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Suppression of the caspase cleavage of β-amyloid precursor protein by its cytoplasmic phosphorylation

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Abstract β-Amyloid precursor protein (APP) is a type I transmembrane protein. Its cleavages by β- and γ-secretases yield β-amyloid, which is the main constituent of senile plaques in Alzheimer's disease (AD). In apoptotic cells and AD brains, APP is alternatively cleaved by caspases in the cytoplasmic region after the Asp664 residue (with respect to the numbering conversion for the APP695 isoform). Caspase-cleaved fragments of APP are cytotoxic and have been implicated in AD pathogenesis; however, the mechanisms regulating the cleavage have not been studied. APP is constitutively phosphorylated at Thr668 in brain. In the present study, we demonstrate that APP phosphorylated at Thr668 is less vulnerable to cytoplasmic cleavage by caspase-3 and caspase-8. This suggests that APP phosphorylation suppresses the generation of caspase-cleaved fragments of APP in the brain and that perturbation of this phosphorylation may be involved in APP-mediated neurotoxicity.

Keywords: β-Amyloid precursor protein; Alzheimer's disease; Caspase; Cyclin-dependent kinase 5; c-Jun N-terminal kinase

1. Introduction

β-Amyloid precursor protein (APP) is a ubiquitously expressed transmembrane protein and is the precursor of β-amyloid peptide (Aβ), which is a principal component of senile plaques. Aβ is hypothesized to induce neuronal toxicity and is implicated in Alzheimer's disease (AD) pathogenesis [1]. Independently of the cleavages that produce Aβ, APP is also cleaved by caspases between Asp664 and Ala665 (numbering for the APP695 isoform) in its cytoplasmic domain (APPcyt) to generate a short cytoplasmic fragment composed of 31 amino acid residues, APP665-695 (termed C31), and a large amino-terminal protein that lacks the 31 residues (termed APPΔC31) [2–5] (see Fig. 1). These caspase-cleaved fragments of APP have been observed in the brains of AD patients but not in controls, implicating this cleavage in AD pathogenesis [2,5,6]. C31 is cytotoxic and induces neuronal cell death, although the molecular mechanism has not been elucidated [5–8]. APPΔC31 has also been reported to induce neuronal cell death [9] and the controversial possibility that caspase-induced cleavage of APP increases Aβ generation has also been proposed [2,7,10,11].

The APPcyt is phosphorylated at Thr668 in vivo specifically in the brain [12]. Cyclin-dependent kinase 5 (Cdk5), a unique member of the Cdk family that is implicated in central nervous system development, participates in this phosphorylation [12]. In cultured cells, APP is also phosphorylated at Thr668 by c-jun N-terminal kinase (JNK), which is a major kinase in signal transduction for cell death and/or cell survival during the cellular stress response [13–16], and by cdc2 kinase during the G2/M phase of the cell cycle [17]. The phosphorylation at Thr668 induces a conformational change of the Thr668–Pro669 bond and alters the overall structure of the cytoplasmic domain to affect the interaction of APP with its binding proteins, such as FE65 [18,19]. Functionally, APP phosphorylation at Thr668 has been associated with neuronal outgrowth, Aβ generation, and APP-associated transcriptional regulation, however, the roles of the phosphorylation in these processes are not fully understood ([20–22], Nakaya, T., Kawaguchi, E., Shigenaga, K., Yoshida, T. and Suzuki, T, manuscript in preparation).

Although involvement of protein phosphorylation in proteolysis has not yet been widely studied, there are several proteins whose susceptibility to proteolysis is inhibited or attenuated by phosphorylation near the proteolytic cleavage site [23]. Because caspase cleavage of APP occurs near the phosphorylation site (see Fig. 1), in the present study we investigated the relationship between APP phosphorylation and caspase cleavage, and found that caspase cleavage of APP is attenuated by its phosphorylation at Thr668.

