

Neuron-Specific Phosphorylation of Alzheimer's β -Amyloid Precursor Protein by Cyclin-Dependent Kinase 5

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Abstract: The mature form of Alzheimer's β -amyloid precursor protein (APP) is phosphorylated specifically at Thr⁶⁶⁸ in neurons. In mature neurons, phosphorylated APP is detected in neurites, with dephosphorylated APP being found mostly in the cell body. In vitro, active cyclin-dependent kinase 5 (Cdk5) phosphorylated the cytoplasmic domain of APP at Thr⁶⁶⁸. Treatment of mature neurons with an antisense oligonucleotide to Cdk5 suppressed Cdk5 expression and significantly diminished the level of phosphorylated APP. The expression of APP was unaffected in antisense-treated neurons. These results indicate that in neurons APP is phosphorylated by Cdk5, and that this may play a role in its localization. **Key Words:** Alzheimer's β -amyloid precursor protein—Alzheimer's disease—Cyclin-dependent kinase 5—Protein phosphorylation.

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The extracellular cerebral deposition of β -amyloid is a characteristic hallmark of patients with Alzheimer's disease (AD). β -Amyloid is derived from β -amyloid precursor protein (APP) (Price et al., 1998), an integral membrane protein (Kang et al., 1987) that is phosphorylated in the cytoplasmic (Suzuki et al., 1994; Oishi et al., 1997; Iijima et al., 1998) and extracellular (Hung and Selkoe, 1994; Walter et al., 1997) domains. Immature APP (imAPP; *N*-glycosylated form) is localized to the endoplasmic reticulum and *cis*-Golgi, whereas mature APP (mAPP; *N*- and *O*-glycosylated form) is localized to compartments following *trans*-Golgi and on the plasma membrane. We previously reported that in cultured cell lines imAPP is preferentially phosphorylated, compared with mAPP, at Thr⁶⁶⁸ (all numbers refer to those of the APP₆₉₅ isoform) within the cytoplasmic domain by Cdc2 kinase during the G2/M phase of the cell cycle (Suzuki et al., 1994; Oishi et al., 1997). Furthermore, our recent studies using a phosphorylation state-specific antibody raised against phospho-Thr⁶⁶⁸ of APP (pAbThr668) demonstrated that APP is phosphor-

ylated in rat brain (Oishi et al., 1997) and in differentiated PC12 cells (Ando et al., 1999). These results suggest that APP can be phosphorylated at Thr⁶⁶⁸ in neuronal cells by a mechanism distinct from that found in cultured cell lines. However, the protein kinase responsible for the phosphorylation of APP in neuronal cells is unknown. In the present study, we have characterized the phosphorylation of APP at Thr⁶⁶⁸ (P_iAPP) in neurons of the CNS. We have identified the protein kinase that phosphorylates Thr⁶⁶⁸ as cyclin-dependent kinase 5 (Cdk5), a neuronal homologue of Cdc2 kinase.

MATERIALS AND METHODS

Antibodies

Polyclonal anti-APP (AbAPP, UT-421) and phosphorylation state-specific (pAbThr668, UT-33) antibodies were described previously (Tomita et al., 1998; Ando et al., 1999). Anti-APP extracellular domain LN-27 and anti- α -tubulin TU-01 monoclonal antibodies were purchased from Zymed Laboratories Inc. (South San Francisco, CA, U.S.A.). Anti-Cdk5 polyclonal antibody, AbCdk5 G-516, was raised against the Cdk5 carboxyl-terminal sequence Cdk5^{277–291}[Cys]. AbCdk5 C-8, which recognizes the last eight carboxyl-terminal amino acid residues of Cdk5, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). These antigenic sequences are not conserved among other Cdc2-related protein kinases (Meyerson et al., 1992). Noncommercial antibodies were affinity-purified (Oishi et al., 1997). Anti-Cdk5 antibodies used in this study,

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Abbreviations used: AD, Alzheimer's disease; APLP2, amyloid precursor-like protein 2; APP, Alzheimer's β -amyloid precursor protein; Cdk5, cyclin-dependent kinase 5; FITC, fluorescein isothiocyanate; GSK3 β , glycogen synthase kinase-3 β ; GST, glutathione *S*-transferase; imAPP, immature APP; mAPP, mature APP; PAGE, polyacrylamide gel electrophoresis; P_iAPP, phosphorylated APP; SDS, sodium dodecyl sulfate; TRITC, tetramethylrhodamine B isothiocyanate.

