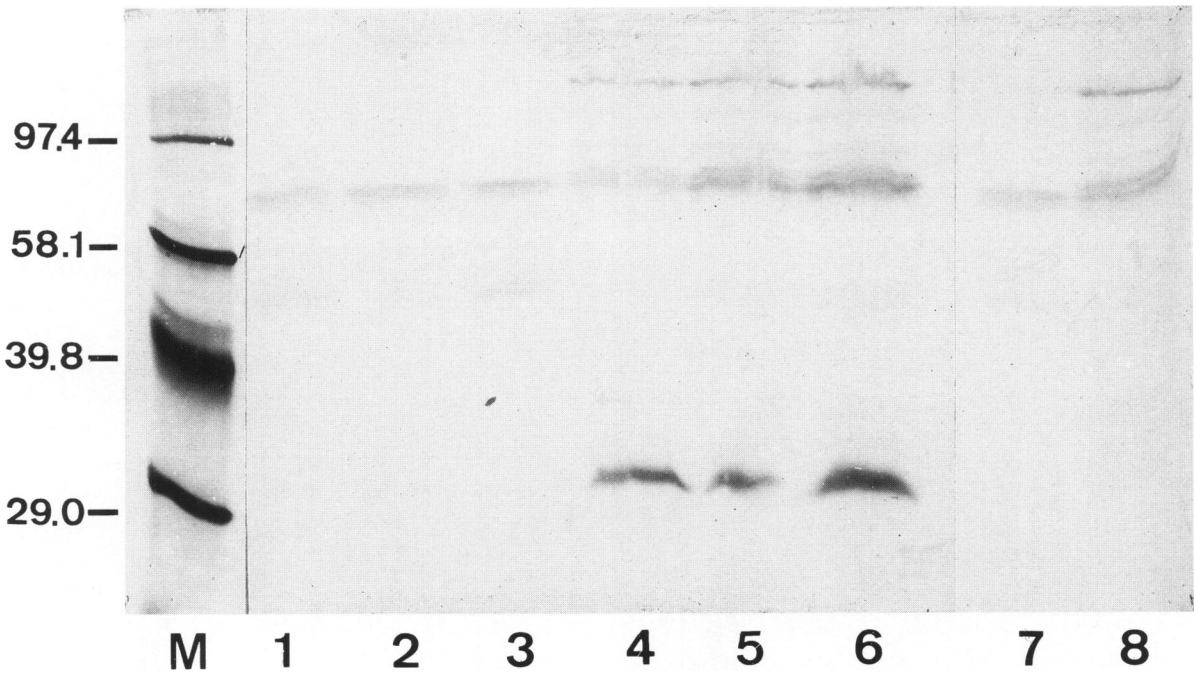


Figure 5. *Immunoblotting of the S1 fraction* (lanes 1, 2, *and* 3) *and the P1 fraction* (lanes 4, 5, *and* 6) *of brain homogenates (temporal cortex) from a control subject* (lanes 1 *and* 4), *a patient with Alzheimer's disease* (lanes 2 *and* 5), *and a patient with DLBD* (lanes 3 *and* 6). *The blot with* lanes 1 *to* 6 *was incubated with the C-8 antibody to cdk5.* Lanes 7 *and* 8 *are blots of the S1* (lane 7) *and P1* (lane 8) *fractions from a control subject incubated only with the detection system. Each lane was loaded with 100 μg of proteins. The* lane M *shows the position of molecular mass markers (the numbers on the left refer to their molecular mass in kilodaltons), ie, from top to bottom are phosphorylase b, catalase, alcohol dehydrogenase, and carbonic anhydrase.*

were also cdk5 immunoreactive; this was observed as well by the two procedures of double immunolabeling (Figure 1). Generally, a complete overlap between ubiquitin and cdk5 immunoreactivities in Lewy bodies was observed. In two DLBD patients, the number of ubiquitin- and cdk5-immunoreactive Lewy bodies were counted on two adjacent sections of the temporal cortex. The labeled Lewy bodies were counted in the same microscopic field corresponding to a length of 13.5 mm of cortical ribbon. In the first DLBD case, the numbers of cdk5- and ubiquitin-positive Lewy bodies were 140 and 122, respectively; in the second case, the numbers of cdk5- and ubiquitin-positive Lewy bodies were 45 and 43, respectively. In DLBD patients, ubiquitin-immunoreactive dystrophic neurites were also observed in the CA2/3 region, as reported previously[31]; these neurites were not labeled by the cdk5 antibodies.

