Regulation of amyloid precursor protein (APP) phosphorylation and processing by p35/Cdk5 and p25/Cdk5

Feng Liu[a], Yuan Su[a], Baolin Li[a], Yan Zhou[a], John Ryder[a], Patricia Gonzalez-DeWhitt[a], Patrick C. May[a], Binhui Ni[a,b,c,*]

[a] *Neuroscience Discovery Research, Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285, USA*
[b] *Department of Psychiatry, Indiana University Medical School, Indianapolis, IN 46202, USA*
[c] *Department of Anatomy and Cell Biology, Indiana University Medical School, Indianapolis, IN 46202, USA*

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Abstract The phosphorylation status of amyloid precursor protein (APP) at Thr668 is suggested to play a critical role in the proteolytic cleavage of APP, which generates either soluble APP_{β} ($sAPP_{\beta}$) and β-amyloid peptide (Aβ), the major component of senile plaques in patient brains inflicted with Alzheimer's disease (AD), or soluble APP_{α} ($sAPP_{\alpha}$) and a peptide smaller than Aβ. One of the protein kinases known to phosphorylate APP^{Thr668} is cyclin-dependent kinase 5 (Cdk5). Cdk5 is activated by the association with its regulatory partner p35 or its truncated form, p25, which is elevated in AD brains. The comparative effects of p35 and p25 on APP^{Thr668} phosphorylation and APP processing, however, have not been reported. In this study, we investigated APP^{Thr668} phosphorylation and APP processing mediated by p35/Cdk5 and p25/Cdk5 in the human neuroblastoma cell line SH-SY5Y. Transient overexpression of p35 and p25 elicited distinct patterns of APP^{Thr668} phosphorylation, specifically, p35 increasing the phosphorylation of both mature and immature APP, whereas p25 primarily elevated the phosphorylation of immature APP. Despite these differential effects on APP phosphorylation, both p35 and p25 overexpression enhanced the secretion of Aβ, $sAPP_{\beta}$, as well as $sAPP_{\alpha}$. These results confirm the involvement of Cdk5 in APP processing, and suggest that p35- and p25-mediated Cdk5 activities lead to discrete APP phosphorylation.

Key words: Cyclin-dependent kinase 5; p35; p25; Amyloid precursor protein; Immature amyloid precursor protein; Mature amyloid precursor protein; Soluble amyloid precursor protein

1. Introduction

The progressive extracellular accumulation of the 4-kDa amyloid β-peptide (Aβ, senile plaques) in the central nervous system is one of the characteristics of Alzheimer's disease (AD) pathology [1,2]. Senile plaques are predominantly composed of Aβ peptides of 40–42 amino acids derived from amyloid precursor protein (APP) [3] via proteolytic cleavage by β- and γ-secretase [4]. APP is a type one transmembrane glycoprotein that is ubiquitously expressed in mammalian tissues [5]. There are three major isoforms of APP (APP_{695}, APP_{751}, and APP_{770}), consisting of 695, 751, and 770 amino acids, respectively [5]. In addition to the generation of Aβ, the cleavage of APP by β- and γ-secretases also gives rise to a soluble N-terminal fragment, $sAPP_{\beta}$. Alternatively, APP is cleaved by α-secretase within the Aβ sequence resulting in $sAPP_{\alpha}$, thus precluding the formation of Aβ [6,7].

In neuronal cells, APP_{695} is phosphorylated at multiple serine and threonine residues [8]. The phosphorylation of Thr668 conveys the most pronounced conformational change in the APP cytoplasmic tail [9], and has been suggested to play important roles in neurite outgrowth [10], APP subcellular localization [11], and APP processing [12]. Several protein kinases are able to phosphorylate APP^{Thr668} in vitro or in vivo [11–14], but the cyclin-dependent kinase 5 (Cdk5) is the key kinase responsible for the APP^{Thr668} phosphorylation in neuronal cells [11]. Cdk5 is a unique member of the small serine/threonine kinase (Cdk) family [15] that plays a critical role in the development of the central nervous system [16]. p35 is the regulatory subunit of Cdk5. The binding of Cdk5 and p35 in neurons triggers Cdk5 enzymatic activity [17]. p35 contains an amino-terminal myristoylation signal motif that anchors the p35/Cdk5 complex to the cell membrane [15]. Therefore, p35 dictates the substrate specificities of Cdk5. p35 is known to be proteolysed to form p25 by Ca^{2+}-dependent protease calpain under neurotoxic conditions [18]. Unlike p35, p25 lacks the amino-terminal myristoylation signal motif. Therefore, p25 is mainly distributed in the cytoplasm. Because p25 is more stable than p35, the accumulation of p25 in cells causes constant association of p25 with Cdk5, which allows the prolonged activation of Cdk5 [19] and the hyperphosphorylation of cellular proteins such as Tau [20]. In this study, we examined the specific effect of p35 and p25 on APP^{Thr668} phosphorylation in neuroblastoma SH-SY5Y and a non-neuronal cell line, HEK293, and we explored the effect of p35 and p25 on APP processing.

