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A Cdk5–p35 Stable Complex Is Involved in the β-Amyloid-Induced Deregulation of Cdk5 Activity in Hippocampal Neurons

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The cdk5 and its activator p35 constitute one of the main tau-phosphorylating systems in neuronal cells. Under normal conditions for neurons, its activity is required for modulating tau involvement in neuronal polarity and in development of the mammalian central nervous system. Recently, we reported that the treatment of rat hippocampal cells in culture with fibrillary β-amyloid (Aβ) results in deregulation of the protein kinase cdk5. The neurotoxic effects of Aβ fibrils were prevented by inhibition of cdk5 activity by butyrolactone I or by using antisense oligonucleotides that control the expression of this kinase. Here, we show that the Aβ-promoted increase of cdk5 activity is associated with changes in tau phosphorylation patterns and in the intraneuronal distribution of tau. In addition to hippocampal cells, deregulation of cdk5 was observed in other cell types. However, butyrolactone I prevented Aβ-induced cell death only in neuronal cells in which cdk5 activation was sensitive to Aβ fibrils. This lost of cdk5 regulation in hippocampal cells exposed to Aβ fibrils appears to be associated with an increase in the cdk5–p35 complex stability. Complex stabilization was sensitive to phosphorylation of cdk5. However, no changes in cdk5 and p35 mRNAs were observed, suggesting that the main effects on cdk5 occur at the posttranslational level. These studies indicate that cdk5 phosphorylation and the formation of an abnormally active cdk5–p35 complex are directly involved in the molecular paths leading to the neurodegenerative process of rat hippocampal neurons triggered by Aβ fibrils. © 2001 Academic Press

Key Words: **protein kinase cdk5; tau phosphorylation; butyrolactone I; cdk5 and p35 antisense; hippocampal cells; Alzheimer's disease; cdk5–p35 complex.**

INTRODUCTION

Alzheimer's disease (AD)[2] is a neurodegenerative disorder that affects the cognitive functions of the human brain. Pathological alterations in AD include an extensive loss of neurons, associated with two major protein aggregates: amyloid plaques, which are deposited extracellularly, and neurofibrillary tangles (NFT), cytoplasmic aggregates that accumulate in the neural cell soma. Both consist of fibers of aggregated protein, the Aβ peptide in the case of plaques, and abnormally phosphorylated tau in the tangles [1, 2]. Tau protein colocalizes with microtubules, and its major cell role is modulating microtubule assembly and their stabilization [3]. In neurons, tau is involved in determining neuronal polarity. Several phosphorylation sites have been identified on the tau molecule, which are targets of different protein kinases [2]. Controlled phosphorylations are crucial in the regulation of tau capacity to promote microtubule assembly. However, abnormal phosphorylations may cause tau conformational changes, thus affecting its interaction with microtubules and with other components of the cytoskeletal network [4–6].

Among kinases involved in tau modifications, two are the most relevant: the glycogen synthase kinase 3β and the cdk5–p35 complex [7–9]. Cdk5 is the catalytic component while p35 is the regulatory protein [9]. This enzyme is characterized as a proline-directed Ser/Thr kinase that contributes to phosphorylation of human tau on Ser^{202}, Thr^{205}, Ser^{235}, and Ser^{404} [9]. The cdk5–p35 system plays a pivotal role in neuronal development as evidenced by the abnormal corticogenesis and perinatal lethality of the cdk5 knockout mice [10] and the disturbances in neuronal migration and early death in p35 knockout mice [11]. There is additional evidence that the neuron-specific activator p35 is directly involved in axonal growth [12]. Recently, we have found that Aβ induces an increase in protein kinase activity of cdk5 on hippocampal cells, a phenom-

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[2] Abbreviations used: Aβ, β-amyloid peptide; AD, Alzheimer's disease; cdk5, cyclin-dependent protein kinase-5; NFT, neurofibrillary tangles.

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enon linked with death of hippocampal neurons [13]; however, no information exists on the sequence of molecular events responsible for cdk5 deregulation. In the present report, we have tackled this important question and advanced further in the analysis of cdk5 deregulation. Here we show that Aβ-induced cdk5 activation appears to be mediated by formation of a stable complex, cdk5–p35, in the pathway leading to neuronal degeneration. It was noteworthy to observe that butyrolactone I, a cdk5 inhibitor [14], prevented Aβ-induced cell death only in neuronal cells. These studies, taken together with several relevant previous findings, suggest that cdk5 may contribute to the formation of NFT in rat hippocampal neurons, thus participating in the cell death process triggered by the fibrillary form of Aβ.

MATERIALS AND METHODS

Cell cultures. Hippocampi from Sprague–Dawley rats at embryonic day 18 were dissected and primary rat hippocampal cultures were prepared as described [15, 16]. Hippocampal cells were seeded in polylysine-coated wells and maintained in MEM supplemented with B27 from Gibco BRL, plus streptomycin and penicillin, for 4 days before the cell treatments. The neuroblastoma N2A and glioma B12 cells used in this study were maintained in DMEM supplemented with 5% fetal bovine serum (FBS). CHO cells were maintained in DMEM supplemented with 10% FBS. N2A cells were induced to undergo differentiation after addition of 5 mM dibutyryl cAMP (Sigma, St. Louis, MO) to the cultures, lowering serum to 0.25% fetal bovine serum, and allowing differentiation for 48 h.

Cytotoxicity assays. The cells were seeded in polylysine-coated 96-well plates at 2.0×10^4 cells/100 μl per well in B27/MEM without phenol red. Then, cells were treated with the β-amyloid fibrils with or without cdk5 inhibitor butyrolactone I. After 24 h of incubation cell viability was measured by the modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [17].