AbCdk5 G-516 and C-8, detected a single polypeptide of 33 kDa when rat brain lysate was analyzed by immunoblot (data not shown). An anti-glycogen synthase kinase-3 β (anti-GSK3 β) polyclonal antibody was kindly provided by Dr. James Woodgett (Woodgett, 1990).

Immunoblot analysis of APP in various tissues

Tissues from adult Wistar rats (7–10 weeks) or brain samples from adult C57BL/6J mice (8 weeks) were homogenized in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] containing 1 μ M microcystin LR, 25 μ g/ml pepstatin, 25 μ g/ml leupeptin, and 25 μ g/ml chymostatin. Samples were centrifuged (10,000 g, 10 min), and APP in the supernatant was immunoprecipitated using UT-421, separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Membranes were probed with appropriate antibodies and ¹²⁵I-protein A (Amersham Pharmacia Biotech). Radioactivity was quantified using a Fuji BAS 2000 Imaging Analyzer. UT-33 was highly specific toward P_iAPP and did not recognize dephospho-APP (Ando et al., 1999). In addition, UT-33 did not recognize the amyloid precursor-like protein 2 (APLP2) phosphorylated at Thr⁷³⁶ (APLP2₇₆₃ isoform numbering), the site homologous to Thr⁶⁶⁸ of APP (Suzuki et al., 1997) (data not shown).

Immunoblot analysis of P_iAPP in glial cells

Glial cells were prepared from cortex of embryonic day 18 rats. Dissociated cells were cultured for 1 week in the presence of fetal calf serum (10%, vol/vol), and the resulting cells, which were highly enriched in glia, were collected and lysed as described previously (Oishi et al., 1997). The enrichment of glial cells was indicated by the presence of glial fibrillary acidic protein, and the absence of the microtubule-associated protein, MAP-2, in the cultures (data not shown). The supernatant was collected, APP was immunoprecipitated with UT-421, and samples were immunoblotted as described above.

Immunocytochemical studies

Rat hippocampal neuronal cultures were prepared from embryonic day 18 rats (Bartlett and Banker, 1984). Dissociated cells (10⁴–10⁵ cells) were plated on glass coverslips coated with poly-L-lysine (1 mg/ml) in culture dishes (12-mm diameter) supplied with Neurobasal medium (Life Technologies) containing B-27 supplement (Life Technologies), 0.5 mM L-glutamine, and 0.01 mg/ml pyruvate, and cultured at 37°C. After culture for the indicated time, cells were fixed for 10 min at room temperature with 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (pH 7.4) containing 4% (wt/vol) sucrose, and permeabilized for 5 min at room temperature with 0.2% (vol/vol) Triton X-100 in phosphate-buffered saline. Cells were further incubated with primary antibody (as indicated) for 12 h at 4°C, washed well, and then incubated for 1 h at room temperature with tetramethylrhodamine B isothiocyanate (TRITC)- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Zymed). The cells were viewed using a confocal microscope, Bio-Rad MRC 600.

In vitro phosphorylation of the cytoplasmic domain of APP by Cdk5

The cytoplasmic domain of human APP₆₉₅ (APP_{COOH}) was subcloned into pGEX-4T-1 (Amersham Pharmacia Biotech) (Tomita et al., 1999). Affinity-purified glutathione *S*-transferase (GST)-APP_{COOH} fusion protein was used as a substrate for Cdk5. Active Cdk5 was recovered from the lysate of embryonic day 18 rat brain by immunoprecipitation with AbCdk5 C-8

[known to precipitate active Cdk5 (Lee et al., 1997) more efficiently than AbCdk5 G-516 (data not shown)]. Brain tissue was homogenized in an extraction buffer (Lee et al., 1997), and the supernatant (~5 mg of protein) of the homogenate was incubated with 5 μ g of C-8 antibody. The Cdk5/antibody complex was recovered by addition of protein A-Sepharose beads. The beads were washed twice with the extraction buffer and twice with Cdk5 reaction buffer containing 20 mM MOPS (pH 7.4) and 30 mM MgCl₂. The beads were incubated with 5 μ g of GST-APP_{COOH} in the reaction buffer containing 100 μ M ATP for 1 h at 30°C. After removal of the resin by centrifugation, GST-APP_{COOH} was recovered by immunoprecipitation with UT-421, subjected to SDS-PAGE [12.5% (wt/vol) polyacrylamide], transferred to a membrane, and incubated with UT-33 or UT-421, and ¹²⁵I-protein A. The resulting membrane was analyzed by autoradiography using a Fuji BAS 2000 Imaging Analyzer.

