FKBP12 regulates the localization and processing of amyloid precursor protein in human cell lines

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One of the pathological hallmarks of Alzheimer's disease is the presence of insoluble extracellular amyloid plaques. These plaques are mainly constituted of amyloid beta peptide (Aβ), a proteolytic product of amyloid precursor protein (APP). APP processing also generates the APP intracellular domain (AICD). We have previously demonstrated that AICD interacts with FKBP12, a peptidyl-prolyl *cis-trans* isomerase (PPIase) ubiquitous in nerve systems. This interaction was interfered by FK506, a clinically used immunosuppressant that has recently been reported to be neuroprotective. To elucidate the roles of FKBP12 in the pathogenesis of Alzheimer's disease, the effect of FKBP12 overexpression on APP processing was evaluated. Our results revealed that APP processing was shifted towards the amyloidogenic pathway, accompanied by a change in the subcellular localization of APP, upon FKBP12 overexpression. This FKBP12-overexpression-induced effect was reverted by FK506. These findings support our hypothesis that FKBP12 may participate in the regulation of APP processing. FKBP12 overexpression may lead to the stabilization of a certain isomer (presumably the *cis* form) of the Thr668-Pro669 peptide bond in AICD, therefore change its affinity to flotillin-1 or other raft-associated proteins, and eventually change the localization pattern and cause a shift in the proteolytic processing of APP.

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1. Introduction

The proteolytic cleavage of amyloid precursor protein (APP) plays a pivotal role in the dysfunction and death of neurons in Alzheimer's disease (AD). Cleavages of APP by β- and α-secretases liberate the soluble APPs and retain the C-terminal fragments (CTFs) (C99 and C83, respectively) in the membrane. These CTFs are further processed by γ-secretase to generate the amyloidogenic Aβ peptide or the non-amyloidogenic P3 peptide, along with the APP intracellular domain (AICD), the extreme C-terminus of APP. The precise cellular function of AICD still remains obscure. Recent studies have shown that APP is physically associated with a lipid raft marker protein, flotillin-1 (Chen *et al.* 2006), and two other raft-associated proteins, caveolin-1 and caveolin-3 (Ikezu *et al.* 1998; Nishiyama *et al.* 1999), through AICD, suggesting that AICD may be involved in the regulation of the localization and processing of APP via its interaction with raft proteins, alongside its potential role in transcriptional regulation as many believe. These results, along with the observation that both BACE (i.e., the β-secretase) and γ-secretase complex are concentrated in lipid rafts, in which approximately a quarter of brain Aβ and a small fraction of cellular APP are also present (Riddell *et al.* 2001; Ehehalt *et al.* 2003; Vetrivel *et al.* 2004; Abad-Rodriguez *et al.* 2004), support the notion that lipid rafts may play certain roles in the proteolysis of APP (Ehehalt *et al.* 2003; Girardot *et al.* 2003).

AICD is phosphorylated, most likely neuron specifically (Iijima *et al.* 2000), at Thr654, Ser655, and Thr668 (based on the numbering system for APP695) (Oishi *et al.* 1997; Lee *et al.* 2003). Among them, Thr668 phosphorylation has received special attention. It is suggested that Thr668 phosphorylation may facilitate BACE cleavage of APP (Lee *et al.* 2003). In support of this hypothesis, Thr668 phosphorylated APP has been shown to preferentially colocalize with BACE

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in enlarged endosomes in AD brains and primary neurons, whereas Thr668 unphosphorylated APP was distributed to different fractions (Lee *et al.* 2003). This observation also echoes the aforementioned notion of the involvement of lipid raft in APP processing. However, contradictory results have also been reported (Sano *et al.* 2006; Barbagallo *et al.* 2010, 2011).

Since the *cis/trans* ratio of the Thr668-Pro669 peptide bond in AICD increases upon Thr668 phosphorylation (Ramelot and Nicholson 2001), it is hypothesized that Thr668 phosphorylation may be participating in the pathogenesis of AD via some isomer-specific mechanism(s). For example, it has been suggested that Pin1, a peptidyl-prolyl isomerase (PPIase) preferentially bound to Thr668-phosphorylated APP or C99 (Akiyama *et al.* 2005; Pastorino *et al.* 2006), is involved in the regulation of APP processing by promoting the *cis* to *trans* isomerization, which presumably favors the non-amyloidogenic APP processing (Pastorino *et al.* 2006).