Preparation of the Aβ fibrils. The $A\beta_{1-40}$ peptide (purchased from Chiron Corp., Emeryville, CA) was subjected to stirring aggregation as described [20]. The Aβ fibrils were concentrated by centrifugation (14,000 rpm for 30 min) and resuspended at 1 mg/ml in B27/MEM. Aβ concentration was evaluated by the BCA protein assay (Pierce) as described by Smith *et al.* [21]. Butyrolactone I (Calbiochem, La Jolla, CA) was prepared in 10 mM DMSO and added together with the Aβ fibrils at the cdk5 inhibitor concentrations indicated.

Immunoprecipitation and cdk5 activity assays. Cells were plated at 1×10^6cells/cm^2 on polylysine-coated 35-mm dishes. Cultured cells were exposed to Aβ fibrils in B27/MEM for 24 h. Afterward, cells were lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 2 μg/ml aprotinin, 2 μM leupeptin, and 1 μg/ml pepstatin). Two hundred micrograms of total cellular protein was used for immunoprecipitation with an anti-cdk5 antibody (C8 antibody; Santa Cruz Biotechnology, Santa Cruz, CA), used at a final dilution of 1:50. For *in vitro* kinase assay, the immunoprecipitates were rinsed three times with RIPA buffer and one time with kinase buffer (50 mM Hepes, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT) plus 1 μM cold ATP. The rinsed agarose beads were incubated with kinase buffer containing 2.5 μg of histone H1 or 4 μg of the purified tau [18] plus 5 μCi [γ-^{32}P]ATP in a final volume of 50 μl for 30 min at 30°C. Enzyme assays using the synthetic tau peptide RTPPKS^{235}PSSAKSR, containing the tau site for cdk5 phosphorylation, as the kinase substrate were also carried out. After incubation, the samples were analyzed by SDS–PAGE and autoradiography [19].

Primary antibodies. The following primary antibodies were used in this study: monoclonal antibody (mAb) against phosphorylated tau at epitopes Ser^{202} and Ser^{205} (clone AT8; Innogenetics, Belgium), mAb against dephosphorylated tau at epitopes Ser^{202} and Ser^{205} (clone Tau-1), mAb against conformational tau epitopes (Tau-5), a polyclonal antibody against tubulin generated in this laboratory [5], polyclonal antibody against p35 (N20; Santa Cruz Biotechnology), and a polyclonal antibody against cdk5 (C8; Santa Cruz). Tau-1 and 5 were donated by Dr. L. Binder.

Immunodetection assays. The immunological reactivity of hippocampal cell extracts treated with Aβ and untreated controls was assayed by Western blots [5] using antibodies that recognize Alzheimer's phosphoepitopes on tau (AT8) and with the antibodies Tau-1 and Tau-5. Western blots (ECL) were developed by using the chemiluminescence (Amersham) procedure. Antibody dilutions in the Western blot assays were AT8, 1:200; Tau-1, 1:1000; Tau-5, 1:1000. Immunoblots for cdk5 and p35 were carried out as indicated [5]. Dilutions of antibodies C8 for cdk5 and N20 for p35 used in the blot assays were 1:1000. In some experiments for the immunodetection of the cdk5–p35 complex, guanidine hydrochloride (GuHCl) was used in order to dissociate the complex, followed by blot analysis of the protein components. For these experiments, the hippocampal cell extracts were boiled for 20 min in the presence of 3 M GuHCl, and samples were analyzed by Western blots [5].

Immunohistochemistry. Hippocampal cells were plated on polylysine-coated coverslips (40,000 cells/cover). After 4 days in B27/MEM, cells were exposed to Aβ fibrils (2.5 μM) for 48 h either in the presence or in the absence of 5 μM butyrolactone I, then were fixed with 4% paraformaldehyde, 1% glutaraldehyde and permeabilized with 0.1% Triton X-100. Immunostaining was done as described [22] using AT8 (1:40) and Tau-1 (1:200) antibodies and a polyclonal anti-tubulin antibody (1:200). The cells were treated with FITC-conjugated anti-mouse antibodies (Sigma, St. Louis, MO) and TRITC-conjugated anti-rabbit antibody (Sigma, St. Louis, MO). Coverslips were mounted in 90% glycerol/PBS and analyzed using a Zeiss confocal microscope.

Phosphorylation of cdk5 and metabolic labeling. The cdk5 phosphorylation assays were done as described [23]; hippocampal cell lysates of the control and Aβ fibril-treated cells (15 μM) were prepared by homogenization in buffer containing 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.1% NP-40, 100 μg/ml PMSF, 2 μg/ml aprotinin, 2 μM leupeptin and 1 μg/ml pepstatin, 0.1 mM okadaic acid, and 1 mM sodium orthovanadate. Cell lysates (10 μl) were used to phosphorylate 25 μg of the bacterially expressed GST-cdk5. The phosphorylation reaction was made in kinase buffer (50 mM Hepes, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT) plus 5 μCi [γ-^{32}P]ATP in a final volume of 50 μl for 30 min at 30°C. GST-cdk5 was recovered by adsorption on glutathione–Sepharose beads followed by three washes with lysis buffer and analyzed by SDS–PAGE and autoradiography. Purification of GST-fusion protein was carried out using glutathione–Sepharose affinity chromatography by standard procedures (Pharmacia, Uppsala, Sweden). For metabolic labeling with $^{32}P_i$, cells were maintained in phosphate-free DMEM/B27 for 2 h before addition of 0.5 mCi/ml [^{32}P]phosphoric acid and Aβ fibrils (15 μM). At different intervals of time the cells were washed three times with cold PBS, before lysis. Then, cdk5 was immunoprecipitated and analyzed by SDS–PAGE and autoradiography.

