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p35^{nck5a} and cyclin-dependent kinase 5 colocalize in Lewy bodies of brains with Parkinson's disease

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Abstract We examined the immunohistochemical localization of p35^{nck5a}, the regulatory subunit of cyclin-dependent kinase 5 (cdk5), in brains obtained postmortem from patients with Parkinson's disease (PD) and controls. We found p35^{nck5a} immunoreactivity in Lewy bodies (LB) in the substantia nigra, locus ceruleus, and neocortex of brains from patients with PD. In addition, p35^{nck5a} was colocalized with cdk5 immunoreactivity in LB. Cdk5 is the kinase most likely to be responsible for the phosphorylation of neurofilament proteins of LB, which is a crucial step for the formation of the insoluble LB fibrils. Since p35^{nck5a} regulates the catalytic activity of cdk5 by forming a heterodimer with cdk5, the present results strongly support the hypothesis that a cdk5-p35^{nck5a} complex is involved in the formation of LB fibrils.

Key words Cyclin-dependent kinase 5 · p35^{nck5a} · cdk5-p35^{nck5a} complex · Lewy body · Parkinson's disease

Introduction

The occurrence of Lewy bodies (LB), neuronal, eosinophilic and hyaline inclusions, is a major histological hallmark of Parkinson's disease (PD) [16]. Ultrastructural studies have shown that LB are composed of aggregates of 8- to 10-nm fibrils [16]. Accumulated evidence has suggested that neurofilament (NF) proteins are integral components of LB fibrils [2, 3, 17]. High (NF-H), intermediate (NF-M), and low (NF-L) molecular weight protein subunits have been identified in LB fibrils [2, 16, 21]. Among these, NF-H and NF-M in LB are abnormally phosphorylated [16, 18, 21]. Although non-phosphorylated NF proteins have been found in LB fibrils, it has been hypothesized that the phosphorylation of NF proteins is a crucial step for the conversion of NF proteins into the insoluble fibrils of LB [15, 16, 18]. Recently cyclin-dependent kinase 5 (cdk5), a kinase that is capable of phosphorylating the lysine-serine-proline (KSP) sequence in NF-H and NF- M [4, 6, 8, 12, 19], was found immunohistochemically in the LB of PD brains [1]. Cdk5 needs to form a heterodimer with p35^{nck5a}, the regulatory subunit of cdk5, to assume an active form [10, 22, 23]. Therefore, if cdk5 is actually involved in phosphorylation of NF in LB, p35^{nck5a} may be associated with LB. Thus, to address this assumption, we examined immunohistochemically whether p35^{nck5a} is associated with LB.

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Materials and methods

Brain specimens obtained postmortem from five patients with idiopathic PD and five control specimens from patients without neurological or neuropathological abnormalities were studied. The mean age of PD patients was 74.5 years and the mean disease duration was 7.0 years. Paraffin sections, 6 μm thick, were prepared from blocks fixed in formalin for 2 weeks at room temperature. Histological samples from the midbrain and pons from the brains of both the control and PD subjects were examined.

Immunohistochemical staining of p35^{nck5a} was performed using a polyclonal antibody raised against a synthetic peptide of the amino-terminal region (Lys^{13}-Thr^{28}-Cys) of human p35^{nck5a} [10, 22, 23]. The synthetic peptide was conjugated with keyhole limpet hemocyanin using 6-maleimido-caproic acid *N*-hydroxysuccinimide ester. After collecting the preimmune serum, the conjugated peptide emulsified with Freund's complete adjuvant was injected intramuscularly into New Zealand white rabbits. After several booster injections with the conjugated peptide emulsified with Freund's incomplete adjuvant at 2-week intervals, sera were collected and affinity-purified using thiopropyl-Sepharose 6B (Pharmacia, USA). Briefly, 2.5 mg synthetic peptide was coupled to 2 ml gel. After deactivation of the remaining active sites, the gel was equilibrated in a physiological buffer at pH 7.2 at room temperature. Five milliliters of crude serum was applied to the gel. After washing, the absorbed protein was eluted with a glycine-HCl buffer at low pH and collected in a sodium hydroxide solution designed to neutralize the elution buffer.