Treatment of mature neurons with antisense/sense oligonucleotide

Antisense oligonucleotide, 5'-TTCTCGTATTTCTGC-3', and sense oligonucleotide, 5'-GCAGAAATACGAGAA-3', corresponding to rat Cdk5⁺⁴⁹⁻⁺⁶³ were designed as described (Pigino et al., 1997). Antisense oligonucleotide, 5'-CCCCGACATGATGGCTCTTC-3', and sense oligonucleotide, 5'-GAAGAGCCATCATGTCCGGGG-3', corresponding to rat GSK3 β ⁻¹¹⁻⁺⁹ were designed as described (Woodgett, 1990). The oligonucleotides were synthesized using phosphorothioate chemistry and purified by HPLC (Nissinbo Co., Tokyo, Japan). The 5'-terminal region of each oligonucleotide was attached to TRITC. Mature neurons, which had been allowed to grow for >96 h in culture after plating, were washed once with culture medium and each oligonucleotide was administered in 200 μ l of culture medium at 10 μ M. The medium was supplemented again with 10 μ M oligonucleotide after 12 and 24 h of culture (Ferreria et al., 1993). Twelve hours after the last addition of oligonucleotide, cells were fixed and permeabilized as described above for immunocytochemical studies.

RESULTS

Neuron-specific phosphorylation of APP

APP was immunoprecipitated with UT-421 from appropriate amounts of various adult rat tissues and analyzed by immunoblotting with UT-421 (Fig. 1A, left) or with UT-33 (Fig. 1A, right). Similar amounts of APP distributed between different isoforms were recovered from the different tissues. P_iAPP was detected only in samples from cerebrum and cerebellum. Mouse brain was also dissected and fractionated. APP from the indicated brain tissues was analyzed with UT-421 (Fig. 1B, left) or with UT-33 (Fig. 1B, right). In all brain regions examined, P_iAPP was detected. These results clearly indicate that basal phosphorylation of APP at Thr⁶⁶⁸ is specific to nervous tissue.

APP from brain exhibits three major isoforms (Fig. 1B, left), and we determined that the two higher molecular weight isoforms are mAPP₆₉₅ variants differing in *O*-glycosylation. When APP recovered from brain was treated with a combination of neuraminidase plus *O*-Glycanase, a process that releases *O*-glycan from mAPP (Tomita et al., 1998), the two APP isoforms of highest molecular weight migrated at the position of imAPP₆₉₅,