Antisense oligonucleotides. Antisense phosphorothioate oligonucleotides (S-modified) described by Paglini *et al.* [12] and Pigino *et al.* [19] were used in the present study. This cdk5 oligonucleotide consists of the sequence 5′ GCATCGCAGCGGCCA 3′, and it is the inverse complement of nucleotides +83/+97 of the cDNA for cdk5. The p35 probe consisted of 5′ CCCTTCGGCCGGAC 3′. The p39 probe consisted of the sequence 5′ AGCCGGCGGTCCCTGTCG 3′.

These probes were purchased from Quality Controlled Biochemicals (Hopkinton, MA). The antisense oligonucleotide was preincubated with 2 μl of Lipofectin Reagent (1 mg/ml; Gibco BRL, Gaithersburg, MD) diluted in 100 μl of serum-free medium. The resulting oligonucleotide suspension was then added to the primary hippocampal cultures that had been grown for 4 days. Four pulses of oligonucleotide (1 μM each) were added every 6 h within a period of 24 h. Control cultures were treated with the same concentration of a scrambled oligonucleotide sequence. Simultaneous with the third pulse of the oligonucleotide (after 12 h treatment), Aβ fibrils (10 μM) were added to hippocampal cells, after the cells' viability had been evaluated.

Total RNA extraction and RT-PCR of primary cultures of rat hippocampal cells. Total RNA was extracted from rat hippocampal cultures of 1×10^6 cells by using the TRIZOL (Gibco BRL) method. The amount of RNA obtained was determined by spectrophotometric measurements at 260 nm. The RT-PCR was carried out with 3 μg of total RNA, to which 100 ng of the random primers was added in a volume of 12 μl. Sample was incubated for 10 min at 70°C followed by addition of 4 μl of 5× transcription buffer, 2 μl of 0.1 mM DTT, 1 μl of 10 mM dNTP, and 200 U of Superscript II (Gibco BRL). The mixture was incubated for 50 min at 42°C. The final volume of the reaction was 20 μl. For PCR amplification, different amounts of synthesized cDNA were analyzed to evaluate the linearity of the reaction. Then, polymerase reaction buffer was added. This solution contained 1.5 mM $MgCl_2$, 200 μM each nucleotide in PCR buffer, 10 pmol of the cdk5 or p35 primers, and 0.25 U *Taq* polymerase. The primers were cdk5 AS, 5′ GCATTGAGTTTGGGCACGACA 3′, and cdk5 SNS, 5′ AAAACCGGGAAACCCATGAGA 3′. The primers for p35 were p35 AS, 5′ GCAACGGTCCCAAAAGGCTT 3′, and p35 SNS, 5′ ACAGCAAGAACGCCAAGGACA 3′. The amplification of p35-actin or cdk5-actin was carried out in the same reaction, maintaining 2:1 and 1:1 ratios for the primer concentrations. Actin primers were SNS, 5′ TCTACAATGAGCTGCGTGTG 3′, and actin AS, 5′ TACATGGCTGGGGTGTTGAA 3′. Amplification was carried out by using the following cycle: initial denaturation at 94°C for 3 min, denaturation at 94°C for 40 s, reassociation at 60°C for 45 s, extension at 72°C for 40 s, and a final extension step of 72°C for 7 min. The number of cycles varied between 26 and 29 and the reassociation temperature was kept the same for p35, cdk5, and actin. The reaction volume was 50 μl and the products were visualized in 2% agarose/TAE gels and stained with ethidium bromide.

RESULTS

The Increase in Cdk5 Activity of Cells Exposed to Aβ Is Linked with Tau Phosphorylation Changes

Figure 1A shows cdk5 activity as measured from immunoprecipitate from control hippocampal cells (lanes 1, 3, and 5) or cells incubated with Aβ fibrils (lanes 2, 4, and 6). The fibrillary form of Aβ amyloid induced a significant increase in cdk5 kinase activity (Fig. 1A) using purified brain tau (lanes 1 and 2), H1 histone (lanes 3 and 4), or the synthetic tau peptide RTPPKS^{235}PSSAKSR (lanes 5 and 6) as cdk5 substrates. In this context, it was of utmost interest to evaluate the effects of cdk5 activation on endogenous tau phosphorylation. Concomitant with the cdk5 activation, fibrillary Aβ also triggered changes in tau phosphorylation, as evaluated by immunoblotting using monoclonal AT8, Tau-1, or Tau-5 antibodies. AT8 recognizes phosphorylated targets for the kinase at Ser^{202}