2. Materials and methods

2.1. Antibodies and reagents

Anti-p35/p25 and Cdk5 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Aβ precursor protein

*Corresponding author. Fax: (1)-317-277 1125.
E-mail address: ni_binhui@lilly.com (B. Ni).

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, β-amyloid peptide; Cdk5, cyclin-dependent kinase 5; imAPP, immature APP; mAPP, mature APP; sAPP, soluble APP; $swAPP_{751}$, APP_{751} bearing the Swedish mutation

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polyclonal antibody (pAb) (CT695) was from Zymed Laboratories (South San Francisco, CA, USA). Anti-APP monoclonal antibody (mAb) 6E10 was obtained from Signet (Dedham, MA, USA), the anti-sAPP$_\beta$ pAb was provided by Mr. E. Johnstone/Dr. S. Little (Eli Lilly and Co), and the anti-phospho-APP (Thr668) antibody was from Cell Signaling (Beverly, MA, USA). The Cdk5, p35, and p25 cDNA constructs were kind gifts from Dr. L. H. Tsai (Harvard Medical School, Boston, MA, USA), while the APP$_{695}$ construct was provided by Mr. E. Johnstone/Dr. S. Little. The transfection reagent FuGENE6 was purchased from Roche (Indianapolis, IN, USA). The protein molecular weight marker (Precision Protein Standards) was from Bio-Rad. All other common reagents and cell culture media were from Invitrogen Life Technologies (Carlsbad, CA, USA) unless specified otherwise.

2.2. Cell culture

Human neuroblastoma SH-SY5Y cells were purchased from ATCC (Manassas, VA, USA) and grown in minimum essential medium Eagle/F-12K (1:1 mixture) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Human embryonic kidney (HEK293) cells stably transfected with APP$_{751}$ [21] were cultured in Dulbecco's modified Eagle's medium-F12 supplemented with 5% FBS, 20 mM HEPES, 300 μg/ml G418 and 1% penicillin–streptomycin. All cell lines were propagated in T75 flasks and subsequently plated in 24-well plates for treatments. Cells were maintained at 37°C in a humidified incubator supplied with 5% CO_2.

2.3. Transient transfection

Cells were seeded in 24-well plates and grown to reach 50–60% confluence at the time of transfection. Plasmid DNAs were first mixed with FuGENE6 at the ratio of 1 μg:3 μl (DNA:FuGENE6) per 100 μl of OPTI-MEM (each well received 0.25 μg DNA). The transfection mixtures were incubated at room temperature for 30 min prior to the addition to the cells. Cells were incubated with DNA mixture for the entire duration of transfection (12–24 h).