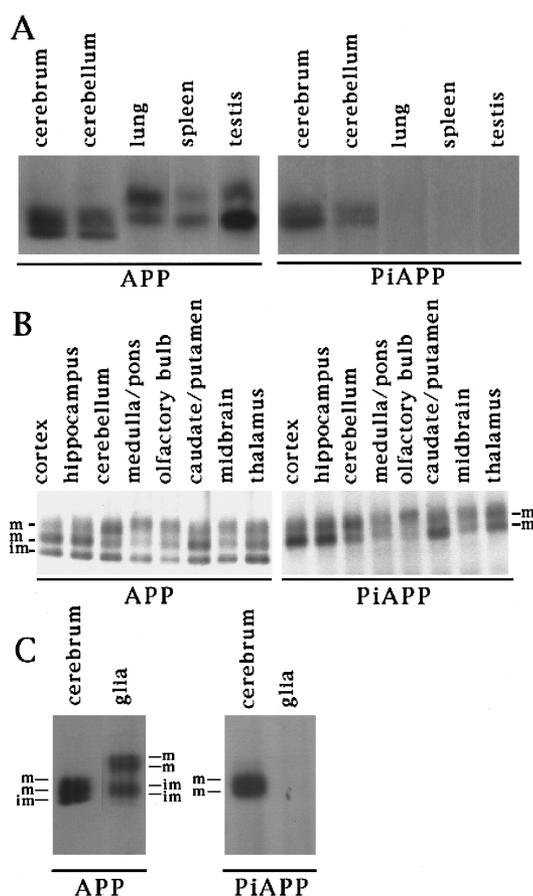


FIG. 1. Neuron-specific phosphorylation of APP at Thr⁶⁶⁸. **A:** APP was isolated from various rat tissues by immunoprecipitation with UT-421, analyzed by SDS-PAGE [7.5% (wt/vol) polyacrylamide], and transferred to a nitrocellulose membrane. The membrane was probed with UT-421 ("APP," left) and UT-33 ("PiAPP," right). **B:** APP was immunoprecipitated from mouse brain lysates with UT-421, analyzed by SDS-PAGE [6% (wt/vol) polyacrylamide], transferred to a nitrocellulose membrane, and probed with UT-421 ("APP," left) and UT-33 ("PiAPP," right). Bars indicate APP₆₉₅ isoforms (m, mAPP₆₉₅; im, imAPP₆₉₅). **C:** APP was isolated from cerebrum or glial cells (as indicated, 2 mg of protein for cerebrum and 600 μ g for glial cells) by immunoprecipitation with UT-421, analyzed by SDS-PAGE [7.5% (wt/vol) polyacrylamide], transferred to a nitrocellulose membrane, and probed with UT-421 ("APP," left) and UT-33 ("PiAPP," right). Bars indicate APP₆₉₅ isoforms for brain and APP₇₇₀ and APP₇₅₁ isoforms for glia cells (m, mature; im, immature).

the lowest isoform (data not shown). As only the two highest molecular weight forms were phosphorylated (Fig. 1B, right), this indicates that only mAPP₆₉₅, but not imAPP₆₉₅, is phosphorylated at Thr⁶⁶⁸ in brain tissues.

To investigate if phosphorylation of APP occurs in neurons but not in glial cells, APP was immunoprecipitated with UT-421 from cerebrum and from a glial cell preparation, and analyzed by immunoblotting with UT-421 (Fig. 1C, left) or with UT-33 (Fig. 1C, right). Two mature and immature APP isoforms, probably APP₇₇₀ and APP₇₅₁, were recovered from glial lysates; however, PiAPP was not detected. These results suggest that phos-

phorylation of APP at Thr⁶⁶⁸ is neuron-specific, and this was investigated in more detail using neuronal cultures as described below.

Localization of APP and PiAPP in cultured rat hippocampal neurons

The localization of APP and PiAPP was analyzed in mature rat hippocampal neurons by immunocytochemistry. Neurons were dissected from embryonic day 18 rat and cultured for 120 h. Mature neurons were double-stained with UT-33 (Fig. 2A), UT-421 (Fig. 2C), or anti- α -tubulin monoclonal antibody TU-01 (Fig. 2B and D). PiAPP was localized mainly to the somatic plasma membrane and to neurites rather than being present within the cell body. In contrast, APP was restricted mostly to the cell body. These observations suggest that the majority of plasma membrane-associated APP is phosphorylated.

Phosphorylation of the cytoplasmic domain of APP by Cdk5 in vitro

We next examined which protein kinase phosphorylates APP at Thr⁶⁶⁸ in neurons. The most likely candidate is Cdk5 because (a) Cdk5 is a neuronal Cdc2-related protein kinase, (b) Cdk5 recognizes an amino acid sequence similar to that of Cdc2 kinase substrates (Meyerson et al., 1992; Lew and Wang, 1994), (c) Cdk5 is active in the brain during late embryonic and adult life (Tsai et al., 1994; Lee et al., 1997), and (d) a recombinant Cdk5/p25 activator complex phosphorylates the APP cytoplasmic peptide, APP₆₉₅⁶⁴⁵⁻⁶⁹⁴, in vitro (J. Bibb and A. C. Nairn, unpublished observation). Active Cdk5 recovered from a lysate of embryonic rat brain was incubated with GST-APP_{COOH} (Fig. 3). GST-APP_{COOH} was phosphorylated by immunoprecipitated Cdk5. Addition of the peptide Cdk5²⁷⁷⁻²⁹² (which includes the amino acids used as antigen) during immunoprecipitation abolished the signal observed for phosphorylation of GST-APP_{COOH}. These results demonstrated that in vitro Cdk5 directly phosphorylates APP at Thr⁶⁶⁸.