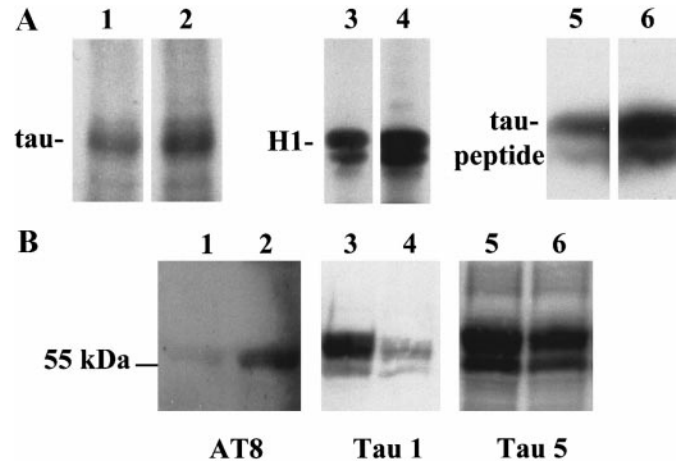

FIG. 1. Effects of Aβ fibrils on the activity of cdk5 and tau phosphorylation in cultured hippocampal neurons. (A) The cdk5 kinase activity in hippocampal neurons exposed to 15 μM Aβ fibrils (lanes 2, 4, and 6) and control cells (lanes 1, 3, and 5) was determined after immunoprecipitation of cell homogenates (200 μg/sample) followed by incubation with [^{32}P]ATP in the presence of purified tau (lanes 1, 2), histone H1 (lanes 3, 4), or the tau peptide containing Ser^{235} (Materials and Methods) as substrates. (B) Western blot analyses of control hippocampal neurons (lanes 1, 3, and 5) and neuronal cells treated with 15 μM Aβ fibrils (lanes 2, 4, and 6) using AT8, Tau-1, and Tau-5 antibodies. Molecular weight of reference according to electrophoretic marker calibration is shown.

and Ser^{205}, while Tau-1 recognizes the same dephosphorylated epitopes. These are sites for *in vitro* tau phosphorylation by cdk5 [8]. An increase in AT8 immunoreactivity was seen in cells incubated with Aβ fibrils (Fig. 1B, lane 2) compared with the control (lane 1). An increase of approximately five times in the AT8 staining of hippocampal cells incubated with Aβ compared with controls was detected after densitometric analysis of blots (lanes 1 and 2). A concomitant decrease in the Tau-1 immunoreactivity was observed in the Aβ-treated cells (Fig. 1B, lane 4) compared with the control (lane 3).In addition, another antibody, Tau-5, which recognizes conformational tau epitopes and is independent of the state of phosphorylation, was used as a control for protein charge (Fig. 1B, lanes 5 and 6). The observation that immunoreactivity for Tau-5 presented only a very minor change indicates that the levels of total tau protein were not significantly modified upon the cell treatment.

The intracellular distribution of phosphorylated forms of tau in Aβ-treated and untreated hippocampal cells was also evaluated. To obtain viable hippocampal cells after the Aβ treatments, cells were incubated for 48 h with lower Aβ concentrations in the culture medium (2.5 μM Aβ). Results of double immunofluorescence staining of hippocampal cells with AT8 or Tau-1 antibodies, and with an anti-tubulin antibody, are shown in Fig. 2. In control cells, immunoreactivity toward AT8 was present both in the neuronal processes