Absence of Labeling of Lewy Bodies with Antibodies to Phosphorylated Tau

Cortical and brainstem-type Lewy bodies were not labeled by the antibodies AT8, AT180, or AT270 antibodies to phosphorylated tau. These antibodies, however, labeled neurofibrillary tangles, neuropil threads, and neuritic plaques in patients with Alzheimer's disease and in the DLBD case showing a moderate number of neurofibrillary lesions (Figure 2B).

Immunoblot Detection of cdk5 in Human Brain

The two rabbit affinity-purified antibodies (C-8 and H-291) to cdk5 labeled a single species with an apparent molecular mass of 33 kd in human brain homogenates (Figure 5). Other bands detected at higher molecular mass correspond to a nonspecific labeling by the detection system (eg, of biotinylated proteins), as they were also present when the primary antibody was omitted. The cdk5 species was much more abundant in the insoluble fraction (P1) than in the soluble fraction (S1) of brain homogenates. For a similar loading of proteins, the intensity of labeling of the cdk5 species was similar in control, in DLBD, and in Alzheimer's disease.

Discussion

In this study, we observed that Lewy bodies were consistently labeled by antibodies to the protein ki-

nase cdk5; cdk5-positive Lewy bodies were as numerous as ubiquitin-positive Lewy bodies, and the cdk5 immunoreactivity was co-localized with the ubiquitin immunoreactivity in Lewy bodies. As ubiquitin immunoreactivity is considered a robust marker of Lewy bodies, allowing the detection of most if not all of them, this suggests that the majority of Lewy bodies were cdk5 positive. This cdk5 immunoreactivity most probably does not result from a nonspecific labeling as it was abolished by preabsorption by the corresponding antigen. Also this labeling does not seem to result from a cross-reactivity with another protein species present in human brain, as the cdk5 antibodies reacted only with a species of 33 kd on Western blots, ie, the reported molecular mass of cdk5.[32,33]

The cdc2 and cdc2-related kinases, designated as cyclin-dependent kinases on the basis of their requirement for association with regulatory subunits known as cyclins, are known to play a key role in the regulation of the cell cycle.[34–36] cdk5 is a protein serine/threonine kinase that has been found to share high homology with cdc2 and cdc2-related kinases. However, contrary to cdc2 and cdk2, which are abundant during brain development and are weakly expressed in the adult brain, the expression of cdk5 increases during brain development and is maximal in the adult brain.[33] cdk5 was also reported to be expressed and active in differentiated neurons,[33,36] whereas cdc2 kinase is abundant in neuroblasts and dividing precursors.[25,33]

The presence of a strong cdk5 immunoreactivity in Lewy bodies suggests that this protein kinase might be responsible for the phosphorylation of molecular components of Lewy bodies. Neurofilament proteins are considered as the main building blocks of Lewy bodies, and phosphorylated neurofilament epitopes have been repeatedly detected in Lewy bodies. Recently, a biochemical analysis of isolated Lewy bodies showed them to be composed of a main protein species of 68 kd, immunoreactive with antibodies to phosphorylated neurofilaments.[37] It seems thus reasonable to speculate that cdk5 might be involved in the phosphorylation of neurofilament in Lewy bodies. Actually, cdk5 purified from bovine brain has been reported to phosphorylate the 200-kd neurofilament protein NF-H *in vitro*.[32] We also observed that cdk5 was more abundant in the particulate fraction (ie, in a fraction rich in neurofilaments) than in the soluble fraction of brain homogenates. Interestingly, most subcortical Lewy bodies showed a peripheral labeling with the cdk5 antibody. A similar staining pattern has been observed with antibodies to tail domains of NF-M and NF-H,[8] containing the multiphosphorylation repeats. This suggests that cdk5 might be concentrated in the peripheral zone of the Lewy-body-containing phosphorylation domains of neurofilaments. Additional studies, eg, of isolated Lewy bodies, will be necessary to establish whether cdk5 is an integral component of Lewy bodies. However, if cdk5 is loosely associated with Lewy bodies, the enzyme might not co-purify with them.

The role of phosphorylation in the initiation of Lewy body formation is unknown. Pale bodies, considered as potential precursors of Lewy bodies, have been reported to be neurofilament and ubiquitin immunoreactive.[38] Pale bodies were also labeled by the anti-cdk5 antibody, thus suggesting that this enzyme might be already present in Lewy bodies at their early stage of formation.