Phosphorylation of APP by Cdk5 in neurons

To investigate whether Cdk5 is the protein kinase responsible for the phosphorylation of APP at Thr⁶⁶⁸ in neurons, fully mature rat hippocampal neurons were treated with antisense or sense oligonucleotides to Cdk5 (Fig. 4A-H). To examine incorporation into the nucleus, we tagged the 5'-termini of the oligonucleotides with TRITC. When sense (Fig. 4A, C, E, and G) or antisense (Fig. 4B, D, F, and H) oligonucleotides to rat Cdk5 were administered, neurons incorporated the oligonucleotide and exhibited strong red fluorescent nuclei. The cells were then stained with G-516 (Cdk5, Fig. 4A and B), UT-33 (PiAPP, Fig. 4C and D), UT-421 (APP, Fig. 4E and F), or TU-01 (α -tubulin, Fig. 4G and H) antibodies, and neurons that had incorporated the oligonucleotide were examined. All cells that had incorporated antisense oligonucleotide exhibited reduced expression of Cdk5 in the somatic cytoplasm (compare green fluorescence in Fig. 4A and B). These cells, in which the expression of

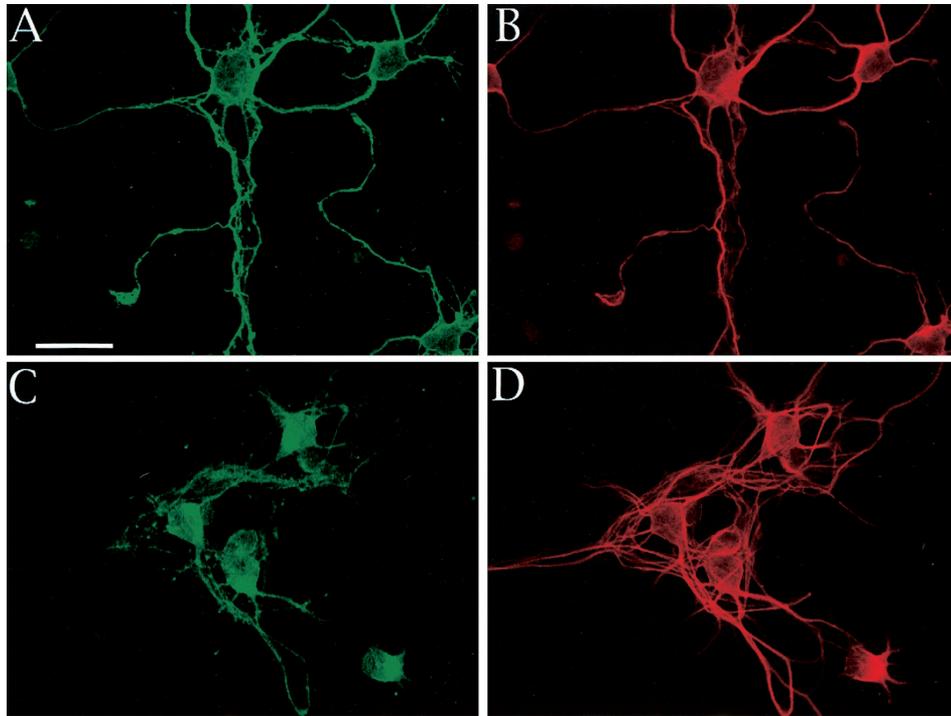


FIG. 2. Localization of APP and P_iAPP in cultured rat hippocampal neurons. Embryonic day 18 hippocampal neurons were cultured for 120 h. Neurons were double-stained with (A) pAbThr668 (UT-33) and (B) anti- α -tubulin antibody (TU-01). Other neurons were double-stained with (C) AbAPP (UT-421) and (D) TU-01. Scale bar = 25 μ m.

Cdk5 was suppressed, displayed a corresponding decrease in the P_iAPP signal (compare green fluorescence in Fig. 4C and D). Neither APP (compare green fluorescence in Fig. 4E and F) nor α -tubulin (compare green fluorescence in Fig. 4G and H) expression was affected in cells that had incorporated antisense oligonucleotides (Fig. 4F and H). Cells that had incorporated sense oligonucleotide showed normal levels of Cdk5 and P_iAPP (Fig. 4A and C). These results suggest strongly that in neurons Cdk5 phosphorylates APP at Thr⁶⁶⁸.