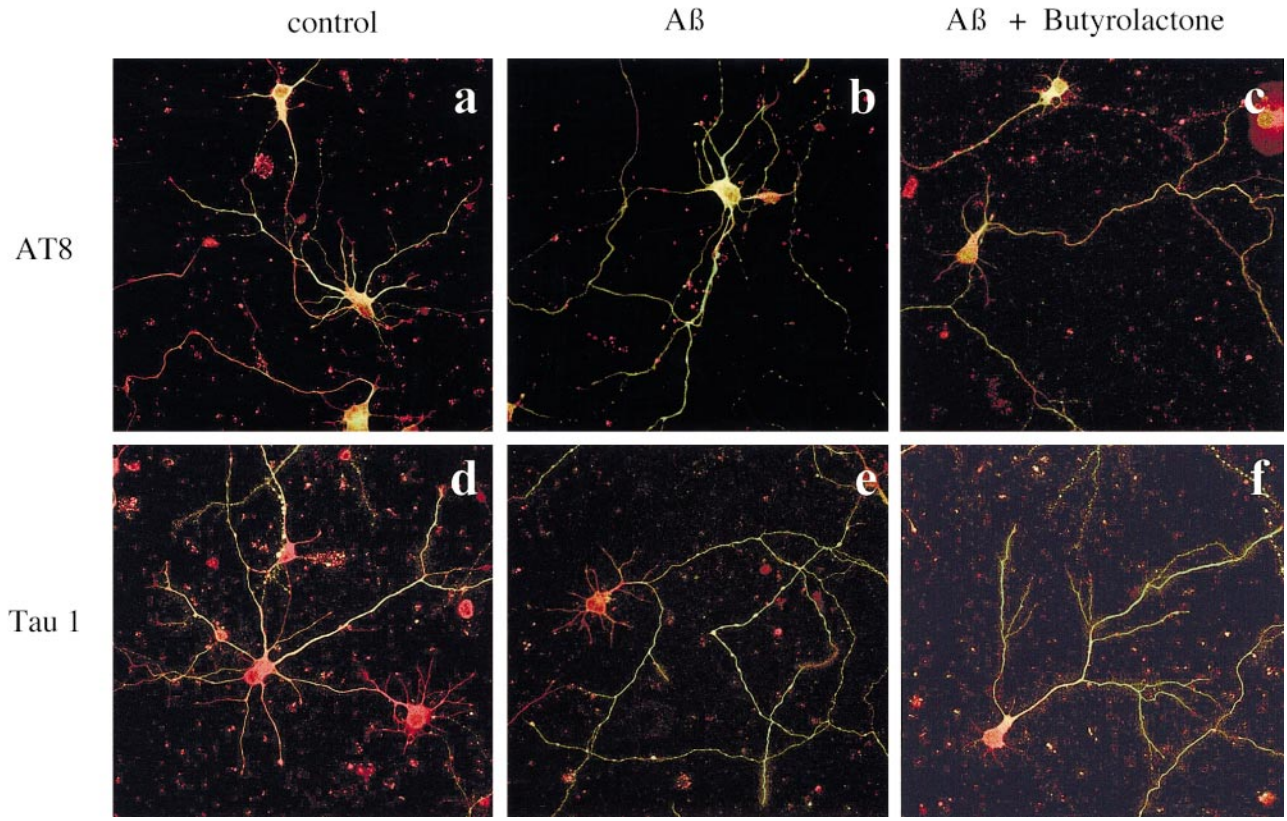

FIG. 2. Effects of Aβ and butyrolactone I on distribution of tau phosphoepitopes in hippocampal cells. Aβ peptide was added in the presence or the absence of butyrolactone I, and cells were analyzed 48 h later by immunofluorescence. Confocal micrographs illustrate hippocampal cells double-stained with polyclonal anti-tubulin and AT8 antibodies (a, b, and c) and with polyclonal anti-tubulin and Tau-1 antibodies (d, e, and f). (a and d) Control neurons. (b and e) Cells treated with 2.5 μM Aβ fibrils. (c and f) Cells treated with 2.5 μM Aβ plus 5 μM butyrolactone. Green staining corresponds to the AT8 antibody-reactive proteins (a–c), while red staining corresponds to tubulin staining. Also, green staining in the double immunofluorescence of d through f corresponds to protein reactive with Tau-1 antibody.

and in part in the cell body (Fig. 2a), while Tau-1 tagged mainly the neuronal processes (Fig. 2d). Tubulin staining was localized in both soma and axonal compartment, colocalizing in certain domains with phosphorylated (Fig. 2a) or dephosphorylated (Fig. 2d) tau epitopes. In contrast, in cells treated for 48 h with Aβ, an increment in AT8 immunoreactivity was observed in the soma in the morphology of Aβ-exposed cells (Fig. 2b), while Tau-1 immunoreactivity maintained its axonal distribution (Fig. 2e). No significant cell death occurred in the Aβ-treated cells. As an additional difference with respect to control neurons, cells treated with Aβ exhibited a bead-like structure along the neuronal processes (Figs. 2b and 2e). However, when butyrolactone I was added to cultures with Aβ, this inhibitor protected neuronal cells against the degenerative changes. The labeling distributions of both AT8 and Tau-1 antibodies in axons and neuronal processes appeared to be similar to those of untreated controls. The AT8 immunostaining pattern of butyrolactone I-treated neurons (Fig. 2c) exhibited a significantly lower intensity in the cell soma compared with AT8 staining of Aβ-exposed cells (Fig. 2b). In addition, preincubation with butyrolactone (Figs. 2c and 2f) prevented formation of bead-like structures observed in neurons treated with Aβ (Figs. 2b and 2e) but in the absence of the cdk5 inhibitor.

Activity of Cdk5 Kinase in Different Neuronal and Nonneuronal Cell Lines

In the context of these studies, it was of interest to examine cell viability and the protection effects of butyrolactone I in different types of cells in culture by using the MTT reduction assay. Previous studies have shown that butyrolactone I protects hippocampal cells from neuronal death induced by Aβ [13]. Figure 3A shows the protective effect of butyrolactone I on cell viability of the differentiated and undifferentiated N2A and B12 cell lines. Aβ fibrils promoted cell death, as revealed by a gradual decrease in the MTT reduction compared with cells treated with Aβ but in the presence of 5 μM butyrolactone I. The MTT levels associated with neuronal survival for cells exposed to

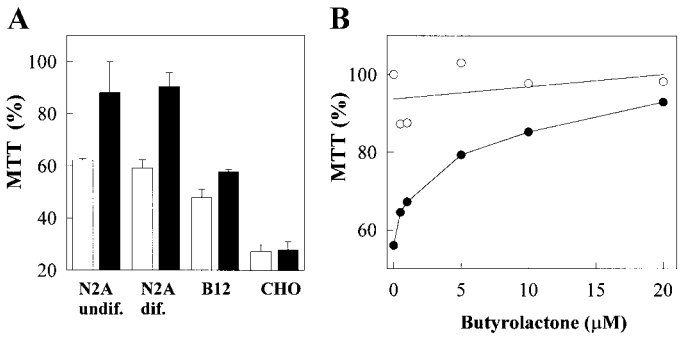

FIG. 3. Cdk5 activity in various cell lines treated with Aβ fibrils. (A) Effects of butyrolactone I on Aβ-induced neurotoxicity in cultured cell lines. Cells were treated with 15 μM Aβ-fibrils alone (open bars) or Aβ fibrils plus 5 μM butyrolactone I (closed bars). Cell viability was evaluated using the MTT reduction assay. Cells incubated in the absence of both Aβ and butyrolactone I were used as control. (B) Concentration-dependent protective effects of butyrolactone I on dibutyryl-cAMP-treated N2A cells against Aβ-induced toxicity. Increasing concentrations of butyrolactone I (●) increased MTT reduction as an expression of viability of neurons treated with Aβ (15 μM). The toxic effect of the Aβ fibrils diminished as concentration of butyrolactone (1–20 μM) increased. As a control, the inhibitor added in the absence of Aβ (○) did not modify the cells' survival.

Aβ in the presence of butyrolactone were higher, as revealed by the two-tailed Student t test of significance, with $n = 5$. No protective effects were observed in CHO cells. Thus, considering that in CHO cells, Aβ induced neuronal death without affecting cdk5, the cdk5 activation seems to be a required step in the butyrolactone I protection against Aβ-induced cell death. The concentration-dependent effect of butyrolactone I on the differentiated neuroblastoma N2A cells treated with Aβ was also shown (Fig. 3B). Protective effects by butyrolactone on B12 cells were also concentration dependent (data not shown).