Paraffin-embedded sections were deparaffinized and incubated in 0.3% hydrogen peroxide in 0.1 M phosphate-buffered saline (PBS) at room temperature for 30 min to block the endogenous

peroxidase. Primary antibodies diluted in PBS were applied onto sections and maintained overnight at room temperature. The affinity-purified antibody against the amino-terminal region of human $p35^{nck5a}$ (p35N) was diluted to 1:100 as a primary antibody for immunohistochemistry. After washing with PBS, sections were incubated with biotinylated anti-rabbit IgG (Vector, USA) diluted (1:200) in PBS for 1 h at room temperature. After rinsing with PBS, they were reacted with an avidin-biotin complex (ABC; Vector) diluted (1:200) in PBS for 1 h at room temperature. Sections were then washed in 0.05 M TRIS-HCl buffer (TB) and stained in 0.02% diaminobenzidine tetrahydrochloride (Dojin, Japan), containing 0.005% hydrogen peroxide (Nacalai Tesque, Japan) and 0.6% nickel acetate (Nacalai Tesque) in 0.05 M TB. Some p35N-immunostained sections were counterstained with hematoxylin and eosin (H&E). To localize cdk5, we immunostained paraffin-embedded sections using anti-cdk5 antibody (C-8, Santa-Cruz Biotechnology, USA) as a primary antibody [13]. To determine the anatomical relationship between $p35^{nck5a}$ and cdk5 immunoreactivities in patients with PD, we examined 2-μm-thick consecutive sections prepared from blocks of the locus ceruleus and substantia nigra from two cases with PD; $p35^{nck5a}$ and cdk5 were immunostained alternatively in adjacent sections.

Immunohistochemical controls were performed by incubating paraffin-embedded sections in preimmune serum for p35N or normal rabbit serum for C-8, instead of primary antibodies. Sections were then treated with biotinylated secondary antibodies and stained as described above. p35N was preabsorbed with the synthetic peptides at concentration of 10 μg/ml and immunostained in an similar manner. For C-8, the diluted antibody was preabsorbed with an excess of the antigen, 10 μg the control peptide (C-8p, Santa Cruz Biotechnology, USA) in 0.2 mg IgG/ml, and used as primary antibody. In these sections, no specific staining was observed.

Characterization of p35N was done using Western blot analysis. Briefly, specimens from the middle frontal gyrus of an autopsy case who died of middle cerebral arterial occlusion (76-year-old man) were homogenized, electrophoresed using Mini-Protean II Ready Gels J (Bio-Rad, USA) and blotted onto a nitrocellulose membrane. The blotted membrane was incubated in p35N diluted (1:20) in PBS containing 0.3% Triton X-100, 2% bovine serum albumin and 5% non-fat milk for 4 h at room temperature. They were then stained using the ABC method as described. As controls, other blotted membranes were incubated in preimmune serum or p35N which was preabsorbed with the antigen as described above.

Results

Western blot analysis showed that p35N recognized a single band of 35 kDa (Fig. 1), in accordance with the predicted molecular mass of human $p35^{nck5a}$ [10, 22]. Positive staining of the 35 kDa band was not observed when the primary antibody was replaced by the preimmune serum or the p35N was preabsorbed with the antigen (Fig. 1). LB were observed in H&E-stained sections of the brain stem nuclei, including the substantia nigra and locus ceruleus, in all PD cases, but were not present in control cases.

In control cases, p35N immunolabeled some neuronal somata (Fig. 2A), varicose fibers (Fig. 2B) and transverse axons. The transverse axons were mainly observed in the white matter, although some positive axons also were found in the gray matter.