It should be pointed out that detection of a cdk5 immunoreactivity in Lewy bodies does not allow one to infer that this enzyme is in an active state. The activation of cdk5 needs its association with a regulatory subunit[35] called p35,[39,40] as is the case for cdc2 and cdk2, the activation of which depends on their binding to cyclins. The p35 regulatory subunit, like cdk5, seems to be highly expressed in postmitotic neurons.[39,40]

The weak cdk5 immunoreactivity in normal cells and even in cytoplasmic areas adjacent to Lewy bodies contrasts with the strong cdk5 immunoreactivity in Lewy bodies. This strong labeling can result from a high focal concentration of this enzyme in Lewy bodies or can be secondary to a trapping phenomenon. Alternatively, a differential sensitivity to fixation of normal or Lewy-body-associated cdk5 epitopes (as observed for normal tau *versus* PHF-tau in neurofibrillary tangles) might explain the intense staining of Lewy bodies. Although the two anti-cdk5 antibodies labeled Lewy bodies, the labeling was stronger with the anti-peptide antibody raised to a sequence mapping at the carboxy terminus of cdk5, possibly indicating that only a fragment of cdk5 is present in Lewy bodies.

By Western blot analysis of cortical samples, we did not observe major differences in the level of cdk5 expression between DLBD cases, Alzheimer cases, and controls. The strong cdk5 immunoreactivity of Lewy bodies might thus result from a redistribution of this enzyme in cells containing these inclusions. Alternatively, a moderate change in cdk5 expression couild occur in neurons containing Lewy bodies but would not be detected by global analysis of a cortical tissue sample, as many neurons in the cortex do not contain Lewy bodies.

Other protein kinases might also be involved in the phosphorylation of Lewy body components; eg, a

labeling of Lewy bodies with antibodies to the calcium/calmodulin-dependent protein kinase II has previously been reported.[41] Our results, however, point to the presence of a selective kinase in Lewy bodies as these inclusions were not labeled by antibodies to the cdc2p34 kinase or to the ERK-1 kinase in our conditions of tissue processing.

PHF-tau species are highly phosphorylated tau species found in paired helical filaments in Alzheimer's disease, and many phosphorylated sites on tau molecules can be generated *in vitro* by several protein kinases with serine/threonine specificities such as extracellular regulated kinases,[42–44] GSK3β kinase (also called tau protein kinase I),[45,46] and cdk5 (also called tau protein kinase II).[47–49] Specific phosphorylation sites on the tau molecules can be recognized by phosphorylation-sensitive antibodies, such as the AT8, AT180, and AT270 antibodies used in this study.[26,27] Lewy bodies were not labeled by these phosphorylation-sensitive antibodies, suggesting that phosphorylated tau is not present in these inclusions. An absence of tau immunoreactivity in Lewy bodies had also been reported in most,[14,15] but not all,[50] previous studies. These immunocytochemical results are in agreement with biochemical studies showing that only a weak level of phosphorylated tau species, by comparison with Alzheimer's disease, could be detected in DLBD.[51] Some antibodies to phosphorylated neurofilament label both neurofibrillary tangles and Lewy bodies, most probably because these antibodies recognize phosphorylated epitopes shared by tau and neurofilament proteins (eg, the KSPV sequences).[52–57]

In conclusion, the present immunocytochemical study provides evidence for a selective concentration of the protein kinase cdk5 in Lewy bodies. This observation suggests that cdk5 might be an enzyme involved in the generation of abnormally phosphorylated molecules in neurons developing Lewy bodies. Additional studies will, however, be necessary to confirm by other methods the presence of cdk5 or a fragment of it in Lewy bodies and to determine whether the kinase is enzymatically active in these inclusions.

Acknowledgments

We are grateful to Dr. A. Vandevoorde (Innogenetics) for providing us with the AT8, AT180, and AT270 antibodies. We also thank Mr. J. L. Conreur for valuable technical assistance.