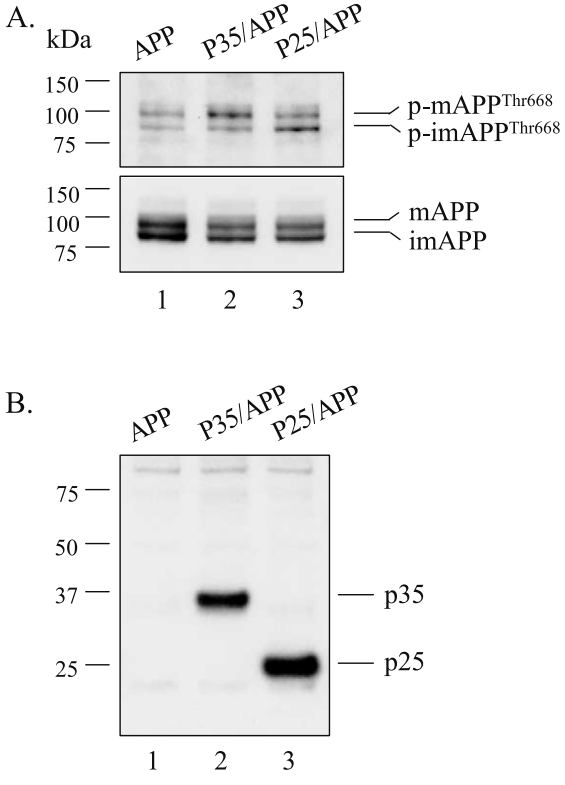

Fig. 1. Overexpression of p35 and p25 distinctly increases APP phosphorylation in SH-SY5Y neuroblastoma cells. A: SH-SY5Y cells were transiently transfected with APP$_{695}$ alone (lane 1), co-transfected with p35 and APP$_{695}$ (lane 2), or co-transfected with p25 and APP$_{695}$ (lane 3). Twenty-four hours following transfection, cells were directly lysed in LDS sample buffer and cell lysates were subjected to Western blotting. The phosphorylated APP^{Thr668} was detected by anti-phospho-APP (Thr668) antibody (top blot), while the total APP was probed with CT695 (bottom blot). B: Protein levels of p35 and p25 in the cell preparations described in A, as detected by antibody C-19, which recognizes both p35 and p25. Data are representative of two experiments.

2.4. Generation of SH-SY5Y cell line constitutively expressing swAPP$_{695}$

SH-SY5Y cells were transfected with a cDNA encoding APP$_{751}$ bearing the Swedish mutation (swAPP$_{695}$) using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. Single cell clones were generated by selection with 250 μg/ml of G418.

2.5. Electrophoresis and Western immunoblotting

The NuPAGE Bis-Tris System (Invitrogen Life Technologies) was utilized for electrophoresis and Western transfer. For the detection of cell-associated proteins, cells were grown, treated in 24-well plates, and lysed with 200 μl and 400 μl of LDS sample buffer for SH-SY5Y and HEK293 cells, respectively. For the detection of APPs released into media, conditioned media were collected, centrifuged at 14 000 rpm for 10 min (4°C) to remove cell debris, and then mixed with LDS sample buffer. Cell lysates or conditioned media were separated on 10% Bis-Tris gels (200 V, 1 h), and transferred to nitrocellulose membranes (0.2 μm; 50 V, 2 h), followed by Western immunoblotting analyses as described previously [22]

2.6. Measurement of Aβ peptides in culture media

The secreted total Aβ and Aβ$_{1-42}$ peptides in conditioned media were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) as previously described [22]. In essence, affinity-purified mAb 266.2 (1.5 μg/well) was applied as the capture antibody for total Aβ, mAb 21F12 (0.5 μg/well) was used as capture antibody for Aβ$_{1-42}$ and biotinylated mAb 3D6 was used as detection antibody. Streptavidin–horseradish peroxidase was purchased from Amersham and TMB Substrate Kit was from Pierce. All assays were performed in 96-well Immulon 4 microtiter plates (Dynex Technologies, Chantilly, VA, USA).

2.7. Statistics

Statistical analysis was performed using the paired *t*-test. Data are presented as mean ± S.E.M., $P < 0.05$ was considered significant.

3. Results and discussion

3.1. p35/Cdk5 and p25/Cdk5 differentially phosphorylate APP

Previous studies from several groups have shown that the phosphorylation of APP at Thr668 is important for certain biological/pathological processes [10]. It has been demonstrated that Cdk5, when activated by its regulating partner p35, directly phosphorylates APP^{Thr668} [11]. p25, the proteolytic product of p35, has been reported to possess the full functionality of p35 [15]. However, the properties of p25 in catalyzing APP phosphorylation have not been characterized. Because p25, but not p35, has recently been proposed to play an important role in AD pathogenesis, it is of great interest to evaluate the characteristics of p35 and p25 in mediating APP phosphorylation. To that end, we transiently co-expressed p35 or p25 with APP$_{695}$ in neuroblastoma SH-SY5Y and evaluated APP phosphorylation by Western analyses using an antibody specifically recognizing the Cdk5 phosphorylation site – Thr668 on APP [12]. Fig. 1A demonstrates that p35 stimulated the phosphorylation of both mature APP (mAPP: *N*- and *O*-glycosylated, tyrosyl-sulfated) and immature APP (imAPP, *N*-glycosylated only), whereas p25 primarily increases the phosphorylation of imAPP (upper panel). The total APP expression levels were not increased by p35 or p25 transfection (lower panel). Fig. 1B shows the comparable and abundant overexpression of p35 and p25 in SH-

SY5Y cells after transfection. These data suggest similar capabilities of p25 and p35 in regulating Cdk5 catalytic activities with preferential substrate selection: p35/Cdk5 targeting both mature and immature APP while p25/Cdk5 prefers the immature form of APP.