In the brains from patients with PD, $p35^{nck5a}$ immunoreactivity was observed in the neuronal somata, varicose fibers, and transverse axons, similar to that seen in controls. However, the conspicuous finding in PD was

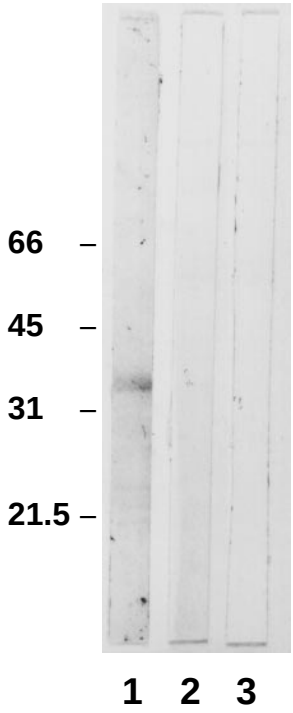

Fig. 1 Western blot analysis of p35N in the postmortem human brain tissue. *Lane 1* p35N (1:20), *lane 2* p35N (1:20) preabsorbed with the antigen at a concentration of 0.1 mg/ml, *lane 3* preimmune serum (1:500)

that LB were immunostained (Fig. 2C). Double staining with p35N and H&E showed that approximately 50% of LB were immunopositive for p35N in the substantia nigra and locus ceruleus. Adjacent sections alternately stained with p35N and C-8 showed the colocalization of $p35^{nck5a}$ and cdk5 (Fig. 2C–F). As seen in Fig. 2E and F, LB that are intensively positive for $p35^{nck5a}$ show strong cdk5 immunoreactivity and vice versa. In the insular cortex of brains with PD, cortical LBs were occasionally observed in the neuronal somata. As seen in Fig. 3, the positive cortical LB showed homogeneous diffuse staining, which is characteristic of the cortical LB [15, 17].

The staining intensity of p35N in neuronal somata of the substantia nigra and locus ceruleus appeared mostly similar to that in controls. In the immunohistochemical controls, preabsorption of p35N with the antigens abolished specific staining (data not shown).

Discussion

p35N recognized a single band of 35 kDa in Western blots of the human brain homogenates. The primary antibody preabsorbed with the antigen did not show any specific staining. Thus, the p35N used appears to stain specifically $p35^{nck5a}$ in the human brain. Recently, an isoform of $p35^{nck5a}$, $p39^{nck5ai}$, has been cloned and sequenced [20]. The isoform showed a sequence similarity to $p35^{nck5a}$ with 57% amino acid identity [20]. The present synthetic peptide was designed as an antigen to eliminate the immunological cross reaction with the isoform by selecting the region of lower sequence homology with 31% identity. Thus, it is unlikely that p35N recognizes $p39^{nck5ai}$.