We have previously demonstrated that AICD interacts with FKBP12, a member of the FK506-binding protein (FKBP) family (Liu *et al.* 2006). FKBP12 has been linked to numerous intracellular signalling pathways through interactions with various cellular partners (Brillantes *et al.* 1994; Wang *et al.* 1994; Cameron *et al.* 1995, 1997). Abnormal expression of FKBP12 has been reported in the brains of patients with neurodegenerative diseases (Avramut and Achim 2002; Sugata *et al.* 2009), suggesting its involvement in the pathogenesis of those diseases. This idea is supported by recent findings that loss of FKBP12 alters mammalian target of rapamycin (mTOR) signalling and enhances synaptic plasticity (Hoeffer *et al.* 2008), preventing Aβ-induced synaptic impairment (Ma *et al.* 2010). While the role of mTOR in the pathogenesis of AD is still a matter of debate (Swiech *et al.* 2008, and references therein; Caccamo *et al.* 2010, 2011; Ma *et al.* 2010), it is possible that FKBP12 simply modulates the amyloidogenesis of APP in a Pin1-like manner since it also has a PPIase activity.

In this study, the effect of FKBP12 overexpression on APP processing was investigated. Our results revealed that APP processing was shifted toward the amyloidogenic pathway, accompanied by a change in the subcellular localization of APP, upon FKBP12 overexpression. Additionally, as FK506 has been shown to block the FKBP12-AICD interaction (Liu *et al.* 2006), whether FK506 influences FKBP12-induced changes in APP processing was also examined.

2. Materials and methods

2.1 *Construction of various APP expression plasmids*

DNA fragment containing the coding sequence of human amyloid precursor protein (APP) was amplified from mRNAs isolated from human prostate adenocarcinoma cells (PC-3) via reverse transcription-polymerase chain reaction (RT-PCR) using primers designed based on a GenBank sequence (GenBank accession number NM_201414) (forward primer: 5′-GGAATTCCATATGCTGCCCGGTTTGG-3′, reverse primer: 5′-CGGGATCCTAGTTCTGCATCTGC-3′) and inserted into the yT&A vector (Yeastern, Taipei, Taiwan). This coding sequence was later subcloned into the pcDNA3.1(+) vector (Invitrogen). pcDNA3.1(+)APPT668V and pcDNA3.1(+)APPT668E were generated by *Dpn* I-mediated site-directed mutagenesis method using pcDNA3.1(+)APP as the template. Sequences of all candidate plasmids were verified by DNA sequencing (Core Facility, College of Medicine, National Taiwan University).

2.2 *Cell culture, transfection, RT-PCR expression analysis and Western blotting*

HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL/Invitrogen) supplemented with 10% fetal bovine serum (FBS), 44 mM $NaHCO_3$, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C, 95% air and 5% CO_2, and replaced with fresh media every two to three days until cells became 70–80% confluent. These cells were then transfected with pcDNA3.1(+)APP and pcDNA3.1(+)FKBP12 (or mock) using calcium phosphate method. Two hundred nM γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine-*t*-butyl ester (DAPT, Merck)(or vehicle) was added 4 h before harvest when necessary. For FK506 treatment experiment, varying concentrations of FK506 (courtesy of Astellas Pharma Inc., Tokyo, Japan) or vehicle were added 16 h post transfection.

Total RNA was isolated 40–48 h post transfection using Trizol™ reagent (Invitrogen) according to manufacturer's instruction, followed by RT-PCR and agarose gel electrophoresis for quantitative analysis. Primer sets for the PCR assay are 5′-AAGCTTCCATGGGAGTGCAGGTGGA-3′ (FKBP12for), 5′-GGTATCCTCATTCCAGTTTAGAAGCTCC-3′ (FKBP12rev), 5′-GGTAGAGGAAGAGGCTGAGGAAC-3′ (APPfor), 5′-CATGACCTGGGACATTCTCTCTC-3′ (APPrev), 5′-AGCAGGCACCACCCCTTGGAACA-3′ (BACEfor), 5′-TCACTTCAGCAGGGAGATGTC ATCAGCA-3′ (BACErev), 5′-GGTGAATATGGCAGA AGGAG-3′ (PS1for), 5′-CTTCCCATTCCTCACTGAAC-3′ (PS1rev), 5′-GTTCGTGGTGCTTCCAGAGG-3′ (PS2for), 5′-ATCACATGCTTCGCTCCGTAT-3′ (PS2rev), 5′-GAAGGTGAAGGTCGGAGTC-3′ (GAPDHfor), and 5′-GAAGATGGTGATGGGATTTC-3′ (GAPDHrev).