To substantiate the observation of the differential effects of p35/Cdk5 and p25/Cdk5 on the phosphorylation of imAPP and mAPP in SH-SY5Y cells, we extended our study to investigate the phosphorylation of mAPP and imAPP by p35/Cdk5 and p25/Cdk5 in HEK293 cells constitutively overexpressing APP_{751} (Fig. 2A) or $swAPP_{751}$ (Fig. 2B), two well established cell systems for studies of APP processing [23–25]. Because these cells express low levels of endogenous Cdk5, cotransfections of p35 and Cdk5 or p25 and Cdk5 were performed. Expressions of p35/Cdk5 and p25/Cdk5 did not alter the protein levels of APP, but increased the phosphorylation of APP in a similar pattern as that of SH-SY5Y cells overexpressing APP695. These results suggest that the differential effects of p35 versus p25 on APP phosphorylation are independent of cell type, APP isoform or the Swedish mutation - even though the Swedish mutation at the APP C-terminus (K670N/M671L) is in the near vicinity of the Cdk5 phosphorylation site (Thr668). Taken together, our results demonstrate that in our cell model systems p35/Cdk5 is able to phosphorylate both mAPP and imAPP, whereas p25/Cdk5 is mostly proficient at phosphorylating imAPP. The

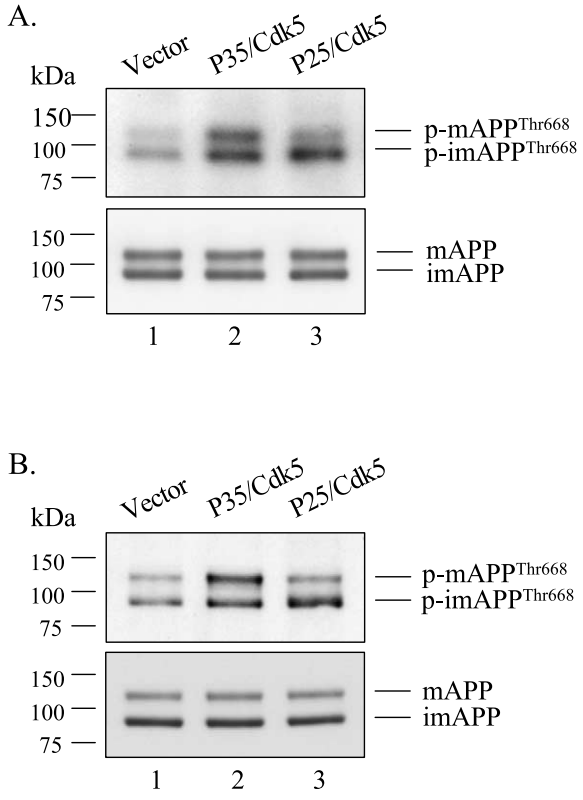

Fig. 2. p35/Cdk5 and p25/Cdk5 distinctly increase APP phosphorylation in HEK293 cells. HEK293 constitutively expressing APP_{751} (A) or $swAPP_{751}$ (B) were transiently transfected with the empty vector (lane 1), the mixture of equal amounts of p35 and Cdk5 cDNA (lane 2), or the mixture of p25 and Cdk5 cDNA (lane 3). Twenty-four hours following transfection, cells were lysed and analyzed by Western blotting. The phosphorylated mature/immature APP^{Thr668} and total APP were detected using the antibodies as described above. Data are representative of three experiments.