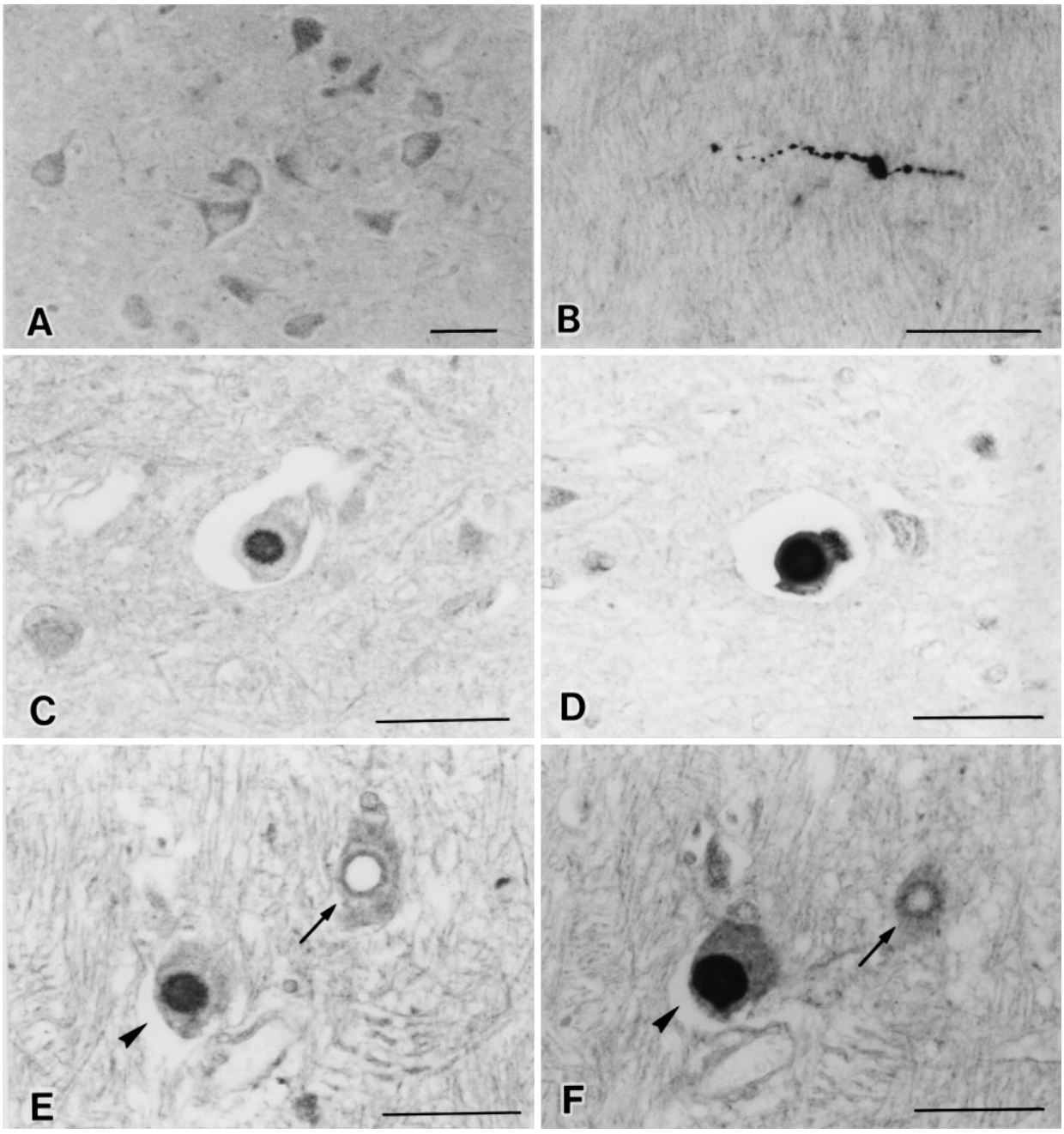

Fig. 2 **A, B** p35N-immunopositive neuronal somata (**A**) and varicose fibers (**B**) in a control case (71-year-old man). **C** In PD, $p35^{nck5a}$-immunoreactive LB is observed (76-year-old man). **D** In the adjacent section to that shown in **C**, the same LB also shows cdk5 immunoreactivity. **E**, **F** Two $p35^{nck5a}$-immunoreactive LB (**E**) show cdk5 immunoreactivity (**F**). Note that the $p35^{nck5a}$-intensive LB (*arrowhead*) shows stronger cdk5 immunoreactivity than the LB (*arrow*) that is less intensely positive for $p35^{nck5a}$ (74-year-old man, PD case) (*PD* Parkinson's disease, *LB* Lewy body, *cdk5* cyclin-dependent kinase 5). *Bars* **A–F** = 20 μm

The neuronal localization of $p35^{nck5a}$ seen in the present study corresponds to the neuronal expression of the messenger RNA (mRNA) of $p35^{nck5a}$ [22]. Unexpectedly, we have found varicose fibers, in addition to the neuronal somata and transverse axons, that were immunopositive for $p35^{nck5a}$. In this context, the cdk5/$p35^{nck5a}$ complex has been shown to be essential for neurite outgrowth during neuronal differentiation [14]. Since the varicose fibers represent presynaptic axon terminals, $p35^{nck5a}$ in varicose fibers might be associated with some plastic reorganization process of axon terminals.