For Western blot analysis, cells collected 40–48 h post transfection were washed with cold phosphate-buffered saline (PBS, 2.7 mM KCl, 137 mM NaCl, 10 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.4), trypsinized with 0.05% trypsin/0.53 mM EDTA (Gibco BRL/Invitrogen), and lysed

by brief sonication in 100 μL of lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM DTT, 1 mM PMSF, and 1x protease inhibitor mixture (0.1 μg/mL chymostatin, 0.5 μg/mL leupeptin, 1 μg/mL pepstatin A, 2.5 μg/mL antipain, and 0.2 mM benzamidine). Cell lysate was obtained by centrifugation at 15,000*g* for 30 min at 4°C and separated by 10 or 14% SDS-PAGE. For the detection of soluble APP N-terminal fragments (sAPPs), conditioned medium was collected and separated by 8% SDS-PAGE. Proteins on the polyacrylamide gel were transferred to a PVDF membrane, and probed with the following antibodies: rabbit anti-APP C-terminal polyclonal (for C99, C83, and total APP, 171610, Calbiochem), mouse anti-APP N-terminal monoclonal (for sAPPs and total APP, 22C11, Chemicon/Millipore), goat anti-FKBP12 polyclonal (sc-6174, Santa Cruz Biotechnology), mouse anti-Aβ(1–17) monoclonal (for C99 and sAPPα, 6E10, Calbiochem), rabbit anti-APP-pT668 polyclonal (UBI-07–700, Upstate/Millipore), or mouse anti-GAPDH monoclonal antibody (MAB374, Chemicon/Millipore) and visualized with ECL Western Blotting Detection Reagent (GE Healthcare) by enhanced chemiluminescence method.

2.3 *Co-immunoprecipitation (Co-IP) and Western blotting*

HEK293T cells grown under conditions described previously were transfected with pcDNA3.1(+)APP (wild-type or Thr668 mutant) and pEGFP-C1-FKBP12 (or mock) using calcium phosphate method. Cells were washed with PBS, trypsinized, and lysed by brief sonication in 100 μL of co-IP lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% CHAPS, 1 mM DTT, 1 mM PMSF, and 1× protease inhibitor mixture). Cell lysate was obtained by centrifugation at 15,000*g* for 30 min at 4°C.

Lysate containing 300 μg of protein was adjusted to 100 mM NaCl and mixed with approximately 15 μL of mouse anti-GFP antibody (sc-9996, Santa Cruz Biotechnology). The mixture was adjusted to a final volume of 500 μL with co-IP buffer (0.1% CHAPS, 100 mM NaCl, 15 mM EGTA, and 50 mM Tris-HCl, pH 7.5), incubated on ice for 90 min with occasional tube inversion, and microcentrifuged at top speed at 4°C for 10 min. The supernatant was transferred to a fresh tube and incubated with 50 μL of 50% protein A Sepharose CL-4B beads slurry (GE Healthcare) for 1 h at 4°C with rotation. The beads were washed with co-IP buffer. Proteins bound to the beads were eluted by boiling in 2x sample buffer (0.1 M Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 2% β-mercaptoethanol, 0.01% coomassie R250) and separated by 10% SDS-PAGE. For Western blot analysis, proteins transferred to a PVDF membrane were probed with anti-APP C-terminal (171610) or anti-GFP monoclonal antibody (sc-9996) and visualized with VisGlow Chemiluminescent Substrate (Visual Protein, Taipei, Taiwan) by enhanced chemiluminescence method.