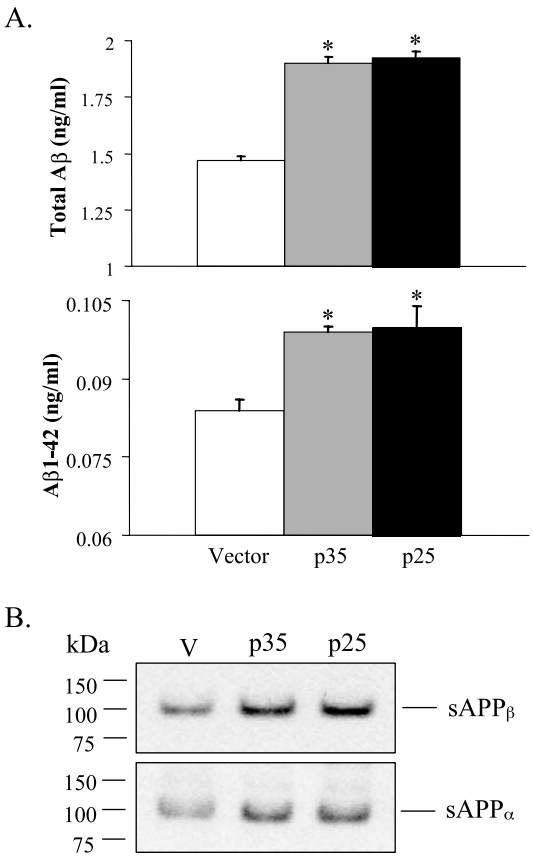

Fig. 3. Both p35 and p25 enhance the production of Aβ and sAPP. SH-SY5Y constitutively expressing $swAPP_{695}$ were transfected with empty vector, p35, or p25. Twelve hours following transfection, the transfection mixture was replaced with fresh culture medium and the cells were further cultured for 24 h. The conditioned media were then collected for the measurement of Aβ and sAPP. A: Total Aβ and $Aβ_{1–42}$ in the conditioned media were quantified by ELISA as described in Section 2. B: sAPP in the conditioned medium was determined by Western immunoblotting. $sAPP_β$ was detected using an antibody specifically recognizing β-secretase-cleaved sAPP fragments (upper blot), and $sAPP_α$ was probed with mAb 6E10 (lower blot). Data are representative of two experiments.

mechanism(s) leading to the distinct manners of APP phosphorylation by p35/Cdk5 and p25/Cdk5 is unknown, but one possibility lies in the different subcellular localization of the two Cdk5 modulators, as well as the localization of the Cdk5 substrates – mAPP and imAPP. p35 contains a highly conserved N-terminal myristoylation signal motif [19] that confers its cell membrane distribution [15,19], which positions p35/Cdk5 in close proximity to the transmembrane APP (both mAPP and imAPP). On the other hand, without the N-terminal membrane anchorage sequence, p25 is destined to where the majority of the immature APP is localized.

3.2. Both p35/Cdk5 and p25/Cdk5 enhance APP processing

The significance of APP^{Thr668} phosphorylation status has been implicated in the regulation of APP processing. Prompted by the observation described above that p35 and p25 mediate APP phosphorylation distinctly, we next investigated the influences of p35 and p25 on APP processing. SH-SY5Y cells constitutively expressing $swAPP_{695}$ were transiently transfected with either vector control or p35 or p25. The secreted $sAPP_α$ and $sAPP_β$ proteins as well as Aβ pep-

tides were detected (Fig. 3). Overexpression of p35 or p25 both significantly increased Aβ levels (Fig. 3A) and the secretion of $sAPP_{\alpha}/sAPP_{\beta}$ (Fig. 3A) in the conditioned media, suggesting that increased Cdk5 activities through p35 and p25 overexpression indeed elevated APP processing. However, p35 and p25 elicited similar enhancements in Aβ and APP secretion i.e. p35 and p25 failed to display differential effects on APP processing. The same results were obtained using HEK293 cells constitutively expressing swAPP751 (data not shown). These results imply that APP^{Thr668} phosphorylation mediated by p35/Cdk5 or p25/Cdk5 plays important roles in the regulation of APP processing, independent of the status of APP maturation in SH-SY5Y and HEK293.

It should be noted, however, that APP processing is rather complex, including APP maturation, secretase cleavage and secretion etc. Multiple kinases involved in each of the steps could participate concomitantly in regulation of the APP processing. For example, a recent report by Phiel et al. demonstrated conclusively that GSK3α facilitates APP processing, likely via regulation of secretase activity, and inhibition of the enzyme by lithium reduces Aβ production [26]. Studies from our laboratory also demonstrate a role for cAMP-dependent protein kinase (PKA) in the regulation of APP processing [27]. It appears that PKA-regulated APP processing is via modulation of APP post-translational modification or maturation [27]. In the present study, however, expression of p35/Cdk5 or p25/Cdk5 did not affect APP maturation although the precise mechanism underlying the regulation of APP by p35/Cdk5 or p25/Cdk5 is unknown.

In summary, p35 and p25 displayed differential preferences in phosphorylation of mAPP and imAPP and might render different physiological functions of APP. p25 is known to be elevated in AD brains and thus conceivably regulates the preferential phosphorylation of imAPP. Although the phosphorylation status of imAPP or the role of imAPP in the pathogenesis of AD awaits further investigation, our findings provide insight into potential mechanisms that underlie the effect of p25 or activation of Cdk5 in neurons.

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