2. Materials and methods

2.1. Antibodies and peptides

The polyclonal anti-phosphoAPP antibody UT-33 [19,24], which selectively recognizes APP phosphorylated at the Thr668 residue, and an end-specific antibody against APPΔC31 SAC [9] have been described. The polyclonal anti-APP cytoplasmic domain A8717 (Sigma), the polyclonal anti-phospho-APP Thr668 (anti-PAPP; Cell Signaling Tech.) and the monoclonal anti-FLAG M2 (Sigma) antibodies were purchased. Peptides encompassing residues 649–695 of APP695 (with or without a phosphate at Thr668) were chemically synthesized and purified [19].

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Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer's disease; APP, β-amyloid precursor protein; APPcyt, the cytoplasmic domain of APP; Cdk5, cyclin-dependent kinase 5; GST, glutathione *S*-transferase; JNK, c-jun N-terminal kinase; PS, presenilin

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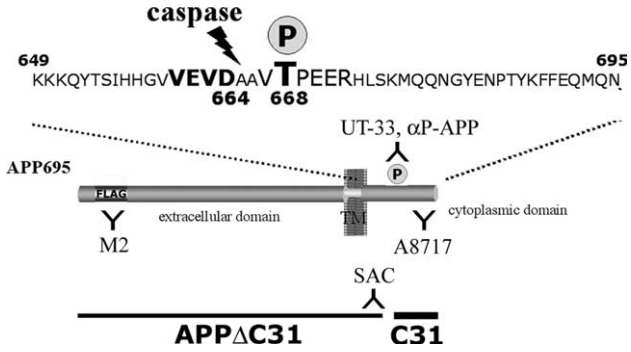

Fig. 1. Schematic representation of APP and the amino acid sequence of the APPcyt. Sites cleaved by caspase and phosphorylated by Cdk5 and JNK, and the extent of the caspase-cleaved fragments APPΔC31 and C31 are indicated. The regions recognized by the antibodies used in this study (anti-FLAG M2, anti-APP cytoplasmic domain A8717, anti-caspase-cleaved fragment of APP SAC, and anti-phosphoAPP UT-33 and PAPP antibodies) are also indicated. The amino acids are numbered according to the APP695 isoform. TM, transmembrane domain.

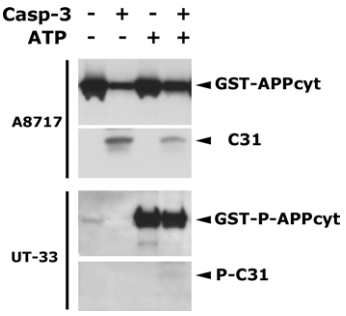

Fig. 2. The APPcyt is cleaved less by caspase-3 in vitro when phosphorylated by Cdk5. Purified GST–APPcyt was subjected to in vitro phosphorylation by an active complex of recombinant p25/Cdk5 in the presence (+) or absence (−) of ATP. The phosphorylated and non-phosphorylated forms were incubated with (+) or without (−) caspase-3 and analyzed by immunoblotting with A8717 to detect intact GST–APPcyt (first panel) and C31 (second panel) and with UT-33 to detect intact GST–APPcyt phosphorylated at Thr668 (GST–P–APPcyt; third panel) and phosphorylated C31 (P-C31; fourth panel).

2.2. Purification and phosphorylation of recombinant protein

Glutathione *S*-transferase (GST)–APPcyt was produced in *Escherichia coli* BL21 transformed with pGEX-4T-1-APPcyt and purified using glutathione–Sepharose 4B (Amersham Biosciences) as described [25,26]. Purified GST–APPcyt was incubated with recombinant Cdk5 in a complex with its activator p25 for 2 h at 30 °C in 30 mM MOPS [pH 7.2] 5 mM $MgCl_2$ containing or not containing 100 μM ATP.