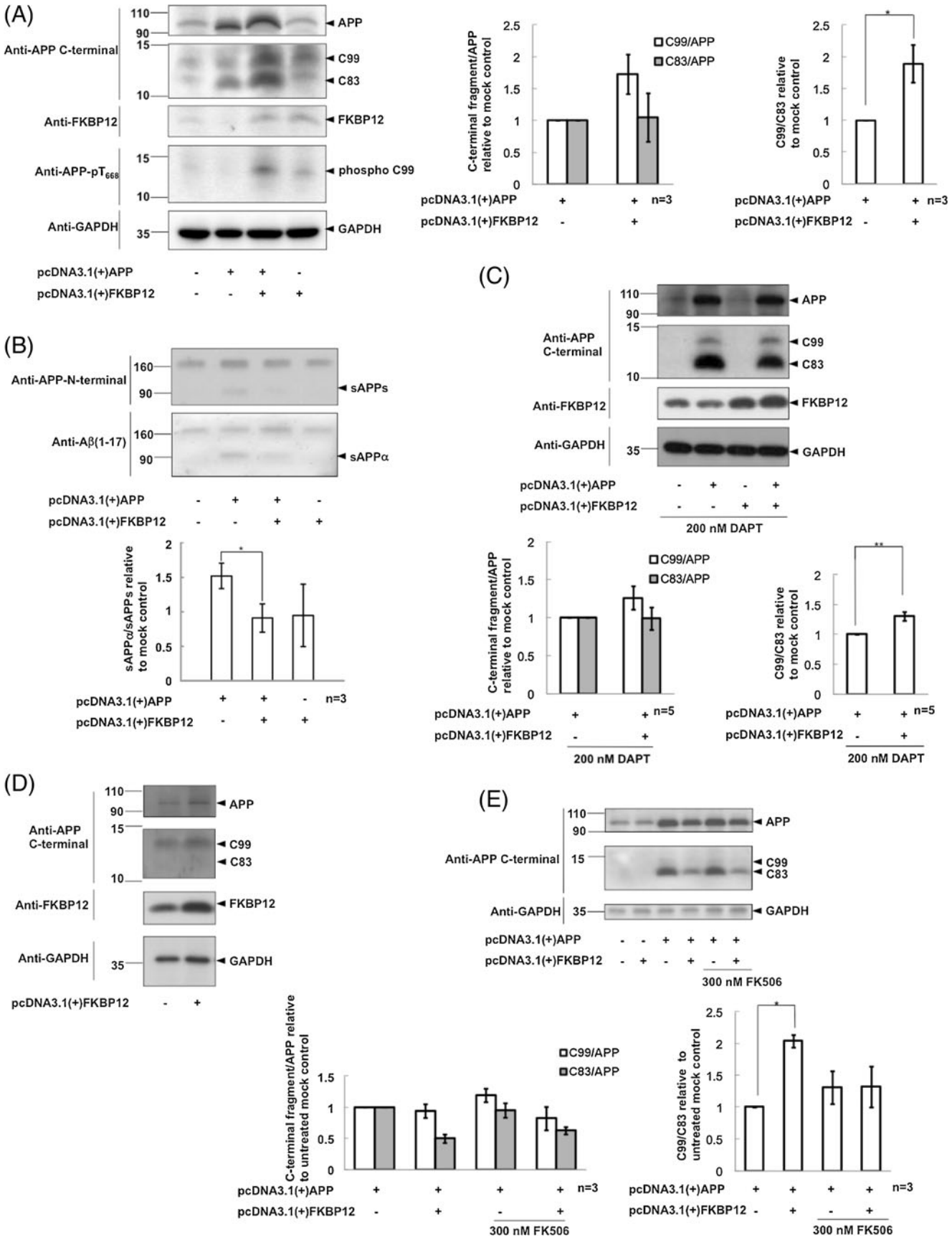
2.4 *Membrane fractionation*

SH-SY5YAPP cells (i.e. APP695-expressing SH-SY5Y cells) were grown in a 1:1 mixture of Ham's F12 nutrients and minimal essential medium (MEM, Gibco BRL/Invitrogen) supplemented with 10% FBS, 1 mM sodium pyruvate, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C, 95% air and 5% CO_2 to 80–90% confluence, and then transfected with pcDNA3.1(+)FKBP12 or mock using calcium phosphate method. Cells were harvested 48 h post transfection as previously described. Briefly, cells were washed with cold PBS, trypsinized, centrifuged, and lysed in 500 μL of protease inhibitor mixture-containing TNET buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100) for 30 min at 4°C. Four hundred and fifty μL of the lysate was then adjusted to 40.7% sucrose by addition of 625 μL of 70% sucrose, placed at the bottom of an ultracentrifuge tube, overlaid with 6 mL of 35% sucrose and 5 mL of 5% sucrose, and centrifuged for 19 h at 39,000 rpm at 4°C in a SW 41 Ti rotor (Beckman Coulter). Twelve fractions were collected starting from the top of the ultracentrifuge tube at the end of centrifugation. Fractionation samples were separated by 8% SDS-PAGE and analysed by Western blot analysis using mouse anti-flotillin-1 monoclonal (Clone18, BD Biosciences Pharmingen), mouse anti-APP N-terminal monoclonal (22C11), mouse anti-CD71 (3B8 2A1) monoclonal (sc-32272, Santa Cruz Biotechnology), or rat anti-GRP78 monoclonal antibody (Santa Cruz Biotechnology).

3. Results

3.1 *FKBP12 overexpression increases the C99/C83 ratio*

To explore the potential roles FKBP12 plays in the pathogenesis of AD, the effect of FKBP12 overexpression on APP processing in cultured cells was studied by transfecting HEK293T cells with both APP695 and FKBP12 cDNA-containing plasmids. Our results revealed that FKBP12 overexpression resulted in an increased C99 to C83 ratio and a lower sAPPα/sAPPs ratio, which may lead to an increase in the amyloidogenic product, Aβ (figure 1A,B). The proportionally higher levels of C99 and C83 in the APP/FKBP12-overexpressing sample appeared in figure 1A are probably due to variations in transfection efficiencies and can be corrected by normalizing to the amount of full-length APP. As further CTF processing may affect CTF levels and C99/C83 ratio as well, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine-*t*-butyl ester (DAPT), a γ-secretase inhibitor, was added to block γ-secretase-mediated CTF processing (figure 1C). A similar pattern of change was observed in DAPT-treated samples, suggesting that FKBP12 affects APP


(A)
Anti-APP C-terminal
APP
C99
C83
Anti-FKBP12
FKBP12
Anti-APP-pT668
phospho C99
Anti-GAPDH