In order to further evaluate the role of cdk5 activation and the subsequent neuronal death, experiments were conducted with antisense oligonucleotides for cdk5 and the regulators p35 and p39 subunits [12, 19]. In the negative control, the treatment of rat hippocampal cells cultured with 10 μM Aβ fibrils with a cdk5 oligonucleotide with scrambled sequence (4 μM) did not prevent neurotoxicity caused by the amyloid. However, in the presence of Aβ fibrils plus cdk5 antisense oligonucleotide (4 μM), neurotoxicity was significantly reduced (Fig. 4). In addition, in the presence of antisense oligonucleotides for p35 and p39 components, Aβ-induced neurotoxicity was also reduced. The significant protection effect exerted by the antisense oligo for the regulatory protein p39 was noteworthy (Fig. 4).

To further analyze the sequence of events that leads to the increase of cdk5 activity of cells exposed to Aβ fibrils, the levels of expression of cdk5 and p35 were analyzed in cells treated with Aβ by using immunodetection with the respective antibodies. No changes in the expression levels were evidenced in either case, using as a protein loading control the expression of actin as a housekeeping gene (Fig. 5A). The levels of cdk5 and p35 mRNAs were analyzed by RT-PCR (Figs. 5B and 5C), and no change was detected in the levels of transcripts, using β-actin mRNA as a control. These studies suggest that changes in cdk5 induced by Aβ respond to posttranslational effects rather than the expression of the enzyme.

In the context of these studies, and to evaluate the regulatory aspects of cdk5 activity, we analyzed the direct effects of posttranslational modifications on cdk5 on the regulation of its activity and the role of p35 in controlling cdk5 activation. The neuronal extracts of Aβ-treated hippocampal cells exhibited a marked increase in endogenous phosphorylation of the GST-cdk5 (Fig. 6A, lane 2) compared with controls (lane 1). In order to analyze the cdk5–p35 interaction, Western blot analysis of electrophoretically separated protein from cell extracts of Aβ-treated (Fig. 6, lanes 2 and 4) and untreated (lanes 1 and 3) hippocampal cells were carried out. A significant increase in formation of a stable cdk5–p35 complex of around 55 kDa MW in the Aβ-treated cells (Fig. 6B, lane 2) compared with untreated cell controls (Fig. 6B, lane 1) was revealed in the blots by using anti-cdk5 antibody. An increase in the complex formation was also evidenced in blots using anti-p35 antibodies (Fig. 6B, lanes 3 and 4). We have observed that together with the increase in cdk5–p35 complex, minor levels of free cdk5 were detected in blots of Aβ-treated cells ($n = 8$), even though free cdk5 is not shown in the experiment of Fig. 6B (lanes 1 and 2).

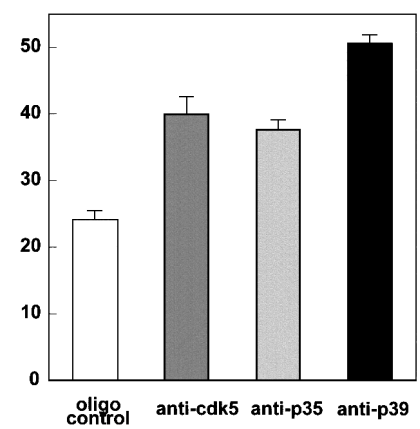

FIG. 4. Effects of antisense probes, against cdk5, p35, and p39 mRNAs, on the viability of hippocampal neurons treated with Aβ. Viability was evaluated on the basis of the MTT reduction assay on cells cultured with 10 μM Aβ. The open bars denote viability of cells treated with the amyloid fibrils plus the scrambled oligonucleotide. The closed bars correspond to the hippocampal cells treated with Aβ in the presence of the cdk5 antisense, p35 antisense, and p39 antisense probes. The standard deviations are shown for each histogram. Each bar corresponds to the mean of three determinations.

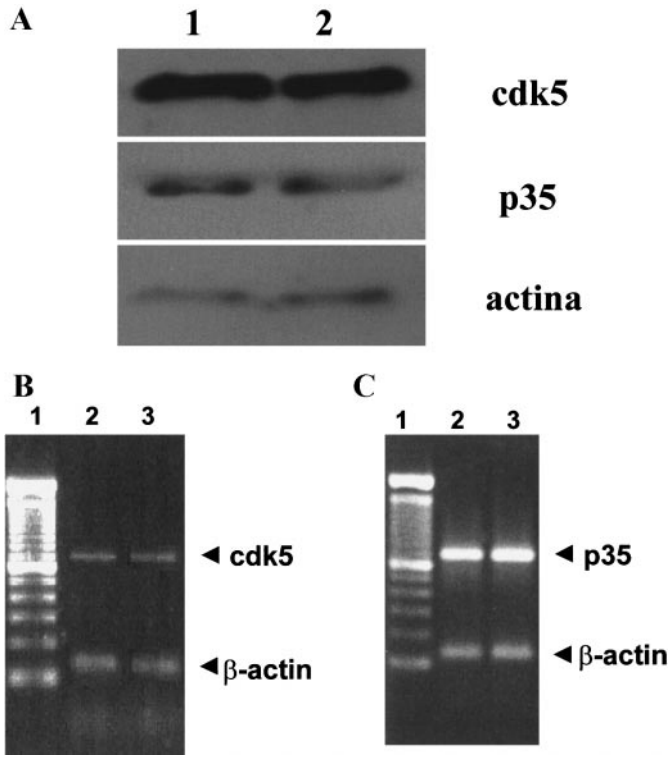

FIG. 5. Expression levels of cdk5 and p35 in hippocampal cells treated with Aβ. (A) Immunodetection by Western blots of cdk5 and p35 in hippocampal cell extracts treated (lanes 1) and untreated (lanes 2) with 15 μM Aβ fibrils. Actin was used as a protein loading control. (B) RT-PCR transcripts of cdk5 from hippocampal cell extracts treated (lane 2) and untreated (lane 3) with 15 μM Aβ fibrils. Lane 1 represents the pattern of a 100 bp DNA ladder standard. (C) RT-PCR transcripts of p35 from hippocampal cell extracts treated (lane 2) and untreated (lane 3) with 15 μM Aβ fibrils. Lane 1 represents the pattern of a 100 bp DNA ladder standard. Arrowheads denote the locations of the respective DNA fragments representing the transcripts (702 bp for cdk5 and 680 bp for p35). The transcript of β-actin was used as an internal control.