2.3. In vitro cleavage of APP by caspases

Purified APP proteins or peptides were incubated with caspase-3 or caspase-8 (BIOMOL Research Laboratories) in caspase reaction buffer (50 mM HEPES [pH 7.4], 10% [v/v] glycerol, 100 mM NaCl, 0.1% [w/v] CHAPS, 1 mM EDTA, 10 mM DTT, and 1 μM Microcystin-LR) for 4 h at 30 °C. The samples were separated by SDS–PAGE and analyzed by immunoblotting using an ECL detection kit (Amersham Biosciences) or Coomassie brilliant blue R250 staining. Quantification was performed with VersaDoc imaging system using Quantity One software (BioRad) or with the Scion Images software program (Scion Corp.), which was calibrated by serial dilution.

2.4. Cell culture and protein purification

Human embryonic kidney 293 (HEK293) cells stably expressing human APP695 or APP695 T668A tagged with FLAG at the amino-terminal were generated and cultured as described [25]. Cells were treated with or without a combination of 0.5 M sorbitol and 2 μM staurosporine (Sigma) in the presence or absence of 50 μM Z-Val–Ala–Asp(OMe)–FMK (Z-VAD; BIOMOL) for 2 h in culture medium. The cells were collected and lysed in radioimmunoprecipitation (RIPA) buffer (50 mM Tris–HCl (pH 8.0), 0.1% [w/v] SDS, 0.5% [w/v] sodium deoxycholate, 1% [v/v] Nonidet P-40, and 0.15 M NaCl) containing 5 μg/ml chymostatin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, and 1 μM Microcystin-LR, and subjected to immunoblot analysis [26]. To purify FLAG-APP, HEK293 cells stably expressing FLAG-APP695 were treated with or without 0.5 M sorbitol for 1.5 h. Cell lysates were subjected to immunoprecipitation with anti-FLAG M2 affinity gel (Sigma). Proteins were eluted with 100 μg/ml FLAG peptide (Sigma) in 50 mM HEPES [pH 7.4], 100 mM NaCl and 1 μM Microcystin-LR and concentrated using a Microcon YM-30 column (Millipore).

3. Results

3.1. The APPcyt is less vulnerable to caspase-induced cleavage when phosphorylated at Thr668

The effect of APP phosphorylation at Thr668 on the caspase cleavage of APP was examined in vitro using the APPcyt and recombinant caspase-3, which has been reported to be the predominant caspase involved in APP cleavage (Fig. 1) [2]. We executed caspase-induced cleavage of APPcyt, which was phosphorylated by Cdk5 in vitro (Fig. 2). In the presence of ATP, a portion of APPcyt was phosphorylated at Thr668 as detected by an anti-phosphoAPP antibody (third panel). Incubation with recombinant caspase-3 decreased the amount of intact GST–APPcyt (first panel) and generated the carboxyl-terminal fragment C31 as detected by an antibody against the carboxyl-terminal region of APP (second panel). GST–APPcyt subjected to phosphorylation (ATP +) yielded C31 less efficiently than GST–APPcyt not subjected to phosphorylation (ATP −). C31 phosphorylated at Thr668 was also generated from phosphorylated GST–APPcyt, however, the amount was too small to be clearly detected (fourth panel). The rate of phosphorylated C31 (fourth panel) to phosphorylated GST–APPcyt (third panel) was much less than that of total C31 (second panel) to GST–APPcyt (first panel). This result suggests that phosphorylated APP is less vulnerable to the cleavage by caspase-3.

We next examined the stoichiometry of the cleavage of APP by caspase-3 using chemically synthesized peptides of the APP cytoplasmic domain with or without phosphate at Thr668 residue. Treatment with caspase-3 dose-dependently decreased the amount of intact APPcyt (Fig. 3A, arrowhead) and generated the cleaved fragment C31 (Fig. 3A, arrow). The amino-terminal half of the cleaved fragment (APP649–664, see Fig. 1) was not detected, probably because it was too small. Compared with the non-phosphorylated peptides, phosphorylated APPcyt was cleaved much less efficiently by caspase-3 (Fig. 3A and B).