References

1. Gibb WRG: Idiopathic Parkinson's disease and the Lewy body disorders. Neuropathol Appl Neurobiol 1986, 12:223–234
2. Trojanowski JQ, Schmidt ML, Shin R-W, Bramblett GT, Rao D, Lee VM-Y: Altered tau and neurofilament proteins in neurodegenerative diseases: diagnostic implications for Alzheimer's disease and Lewy body dementias. Brain Pathol 1993, 3:45–54
3. Pollanen MS, Dickson DW, Bergeron C: Pathology and biology of the Lewy body. J Neuropathol Exp Neurol 1993, 52:183–191
4. Gibb WRG, Esiri MM, Lees AJ: Clinical and pathological features of diffuse cortical Lewy body disease (Lewy body dementia). Brain 1985, 110:1131–1153
5. Dickson DW, Davies P, Mayeux R, Crystal H, Horoupian DS, Thompson A, Goldman JE: Diffuse Lewy body disease: neuropathological and biochemical studies of six patients. Acta Neuropathol (Berl) 1987, 75:8–15
6. Perry RH, Irving D, Blessed G, Fairbairn A, Perry EK: Senile dementia of Lewy body type: a clinically and neuropathologically distinct form of Lewy body dementia in the elderly. J Neurol Sci 1990, 95:119–139
7. Hansen L, Salomon D, Galasko D, Masliah E, Katzman R, DeTeresa R, Thal L, Pay MM, Hofstetter R, Klauber M, Rice V, Butters N, Alford M: The Lewy body variant of Alzheimer's disease: a clinical and pathological entity. Neurology 1990, 40:1–8
8. Hill WD, Lee VMY, Hurtig HI, Murray JM, Trojanowski JQ: Epitopes located in spatially separate domains of each neurofilament subunit are present in Parkinson's disease Lewy bodies. J Comp Neurol 1991, 309:150–160
9. Galloway PG, Mulvihill P, Perry G: Filaments of Lewy bodies contain insoluble cytoskeletal elements. Am J Pathol 1992, 140:809–822
10. Goldman JE, Yen SH, Chiu FC, Peress NS: Lewy bodies of Parkinson's disease contain neurofilament antigens. Science 1983, 221:1082–1084
11. Kahn J, Anderton BH, Gibb WRG, Lees AJ, Wells FR, Marsden CD: Neuronal filaments in Alzheimer's, Pick's, and Parkinson's diseases. N Engl J Med 1985, 313: 520–521
12. Forno LS, Sternberger LA, Sternberger NH, Strefling AM, Swanson K, Eng LF: Reaction of Lewy bodies with antibodies to phosphorylated and non-phosphorylated neurofilaments. Neurosci Lett 1986, 64:253–258
13. Pappolia MA: Lewy bodies of Parkinson's disease. Arch Pathol Lab Med 1986, 110:1160–1163
14. Galloway PG, Grundke-Iqbal I, Iqbal K, Perry G: Lewy bodies contain epitopes both shared and distinct from Alzheimer neurofibrillary tangles. J Neuropathol Exp Neurol 1988, 47:654–663
15. Bancher C, Lassmann H, Budka H, Jellinger K, Grundke-Iqbal I, Iqbal K, Wiche G, Seitelberger F, Wisniewski HM: An antigenic profile of Lewy bodies: immunocytochemical indication for protein phosphory-