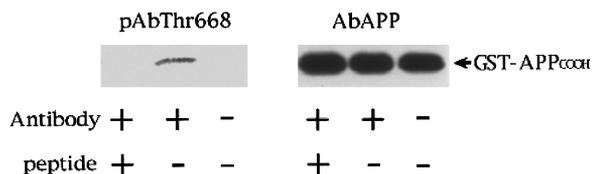


FIG. 3. Phosphorylation of the cytoplasmic domain of APP at Thr⁶⁶⁸ by Cdk5. Cdk5 was immunoprecipitated from rat embryonic brain with (antibody "+") or without (antibody "-") AbCdk5 (C-8) in the presence (peptide "+") or absence (peptide "-") of a peptide that includes the amino acids used as antigen. The resulting Cdk5/C-8 complex coupled to resin was incubated with GST-APP_{COOH} fusion protein (arrow). GST-APP_{COOH} was recovered by immunoprecipitation with UT-421, and samples were analyzed by immunoblot using UT-33 (left) or UT-421 (right).

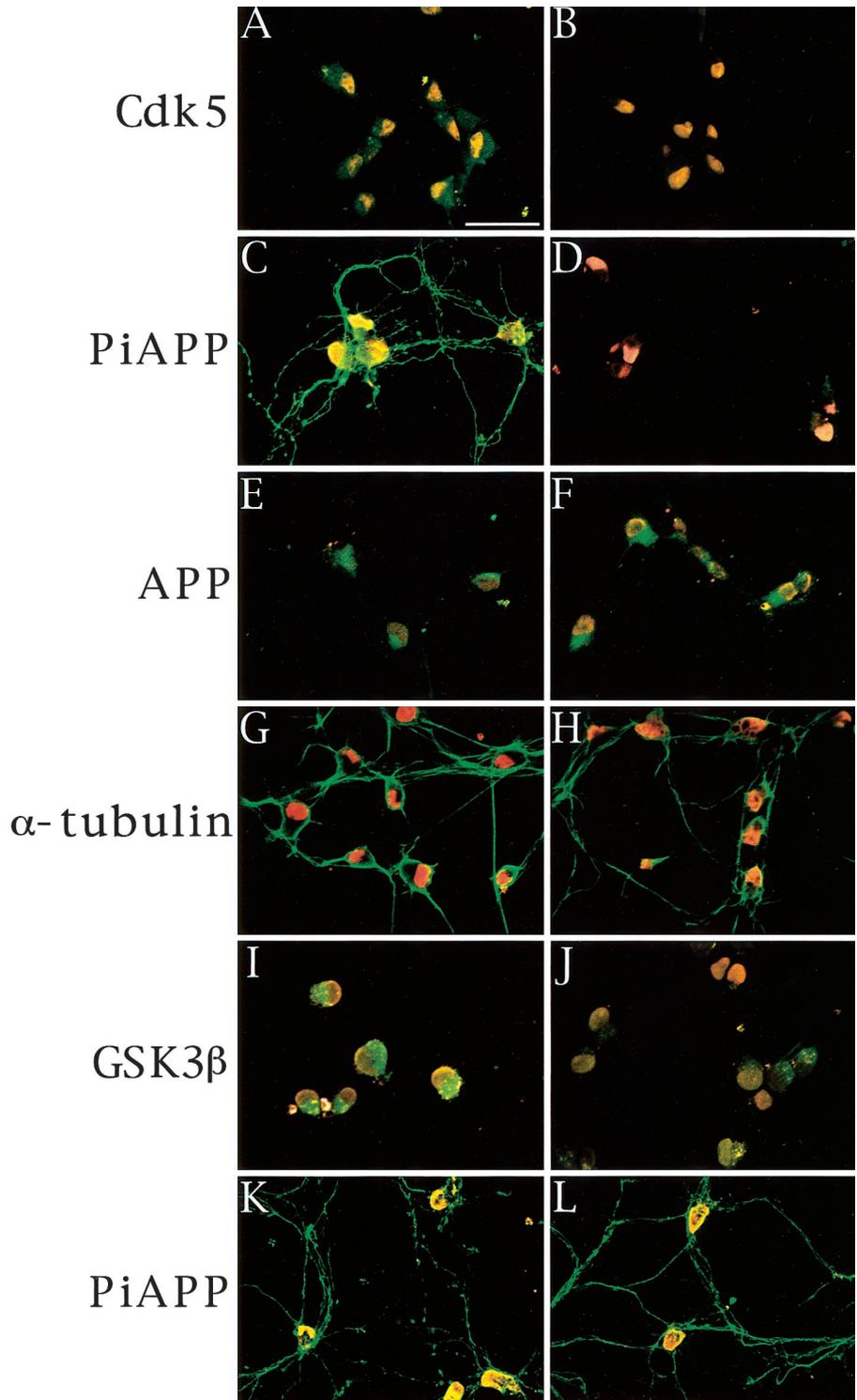
GSK3 β does not phosphorylate APP at Thr⁶⁶⁸ in neurons