Caspase-3 was initially identified as the protease responsible for the cleavage of APPcyt [2], but caspases-6, -8, and -9 have also been reported to cleave APP between Asp664 and Ala665 [3–5]. Caspases are classified into three groups on the basis of their substrate specificity; caspase-3 belongs to the group II caspases, and caspases-6, -8, and -9 belong to group III [27]. To elucidate whether phosphorylation at Thr668 also attenuates the cleavage by group III caspases, we examined the cleavage of phosphorylated and non-phosphorylated APP by caspase-8 (Fig. 3C). Phosphorylated APP was cleaved less efficiently by caspase-8 than non-phosphorylated APP, although

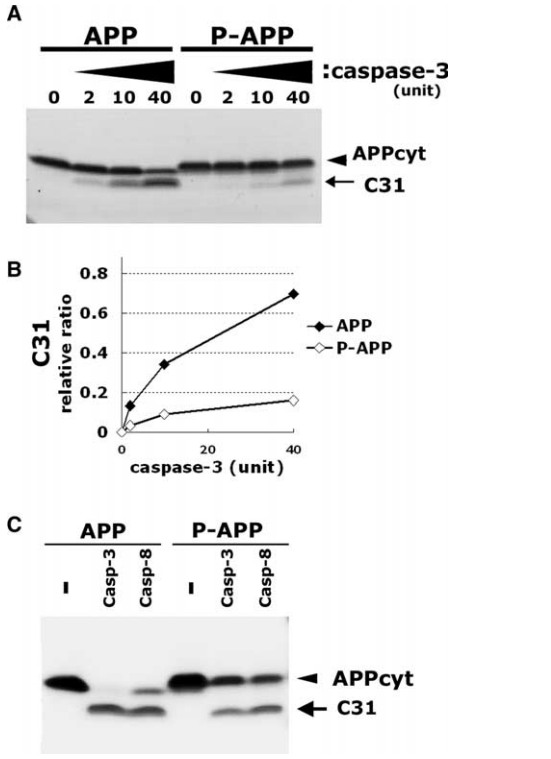

Fig. 3. Phosphorylation of APP at Thr668 prevents its cleavage by caspase-3 and caspase-8. (A) and (C) Cleavage of the APPcyt peptide with or without phosphate at Thr668 by caspase-3 and caspase-8. Synthetic APPcyt-(649–695) peptides (0.5 nmol) with (P-APP) or without (APP) a phosphate at Thr668 were incubated with caspase-3 (0, 2, 10, and 40 units in A, 50 units in C) or caspase-8 (50 units in C), and the resulting fragments were detected with SDS–PAGE followed by staining with Coomassie brilliant blue R250. (B) The amounts of C31 (arrow in A) were quantified and are displayed as a ratio relative to the level of APPcyt without caspase-3 (set to 1.0).

the difference of sensitivity to cleavage by caspase-8 between APPcyt and phospho-APPcyt was not as remarkable as that by caspase-3.

3.2. Phosphorylation of APP695 at Thr668 attenuates its caspase-induced cleavage

To further examine the role of APP phosphorylation in caspase cleavage, we performed a cellular analysis. First, we examined whether full-length APP phosphorylated at Thr668 in cells is less vulnerable to caspase-induced cleavage in vitro (Fig. 4). Comparable amounts of full-length APP were isolated from cells, which are stably expressing FLAG-tagged APP, with or without treatment with hyperosmotic stress which induces APP phosphorylation effectively [14,16] (Fig. 4, left and middle panels). Phosphorylation of APP at Thr668 was observed in APP isolated from treated cells (open arrowheads in middle panel), but not in APP from non-treated cells. Incubation of the isolated APP with caspase-3 decreased the amount of intact APP (arrowheads in upper right panel) and generated the cleaved fragment C31 (arrow in lower right panel). Because in vitro caspase-3 artificially cleaves APP in the extracellular domain in addition to the cleavage site in the cytoplasmic domain [2], we compared the generation of C31 rather than the decrease of intact APP to estimate the effect of APP phosphorylation. The amount of C31 generated from