The cdk5–p35 complex could be dissociated by treatment of the Aβ-exposed cell extracts with 3 M GuHCl, and only free cdk5 was detected in the blot analyses (Fig. 6B, lane 5). This experiment was repeated several times ($n = 5$) with identical results, and no complex was detected after GuHCl treatment. Similarly, free p35 and no remaining complex after GuHCl treatment was also detected by using anti-p35 antibody (data not shown).

It was important to evaluate the effects of endogenous phosphorylation of cdk5 on the cdk–p35 complex formation. For this purpose free and complexed cdk5 was trapped by immunoprecipitation with anti-cdk5, followed by autoradiography. Thus, it was observed that the highest phosphorylation levels of the cdk5–p35 complex were attained after 12 h of treatment with Aβ (Fig. 6C, left), aspects that merit further investigation in the light of the sensitivity of cdk5 to phosphorylation. Moreover, the phosphorylation pattern of free cdk5 (Fig. 6C, right) showed similar features compared with that observed for the cdk5 complex.

DISCUSSION

We have shown that the treatment of hippocampal cells with amyloid filaments induces a deregulation of the protein kinase cdk5 [13]. On the basis of this finding, we focused on the relationships between the cdk5 activation and tau phosphorylation and the molecular signals that determine cdk5 deregulation. Here, we report that cdk5 activation is concomitant with the hyperphosphorylation of one of its major neuronal substrates, tau protein, at the epitopes serine 202 and threonine 205. This was evidenced by the marked in-

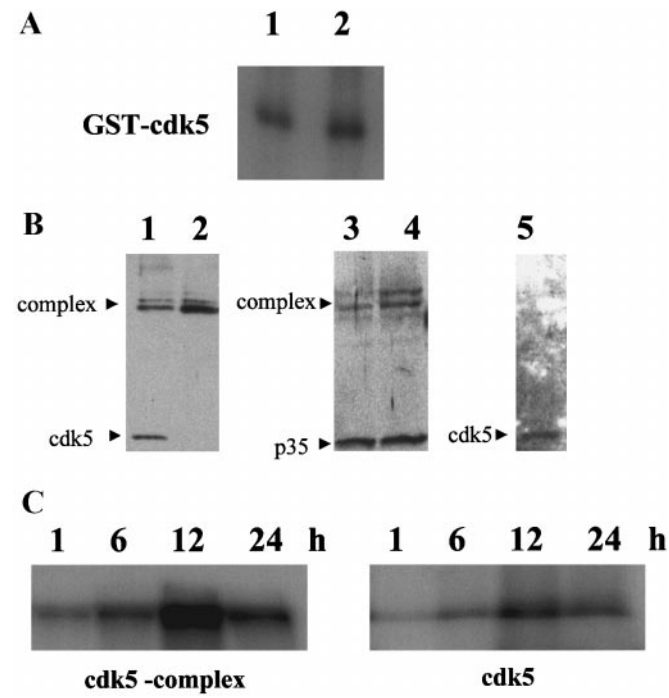

FIG. 6. Effects of endogenous phosphorylation of cdk5 on the complex formation. (A) Phosphorylation of the construct of glutathione transferase-cdk5 for the hippocampal extract from cells untreated (lane 1) and treated with 15 μM Aβ (lane 2). The exogenous GST-cdk5 construct was incubated with the neuronal extract in the presence of [γ-^{32}P]ATP, then the GST-cdk5 was purified and analyzed by autoradiography. (B) Immunodetection by Western blots of the complex cdk5–p35 (arrowhead) by using both the anti-cdk5 and the anti-p35 antibodies. Lanes 1 and 3, control hippocampal cells; lanes 2 and 4, hippocampal cells treated with 15 μM Aβ fibrils for 48 h. The free cdk5 is also indicated by an arrowhead. Lane 5 corresponds to hippocampal cell extracts treated with GuHCl and stained with anti-cdk5. (C) Endogenous phosphorylation of the cdk5–p35 complex and free cdk5. Hippocampal cells were subjected to metabolic labeling with $^{32}P_i$, in the presence of 15 μM Aβ fibrils, at different time intervals, and the cdk5 was immunoprecipitated with anti-cdk5 and revealed by autoradiography. Left: cdk5–p35 complex. Right: free remaining labeled cdk5.

crease in cdk5 reactivity toward AT8 antibody and a decrease with Tau-1. This epitope has been described as a major target for cdk5 [24]. Furthermore, this epitope has been characterized as a major epitope of tau from paired helical filaments. The increase in tau phosphorylation in serine 202 and threonine 205 was associated with changes in the distribution of tau phosphoepitopes as assessed by immunofluorescence using both anti-tau antibodies. This change in phosphorylated tau distribution was probably driven by the increment in cdk5 activity, since it was possible to revert the distribution pattern recovering the normal intraneuronal tau organization by inhibiting cdk5 with butyrolactone I. This effect was mainly due to the inhibitory action of this drug on cdk5, since no detectable amounts of other cyclin-dependent protein kinases were found in the hippocampal cell cultures [13]. Recently, hyperphosphorylation of tau has been described in mice that overexpress p25 activator of cdk5 [25]. Furthermore, the p25 transgenic mice display a cytoskeletal disruption reminiscent of Alzheimer's disease and other neurodegenerative disorders [25].