GAPDH
pcDNA3.1(+)APP
pcDNA3.1(+)FKBP12
C-terminal fragment/APP relative to mock control
C99/APP
C83/APP
n=3
C99/C83 relative to mock control
(B)
Anti-APP-N-terminal
sAPPs
Anti-Aβ(1-17)
sAPPα
sAPPα/sAPPs relative to mock control
(C)
Anti-APP C-terminal
200 nM DAPT
(D)
(E)
300 nM FK506
C-terminal fragment/APP relative to untreated mock control
C99/C83 relative to untreated mock control

◀ **Figure 1.** Overexpression of FKBP12 alters the C99/C83 ratio. (**A**) Lysates from HEK293T cells transfected with indicated expression vectors were separated by 14% SDS-PAGE and analysed by Western blot. GAPDH was used as a loading control. The C99/APP, C83/APP, and C99/C83 ratios were quantified (right panel). Values of cells transfected with pcDNA3.1(+)APP only were arbitrarily set as 1. Results are given as the means from 3 independent experiments. Error bars represent standard errors of the means. Asterisk indicates statistical significance (Student's *t*-test, $P < 0.05$). (**B**) Soluble APPs and soluble APPα were detected in the conditioned medium from A. The sAPPα/sAPPs ratio was quantified (lower panel). Values of mock-transfected cells were arbitrarily set as 1. Results are given as the means from 3 independent experiments. Error bars represent standard errors of the means. Asterisk indicates statistical significance (Student's *t*-test, $P < 0.05$). (**C**) Western blot analysis of lysates from transiently transfected HEK293T cells treated with DAPT 4 h before harvest. The C99/APP, C83/APP, and C99/C83 ratios were quantified (lower panel). Values of cells transfected with pcDNA3.1(+)APP only were arbitrarily set as 1. Results are given as the means from 5 independent experiments. Error bars represent standard errors of the means. Double asterisk indicates statistical significance (Student's *t*-test, $P < 0.01$). (**D**) Western blot analysis of lysates from SH-SY5YAPP cells transiently transfected with pcDNA3.1(+)FKBP12. (**E**) The effect of FKBP12 overexpression on C99/C83 ratio was reverted in the presence of 300 nM FK506. HEK293T cells transfected with combinations of expression vectors as indicated were treated with 300 nM FK506 16 h post-transfection and harvested 48 h post-transfection. Total lysates were separated by 14% SDS-PAGE and analysed by Western blot. GAPDH was used as a loading control. The C99/APP, C83/APP, and C99/C83 ratios were quantified (lower panel). Values of vehicle-treated cells transfected with pcDNA3.1(+)APP only were arbitrarily set as 1. Results are given as the means from 3 independent experiments. Error bars represent standard errors of the means. Asterisk indicates statistical significance (Student's *t*-test, $P < 0.05$).