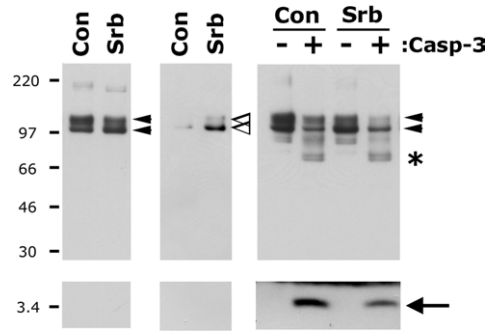

Fig. 4. APP695 phosphorylation at Thr668 induced by stress produces fewer caspase-cleaved fragments. APP was isolated from HEK293 cells stably expressing FLAG-APP695 following no treatment (indicated as *Con*) or treatment with 0.5 M sorbitol (indicated as *Srb*) for 1.5 h. Isolated APP was analyzed by immunoblotting with the anti-APP cytoplasmic domain antibody A8717 (arrowheads in left panel, total APP) and UT-33 (open arrowheads in middle panel, phosphorylated APP). Isolated APP incubated with (+) or without (−) caspase-3 were also analyzed by immunoblotting with A8717 (right panels). Intact APP (arrowheads) and C31 (arrow) were indicated. Asterisk indicates artificially digested product of APP in vitro. The numbers refer to the molecular mass (kDa) of the protein standards.

APP isolated from phosphorylation-induced cells was smaller than that from not induced cells. C31 phosphorylated at Thr668 could not be detected by immunoblotting with the anti-phosphoAPP antibody (data not shown). These suggest that phosphorylation of full-length APP at Thr668 in human cells attenuates the cleavage by caspases.

Finally, we examined whether Thr668 residue is actually involved in caspase-induced cleavage of APP in cells. HEK293 cells stably expressing wild-type APP (WT) or APP carrying Ala substitution for Thr668 (T668A) were treated with a combination of sorbitol and staurosporine, an apoptosis inducer (Fig. 5). APP WT was remarkably phosphorylated at Thr668 after the stimulation (middle panel). APPΔC31 was observed after the stimulation using an endo-specific antibody SAC, whereas it was not observed when cells were treated with

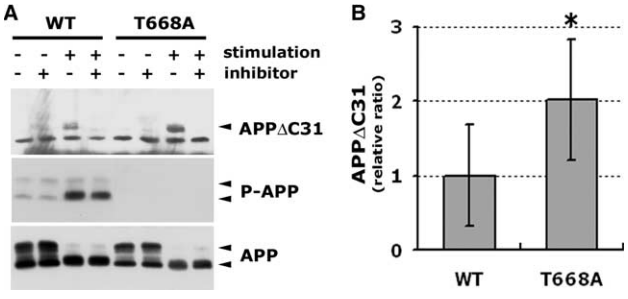

Fig. 5. Thr668 residue of APP is involved in the regulation of caspase-cleavage in cells. (A) HEK293 cells stably expressing FLAG-APP (WT) or FLAG-APP T668A (T668A) were treated with or without a combination of 0.5 M sorbitol and 2 μM staurosporine (stimulation) in the presence or absence of 50 μM Z-VAD (inhibitor) for 2 h. Cell lysates were analyzed by immunoblotting with SAC (upper panel), anti-PAPP (middle panel) and M2 (lower panel) antibodies. (B) The amounts of APPΔC31 detected by SAC and total APP detected by M2 were quantified, and the ratio of APPΔC31 to total APP was compared. Average of experiments with seven independent cell lines either expressing WT or T668A is displayed as a ratio relative to the mean of the cell lines expressing WT (set to 1.0). Bars indicate the means ± S.D. ($^*P < 0.05$).

general caspase inhibitor Z-VAD, demonstrating that caspase-dependent cleavage of APP was induced effectively by the stimulation in both cell lines (upper panel). However, compared with cell lines expressing APP WT, cell lines expressing APP T668A tended to produce significantly larger amount of APPΔC31. T668A was not phosphorylated after the stimulation as APP WT was (middle panel), whereas the sensitivity to caspase cleavage in vitro of T668A is same as that of non-phosphorylated APP WT as far as we examined (data not shown). Therefore, this result suggests that the phosphorylation of APP at Thr668 can attenuate the caspase-induced cleavage of APP in cell.