In vitro, GSK3 β phosphorylates APP_{COOH} at Thr⁶⁶⁸ (Aplin et al., 1996). Therefore, we examined whether antisense oligonucleotide specific for GSK3 β might decrease P_iAPP in neurons (Fig. 4I–L). Mature rat hippocampal neurons were treated with antisense (Fig. 4J and L) or sense (Fig. 4I and K) oligonucleotides to rat GSK3 β that had been tagged with TRITC at their 5'-termini. Neurons that had incorporated antisense oligonucleotide exhibited decreased GSK3 β expression (compare Fig. 4I and J); however, the level of P_iAPP in neurites was not affected (compare Fig. 4K and L). Treatment with sense oligonucleotide had no effect on the expression of GSK3 β and P_iAPP (Fig. 4I and K). Longer incubation of mature neurons with antisense oligonucleotide to GSK3 β resulted in cell death, but even as cell lysis began to occur, P_iAPP was detected (data not shown). Moreover, treatment of neurons with lithium chloride (10 mM), which inhibits GSK3 β activity specifically (Stambolic et al., 1996), had no effect on the P_iAPP signal (Fig. 5). These observations indicate that GSK3 β does not make a major contribution to the phosphorylation of APP at Thr⁶⁶⁸ in neurons.

DISCUSSION

We have identified that the mature form of APP, mAPP₆₉₅, but not imAPP₆₉₅, is phosphorylated constitu-

FIG. 4. Treatment of mature rat hippocampal neurons with antisense/sense oligonucleotides to Cdk5 or GSK3 β . Mature rat hippocampal neurons were treated for 36 h with sense (**A, C, E, and G**) or antisense (**B, D, F, and H**) oligonucleotides specific for rat Cdk5. Neurons were stained with AbCdk5 (G-516) (**A** and **B**), UT-33 (**C** and **D**), UT-421 (**E** and **F**), or TU-01 (**G** and **H**) and visualized with FITC-conjugated secondary antibody (green). Neurons were also treated with sense (**I** and **K**) or antisense (**J** and **L**) oligonucleotides specific for rat GSK3 β . Neurons were stained with AbGSK3 β (**I** and **J**) or UT-33 (**K** and **L**) and visualized with FITC-conjugated secondary antibody (green). The oligonucleotides were labeled with TRITC, and the incorporation of oligonucleotide into the nucleus was confirmed by the observation of red fluorescence. Scale bar = 25 μ m. In all cases, the antisense and sense studies used 2×10^5 cells and were performed at least five different times. Similar results were obtained each time. When incorporation of antisense Cdk5 oligonucleotides was detected in the nucleus, all cells (100%) demonstrated a reduction in the level of P_iAPP. Neurons that did not incorporate oligonucleotides sufficiently into the nucleus exhibited normal immunostaining for the various proteins studied.



tively in the neurons in brain. Notably, APP is not basally phosphorylated in nonneuronal tissues, suggesting that phosphorylation of Thr⁶⁶⁸ may be involved in a neuron-specific aspect of APP metabolism and/or function. Based on biochemical and pharmacological studies and studies using antisense oligonucleotides, our results

suggest that Cdk5, but not GSK3 β , phosphorylates APP at Thr⁶⁶⁸ in neurons.