With the purpose of evaluating if this activation phenomenon is conserved in other cell types, different from hippocampal cells, of the central nervous system, we analyzed the effects of Aβ on cell viability in cell lines such as neuroblastoma N2A and glioma B12. CHO cells were used as a model for nonneuronal cells. These different cells were treated with Aβ in the presence of butyrolactone I. After assessing the viability of these cells, it was observed that butyrolactone I exerted protection in both differentiated and undifferentiated N2A and glioma cells, but no protection by the cdk5 inhibitor was found in CHO cells. This suggests that in CHO cells, cellular death was not associated with a signal transduction pathway involving cdk5. Concomitantly, activation of cdk5 was observed in neuronal and glial cells but it was not detected in CHO cells (data not shown). Even though cdk5 activation was not observed, CHO cells were sensitive to the neurotoxic effects of fibrillary Aβ. The fact that Aβ promoted cell death in the CHO system, which lacks tau protein, suggests that the effects of Aβ on those cells could be mediated by a different pathway. It has been reported that cdk5 has been detected in CHO cells but it does not seem to have a significant activity [26]. The substrates for this kinase in CHO cells have not been identified yet. Our studies showed that antisense oligonucleotides for cdk5 and for the activators p35 and p39 partially neuroprotected hippocampal cells against Aβ toxicity. Even though these neuroprotective effects were lower than those observed for butyrolactone [13], it is worth pointing out the action of p35 and p39 antisense in reducing neuronal death promoted by Aβ fibrils.

In order to evaluate the molecular aspects underlying the Aβ-induced increase of cdk5 activity, it was important to determine the levels of expression of cdk5 and the regulator p35 in hippocampal cells treated with Aβ. No changes were observed in expression either at the level of mRNA or at the protein level. In this context, we evaluated the possible posttranslational regulation of cdk5 activity. It has been described [23] for other cyclin-dependent kinases that the main regulatory event is the association of the catalytic subunit with the regulator, interactions that seem to depend on phosphorylation that occurs in both proteins. In this context, it has been reported that the cdk5–p35 complex formation is dependent upon previous phosphorylation on cdk5, presumably on Ser^{159} [23]. In the case of PC12 cells, the kinase phosphorylating cdk5 corresponds to casein kinase I, an effect that results in activation of cdk5 [23]. We also found phosphorylation of cdk5 and this modification increased the complex formation. Most interestingly this modification increased the activity of cdk5. In this context, we detected a stabilized form of cdk5–p35 complex upon treatment of hippocampal cells with fibrillary Aβ. This complex was resistant to dissociation by SDS, and it was dissociated only in the presence of chaotropic agents such as guanidine hydrochloride. The marked increase in the stability of the cdk5–p35 complex was concomitant with the uncontrolled increase in cdk5 activity and with the endogenous phosphorylation of this enzyme, suggesting that cdk5 phosphorylation is a critical step in the abnormal deregulation of cdk5 upon Aβ treatment of hippocampal cells. On the other hand, it is worth pointing out that the posttranslational modifications on cdk5 involved in regulation of neuronal development appear to be linked to changes in its dephosphorylation patterns. In this context, a recent publication has revealed that cdk5 can be also phosphorylated on Tyr^{15} and that this reaction is stimulated by the activity of cdk5–p35 complex [27]. This information along with the present data on the regulatory aspects of cdk5 and interactions with regulatory proteins such as p35 and p39 point to the unique features of this protein kinase, which are distinct from the other cyclin-dependent protein kinases.

The electrophoretic, Western blot, and complex dissociation studies indicate that the protein kinase complex is mainly formed by cdk5 and p35. Under the present conditions, a specific antibody against cdk5 and a polyclonal antibody against the N-terminal domain of p35 were used. However, a significant increment of a cleavage product of p35, the fragment p25, in Alzheimer's brains has recently been described [28] and the protease involved in this cleavage seems to be calpain [29]. In those reports [28, 29] it was postulated that the increase in a potential complex with p25 leads to an increase of cdk5 activity, which may result from an increment in the half-life of p25 fragment. Whether

p25 is a component in the complex remains to be elucidated. Under our present experimental conditions, and in the model system of primary cultures from rat hippocampal cells, p25 has not been detected at all in the recovered complex. This may be due to the antibody used in the study or it may imply that in response to the effects of Aβ, the membrane-associated p35 stabilizes a complex with cdk5. However, in the hippocampal cell system, generation of a complex including p25 (cdk5–p25) and its involvement in the increase of activity upon Aβ treatment cannot be discarded. The data in Fig. 6B show a minor proportion of a higher molecular weight product upon association of cdk5 with p35, an aspect that remains to be investigated in depth.

In summation, studies strongly suggest that changes in hyperphosphorylated tau distribution occur upon treatment of hippocampal cells with fibrillary Aβ and that formation of a stable complex between a post-translationally modified form of cdk5 and its activator p35 could be responsible for the sequence of events that result in an uncontrolled cdk5 activation upon Aβ treatment. This activated cdk5 may be responsible for some abnormal tau hyperphosphorylation, which in conjunction with other factors, i.e., changes in tau conformation and glycation reactions [5], leads to neuronal degeneration.

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