lation and ubiquitination. J Neuropathol Exp Neurol 1989, 48:81–93
16. Schmidt ML, Murray J, Lee VMY, Hill WD, Wertkin A, Trojanowski JQ: Epitope map of neurofilament protein domains in cortical and peripheral nervous system Lewy bodies. Am J Pathol 1991, 139:53–65
17. Kuzuhara S, Mori H, Izumiyama N, Yoshimura M, Ihara Y: Lewy bodies are ubiquitinated: a light and electron microscopic immunocytochemical study. Acta Neuropathol (Berl) 1988, 75:345–353
18. Love S, Saitoh T, Quijada S, Cole GM, Terry RD: Alz-50, ubiquitin and tau immunoreactivity of neurofibrillary tangles, Pick bodies and Lewy bodies. J Neuropathol Exp Neurol 1988, 47:393–405
19. Lowe J, Blanchard A, Morrell K, Lennox G, Reynolds L, Billett M, Landon M, Mayer RJ: Ubiquitin is a common factor in intermediate filament inclusion bodies of diverse type in man, including those of Parkinson's disease, Pick's disease, and Alzheimer's disease, as well as Rosenthal fibres in cerebellar astrocytomas, cytoplasmic bodies in muscle, and Mallory bodies in alcoholic liver disease. J Pathol 1988, 155:9–15
20. Manetto V, Perry G, Tabaton M, Mulvihill P, Fried VA, Smith HT, Gambetti P, Autilio-Gambetti L: Ubiquitin is associated with abnormal cytoplasmic filaments characteristic of neurodegenerative diseases. Proc Natl Acad Sci USA 1988, 85:4501–4505
21. Forno LS: The neuropathology of Parkinson's disease: the Lewy body as a clue to the nerve cell degeneration. Progress in Parkinson's Research. Edited by F Hefti, JW Weiner. New York, Plenum Press, 1988, pp 11–21
22. Kahn J, Anderton BH, Brion JP, Cowell I, Dale G, Kilford L, Marsden CD, Parke J, Robinson P Immunocytochemical staining of Lewy bodies with antibodies to cytoskeletal proteins. Recent Developments in Parkinson's Disease. Edited by S Fahn, CD Marsden, D Calne, M Godstein. Florham Park, New Jersey, Macmillan, 1987, pp 15–23
23. Nixon RA, Sihag RK: Neurofilament phosphorylation: a new look at regulation and function. Trends Neurosci 1991, 14:501–506
24. Braak H, Braak E: Neuropathological staging of Alzheimer-related changes. Acta Neuropathol (Berl) 1991, 82:239–259
25. Brion JP, Octave JN, Couck AM: Distribution of the phosphorylated microtubule-associated protein tau in developing cortical neurons. Neuroscience 1994, 63: 895–909
26. Goedert M, Jakes R, Crowther RA, Six J, Lübke U, Vandermeeren M, Cras P, Trojanowski JQ, Lee VM-Y: The abnormal phosphorylation of tau protein at Ser-202 in Alzheimer disease recapitulates phosphorylation during development. Proc Natl Acad Sci USA 1993, 90:5066–5070
27. Goedert M, Jakes R, Crowther RA, Cohen P, Vanmechelen E, Vandermeeren M, Cras P: Epitope mapping of monoclonal antibodies to the paired helical filaments of Alzheimer's disease: identification of phosphorylation sites in tau protein. Biochem J 1994, 301: 871–877
28. Brion JP, Guilleminot J, Couchie D, Nunez J: Both adult and juvenile tau microtubule-associated proteins are axon specific in the developing and adult rat cerebellum. Neuroscience 1988, 25:139–146
29. Hyman BT, Elvhage TE, Reiter J: Extracellular signal regulated kinases: localization of protein and mRNA in the human hippocampal formation in Alzheimer's disease. Am J Pathol 1994, 144:565–572
30. Ino H, Mochizuki T, Yanaihara N, Chiba T: $p34^{cd2}$ homologue is located in nucleoli of the nervous and endocrine systems. Brain Res 1993, 614:131–136
31. Dickson DW, Schmidt ML, Lee VM-Y, Zhao M-L, Yen S-H, Trojanowski JQ: Immunoreactivity profile of hippocampal CA2/3 neurites in diffuse Lewy body disease. Acta Neuropathol (Berl) 1994, 87:269–276
32. Lew J, Winkfein RJ, Paudel HK, Wang JH: Brain proline-directed protein kinase is a neurofilament kinase which displays high sequence homology to p34cdc2. J Biol Chem 1992, 267:25922–25926
33. Tsai LH, Takahashi T, Caviness VS Jr, Harlow E: Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. Development 1993, 119:1029–1040
34. Meyerson M, Enders GH, Wu CL, Su LK, Gorka C, Nelson C, Harlow E, Tsai LH: A family of human cdc2-related protein kinases. EMBO J 1992, 11:2909–2917
35. Lew J, Beaudette K, Litwin CM, Wang JH: Purification and characterization of a novel proline-directed protein kinase from bovine brain. J Biol Chem 1992, 267: 13383–13390
36. Hellmich MR, Pant HC, Wada E, Battey JF: Neuronal cdc2-like kinase: a cdc2-related protein kinase with predominantly neuronal expression. Proc Natl Acad Sci USA 1992, 89:10867–10871
37. Pollanen MS, Bergeron C, Weyer L: Characterization of a shared epitope in cortical Lewy body fibrils and Alzheimer paired helical filaments. Acta Neuropathol (Berl) 1994, 88:1–6
38. Dale GE, Probst A, Luthert P, Martin J, Anderton BH, Leigh PN: Relationships between Lewy bodies and pale bodies in Parkinson's disease. Acta Neuropathol (Berl) 1992, 83:525–529
39. Tsai L-H, Delalle I, Caviness VS Jr, Chae T, Harlow E: p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. Nature 1994, 371:419–423
40. Lew J, Huang Q-Q, Qi Z, Winkfein RJ, Aebersold R, Hunt T, Wang JH: A brain-specific activator of cyclin-dependent kinase 5. Nature 1994, 371:423–426
41. Iwatsubo T, Nakano I, Fukunaga K, Miyamoto E: Ca^{2+}/calmodulin-dependent protein kinase II immunoreactivity in Lewy bodies. Acta Neuropathol (Berl) 1991, 82:159–163
42. Drewes G, Lichtenberg-Kraag B, Döring F, Mandelkow E-M, Biernat J, Goris J, Dorée M, Mandelkow E: Mitogen activated protein (MAP) kinase transforms tau pro-