4. Discussion

In this study, we demonstrated that APP phosphorylation at the Thr668 residue attenuates caspase-induced cleavage of APP after the Asp664 residue. Cleavage at this site generates cytotoxic fragments and is implicated in AD pathogenesis [2–9]. To our knowledge, this is the first report concerning the regulation of caspase-induced cleavage of APP.

Caspases are widely known to cleave protein substrates during apoptotic cell death; however, the involvement of phosphorylation of substrates in regulating this caspase-induced cleavage has been demonstrated in only a few molecules, including presenilin (PS) 1, PS2, Bid, MST1, and IκB [29–33]. PS1 and PS2 are causative factors of familial AD and are responsible for the γ-cleavage of APP [1]. PS1 is cleaved between Asp345 and Ser346 by caspase-3, and its phosphorylation at Ser346 by PKC inhibits the cleavage [29]. PS2 is also cleaved by caspase-3, between Asp329 and Ser330 and between Asp326 and Ser327, and phosphorylation of the Ser residues inhibits the cleavage [30]. Phosphorylation of IκB and MST1 at Ser residues located adjacent to caspase cleavage sites also prevents cleavage [32,33]. Our results suggest that cleavage of APP is also likely to be regulated by phosphorylation, although APP is different from these previously reported proteins in that the phosphorylation occurs at a Thr residue located several residues away from the cleavage site. Thr668 of APP is located in the motif 667-VTPEER-672, which forms a type I β-turn and an amino-terminal helix-capping box structure [18]. Phosphorylation of Thr668 induces significant conformational changes in the cytoplasmic domain to affect the interaction of APP with its binding proteins [19]. Therefore, we suppose that the prevention of caspase cleavage in APP may be mediated by direct steric hindrance by the phosphate group itself or by the conformational change induced by the phosphorylation.

In our cellular model, phosphorylation of APP was implicated in suppressing caspase-induced APP cleavage. In brain, APP is suggested to be phosphorylated by JNK under certain stress conditions such as ischemia and injury as in cultured cells, although it has not been well studied yet. In addition, a portion of APP is constitutively phosphorylated by Cdk5 [12,22]. These constitutive and/or transient phosphorylation may lower the risk of cytotoxicity in neurons by reducing the generation of C31 or APPΔC31. Phosphorylation of PS1, PS2, and Bid is thought to reduce the progression of apoptosis by suppressing their caspase-induced cleavage [28–30], and the regulation of caspase-induced protein cleavage by phosphorylation might therefore be a widely used mechanism for regulating cell death.

Caspase-induced cleavage of APP is implicated in AD progression. Higher expression of activated caspases is observed in the brains of patients with AD than in age-matched controls [2,5]. Moreover, caspase-cleaved products of APP are detectable in AD brains but not in controls [2,5,6]. Lu et al. [8] proposed that Aβ-induced cell death is associated with the caspase cleavage of APP. These observations suggest that the cytotoxicity of caspase-cleaved products of APP could contribute to the neurodegeneration of AD that is triggered by the abnormal generation and accumulation of Aβ. Although the role of APP phosphorylation in AD progression has not been well characterized, a recent report suggested that Thr668-phosphorylated APP is present in high levels and accumulates in some cellular structures in the AD brain [22]. Therefore, APP phosphorylation may decrease the toxicity induced by caspase-dependent cleavage. Conversely, neurodegeneration in AD pathogenesis might be accelerated in neurons in which APP phosphorylation is perturbed. Further elucidation of mechanisms regulating the phosphorylation of APP and detailed profiling of the relationship between phosphorylated APP and caspase-cleaved APP in the AD brain may contribute to understanding the neuronal degeneration process and may help to identify novel therapeutic targets for the treatment of AD.

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