Cyclin-dependent kinases are essential for cell-cycle control [9, 11]. On the basis of sequence homology screening, seven types of cdk have been identified [9, 11]. Among these protein kinases, cdk5 is unique in that mRNA for this kinase has been shown to be relatively abundant in neurons that are post-mitotic and in the cell cycle of G_0 [9, 11]. Another unique characteristic is that the protein kinase activity of cdk5 in brain is regulated by

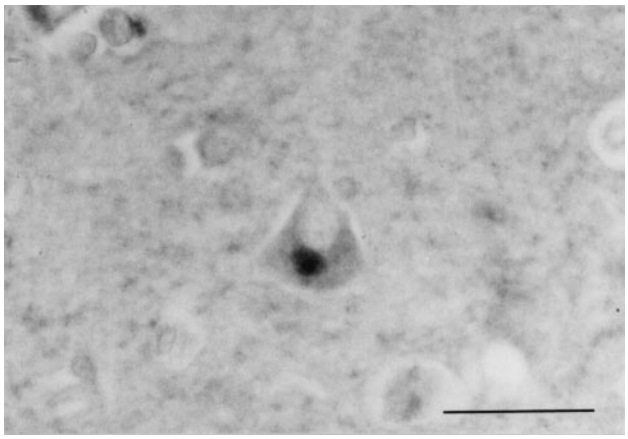

Fig. 3 A cortical LB is also immunoreactive for p35N in the insular cortex of a patient with PD (79-year-old woman). *Bar* = 20 μm

a regulatory subunit, $p35^{nck5a}$, which is distinct from cyclins [10, 22, 23]. Members of the cdk family need to associate with cyclins to assume an active form [9, 11]. However, $p35^{nck5a}$ displays no sequence homology with cyclins, and the mRNA for $p35^{nck5a}$ is expressed exclusively in the brain [10, 22, 23]. Based on these findings, cdk5 has been suggested to be associated with neuronal functions unrelated to the cell cycle in neurons [9].

Mammalian NF are composed of three subunits with high (-H), intermediate (-M), and low (-L) molecular weight protein subunits. Each subunit consists of three domains, the amino-terminal globular domain, an α-helical central core, and a carboxyl-terminal extension of various length that contributes to the differences in molecular weights among subunits [13]. The long carboxyl-terminal domains of human NF-H and NF-M show multiple tandemly repeated sequences of a lysine-serine-proline (KSP) motif, which are phosphorylated in vivo [15]. It has recently been demonstrated that purified cdk5 phosphorylates the carboxyl-terminal domain of NF-H and NF-M in vitro [4, 8, 12, 19] and, in particular, their KSP motifs [6, 8, 12]. The major components of LB are NF-H and NF-M. Importantly, KSP motifs in the carboxyl-terminal domain of NF-H and NF-M of LB are highly phosphorylated [16, 18, 21].

A previous study has demonstrated the colocalization of cdk5 immunostaining and LB [1]. However, this evidence does not necessarily indicate an involvement of cdk5 in LB formation, since cdk5 needs $p35^{nck5a}$ as the regulatory subunit to function as an active protein kinase in the brain. The present study has identified the colocalization of immunoreactivity for both $p35^{nck5a}$ and cdk5 in LB. While the precise mechanism of the conversion of normal NF proteins to LB fibrils is still unclear, the phosphorylation of NF proteins is thought to be an important step [15, 16]. The present results support the hypothesis that the cdk5-$p35^{nck5a}$ complex is involved in the phosphorylation of NF proteins in LB, which may lead to the formation of the insoluble LB fibrils.

A recent in vitro study [7] has shown the presence of the macromolecular cdk5-$p35^{nck5a}$ complex in bovine brain in which the kinase activity appears to be inactive. As yet no similar macromolecular complex has been documented in the human brain. If it were present, it is not known whether the cdk5-$p35^{nck5a}$ complex in LB would be in an active heterodimeric form or an inactive macromolecular complex. It has been suggested for the bovine case [7] that a low molecular weight inhibitor may be associated with the complex, which may inhibit the kinase activity of the macromolecular complex. Given that LB contain the cdk5-$p35^{nck5a}$ macromolecular complex, the further study designed to investigate whether or not a low molecular weight kinase inhibitor is associated with LB should be performed.

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