tein into an Alzheimer-like state. EMBO J 1992, 11: 2131–2138
43. Ledesma MD, Correas I, Avila J, Díaz-Nido J: Implication of brain cdc2 and MAP2 kinases in the phosphorylation of tau protein in Alzheimer's disease. FEBS Lett 1992, 308:218–224
44. Goedert M, Cohen ES, Jakes R, Cohen P: p42 MAP kinase phosphorylation sites in microtubule-associated protein tau are dephosphorylated by protein phosphatase 2A1: implications for Alzheimer's disease. FEBS Lett 1992, 312:95–99
45. Hanger DP, Hughes K, Woodgett JR, Brion JP, Anderton BH: Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filaments epitopes and neuronal localization of the kinase. Neurosci Lett 1992, 147:58–62
46. Mandelkow E-M, Drewes G, Biernat J, Gustke N, Van Lint J, Vandenheede JR, Mandelkow E: Glycogen synthase kinase-3 and the Alzheimer-like state of microtubule-associated protein tau. FEBS Lett 1992, 314:315–321
47. Paudel HK, Lew J, Ali Z, Wang JH: Brain proline-directed protein kinase phosphorylates tau on sites that are abnormally phosphorylated in tau associated with Alzheimer's paired helical filaments. J Biol Chem 1993, 268:23512–23518
48. Baumann K, Mandelkow E-M, Biernat J, Piwnica-Worms H, Mandelkow E: Abnormal Alzheimer-like phosphorylation of tau-protein by cyclin-dependent kinases cdk2 and cdk5. FEBS Lett 1993, 336:417–424
49. Kobayashi S, Ishiguro K, Omori A, Takamatsu M, Arioka M, Imahori K, Uchida T: A cdc2-related kinase PSSALRE/cdk5 is homologous with the 30 kDa subunit of tau protein kinase II, a proline-directed protein kinase associated with microtubule. FEBS Lett 1993, 335:171–175
50. Galloway PG, Bergeron C, Perry G: The presence of tau distinguishes Lewy bodies of diffuse Lewy body disease from those of idiopathic Parkinson disease. Neurosci Lett 1989, 100:6–10
51. Harrington CR, Perry RH, Perry EK, Hurt J, McKeith IG, Roth M, Wischik CM: Senile dementia of Lewy body type and Alzheimer type are biochemically distinct in terms of paired helical filaments and hyperphosphorylated tau protein. Dementia 1994, 5:215–228
52. Miller CCJ, Brion JP, Calvert R, Chin TK, Eagles PAM, Downes MJ, Haugh M, Kahn J, Probst A, Ulrich J, Anderton BH: Alzheimer paired helical filaments share epitopes with neurofilaments side arms. EMBO J 1986, 5:269–276
53. Nukina N, Kosik KS, Selkoe DJ: Recognition of Alzheimer paired helical filaments by monoclonal neurofilament antibodies is due to crossreaction with tau protein. Proc Natl Acad Sci USA 1987, 84:3415–3419
54. Ksiezak-Reding H, Dickson DW, Davies P, Yen SH: Recognition of tau epitopes by anti-neurofilament antibodies that bind to Alzheimer neurofibrillary tangles. Proc Natl Acad Sci USA 1987, 84:3410–3414
55. Lee VMY, Otvos L, Schmidt ML, Trojanowski JQ: Alzheimer disease tangles share immunological similarities with multiphosphorylation repeats in the two large neurofilament proteins. Proc Natl Acad Sci USA 1988, 85:7384–7388
56. Lichtenberg-Kraag B, Mandelkow E-M, Biernat J, Steiner B, Schröter C, Gustke N, Meyer HE, Mandelkow E: Phosphorylation-dependent epitopes of neurofilament antibodies on tau protein and relationship with Alzheimer tau. Proc Natl Acad Sci USA 1992, 89:5384–5388
57. Brion JP, Couck AM, Robertson J, Loviny TLF, Anderton BH: Neurofilament monoclonal antibodies RT97 and 8D8 recognize different modified epitopes in PHF-tau in Alzheimer's disease. J Neurochem 1993, 60: 1372–1382