In mature neurons, P_iAPP was located mainly in neurites, whereas APP was located mainly within the cell body. The molecular basis for this distribution of P_iAPP is not known, but this may be related to the cellular

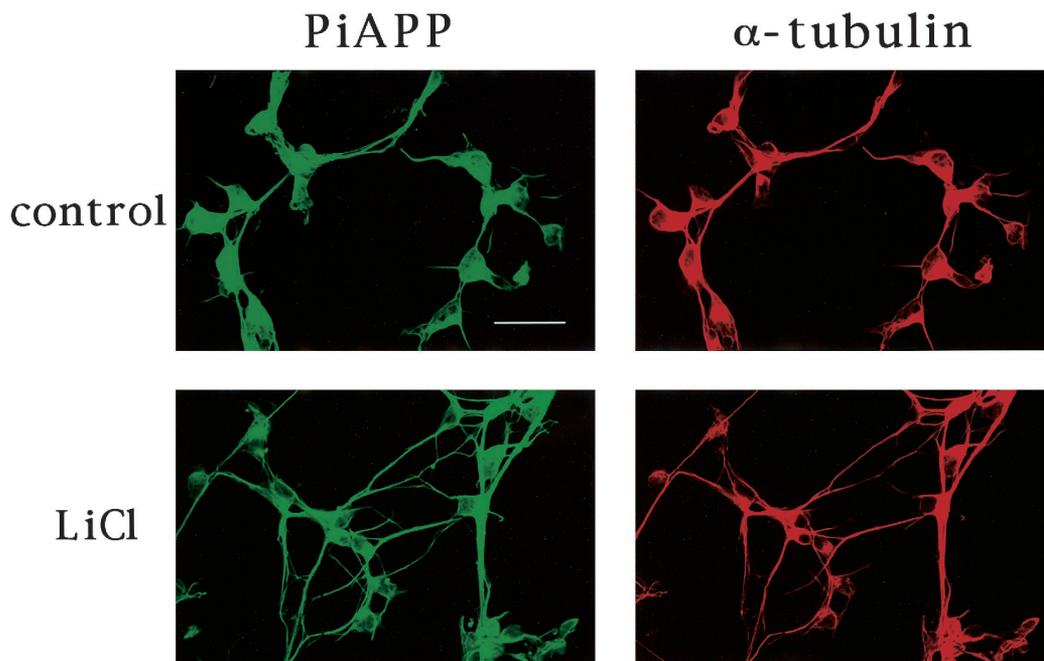


FIG. 5. Treatment of neurons with lithium chloride has no effect on APP phosphorylation. Mature rat hippocampal neurons were treated for 2.5 h with 10 mM lithium chloride (LiCl) or sodium chloride (control). Neurons were double-stained with UT-33 (P_iAPP) and TU-01 (α -tubulin) and visualized with FITC- and TRITC-conjugated secondary antibody. Scale bar = 25 μ m.

distribution of Cdk5 and/or its activator protein p35, or of the protein phosphatase(s) that dephosphorylate Thr⁶⁶⁸. Together with our recent studies using PC12 cells (Ando et al., 1999), the present results suggest that phosphorylation of APP at Thr⁶⁶⁸ may play a specific role in regulation of APP function in neurites. Possibly, a decrease in P_iAPP level and/or unregulated phosphorylation of APP may induce retraction of neurites and may result in damage of neuronal circuits in the adult brain. In fact, we have observed that prolonged incubation of neurons with antisense oligonucleotide to Cdk5 brings about the retraction of neurites (data not shown). The disappearance of P_iAPP in neurons treated with antisense oligonucleotide to Cdk5 occurred before the retraction of neurites.

Cdk5 is activated by the regulatory subunit, p35 (Ishiguro et al., 1994; Lew et al., 1994; Tsai et al., 1994). A recent study has indicated that a truncated form of p35, termed p25, accumulates in postmortem brain samples obtained from patients with AD (Patrick et al., 1999; Ahljianian et al., 2000). The interaction of p25 with Cdk5 results in the constitutive activation of the kinase and increased phosphorylation of tau, a microtubule-binding protein that has been implicated in paired-helical filament formation in AD (Patrick et al., 1999). As our present studies indicate that APP is phosphorylated at Thr⁶⁶⁸ in a neuron-specific manner by Cdk5, increased phosphorylation of APP in brain of AD patients might be expected. However, in preliminary studies, we have observed a slight decrease in the level of phosphorylation of APP in brain tissue from AD patients (Suzuki et al., unpublished observation). Active Cdk5 may possibly be

localized to a specific subcellular location within neurons, or may be specifically localized together with tau in the brain of AD patients. In any case, our results and the results from Patrick et al. (1999) indicate that APP and tau, two proteins implicated in AD, are physiological substrates for Cdk5. It is hoped that further studies of the phosphorylation of APP by Cdk5 will contribute to our understanding of the role of APP in neuronal differentiation and possibly of the role of APP in the pathogenesis of AD.

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