processing by shifting it toward a more pathogenic pathway, rather than by selectively modulating γ-secretase activity. The presence of DAPT prevents both C83 and C99 from further proteolytic processing, justifying the utilization of the C99/C83 ratio as an indicator for the amyloidogenic pathway and eliminating the need for cumbersome and costly quantification of both the secreted and intracellular Aβ. This FKBP12-overexpression-induced change in C99/C83 ratio was also observed in APP overexpressing SH-SY5Y cells (figure 1D), making it more likely a physiologically and pathologically relevant phenomenon.

3.2 *FK506 treatment reverses the C99/C83 ratio change*

We have previously demonstrated that FK506, a known PPIase inhibitor of FKBPs (Harding *et al.* 1989; Siekierka *et al.* 1989), is capable of interfering with FKBP12-AICD interaction in a dose-dependent manner (Liu *et al.* 2006). To assess the effect of FK506 on FKBP12-overexpression-induced change in C99/C83 ratio, the production of APP CTFs in HEK293T cells cotransfected with pcDNA3.1(+)APP and pcDNA3.1(+)FKBP12 (or mock) and cultured in the absence or presence of 300 nM FK506, a concentration likely to be sufficient to inhibit FKBP12 PPIase activity as suggested by others (Edlich *et al,* 2005; Deleersnijder *et al.* 2011) and interfere with the interaction between AICD and FKBP12 (Liu *et al.* 2006), was examined. FK506 was not cytotoxic at concentrations up to 500 nM (data not shown). It is clearly shown in figure 1E that the increase in the level of C99 upon FKBP12 overexpression was reverted by FK506, underscoring the importance of APP-FKBP12 interaction in the regulation of APP processing. Reverse transcription PCR analysis indicated that FK506 treatment did not significantly alter the mRNA levels of APP and FKBP12 compared to vehicle-treated controls (figure 2B). Whether this reversion effect of FK506 is produced by hindering the interaction between FKBP12 and AICD or by inhibiting the PPIase activity of FKBP12 directly, however, remains an open question.

3.3 *mRNA levels of BACE and presenilins are not changed upon FKBP12 overexpression*

To examine the possibility that the observed changes in C99/C83 ratio caused by FKBP12 overexpression might be at least partly due to altered expression levels of BACE and/or presenilins (PSs), RNAs isolated from pcDNA3.1(+)APP/pcDNA3.1(+)FKBP12 cotransfected HEK293T cells were quantitatively analysed using reverse transcription-PCR. The primer set for APP is specifically designed to flank the alternatively spliced exons 7 and 8 so that all three major isoforms of APP (APP770, APP751 and APP695) can be detected simultaneously. As shown in figure 2A, mRNA levels of BACE, PS1, and PS2 all remain relatively constant regardless experimental conditions. Whether the activities of these secretases are affected by overexpression of FKBP12 is yet to be answered. This result, however, reveals that the observed changes in C99/C83 ratio is less likely to be mediated by a transcriptional regulation mechanism.

3.4 *FKBP12 is preferentially associated with Thr668 phosphorylated APP*

It should be noted that C99 is the major Thr668 phosphorylated CTF, as reported by others (Lee *et al.* 2003), and that a higher level of Thr668 phosphorylated CTFs was observed upon FKBP12 overexpression (figure 1A). The Thr668

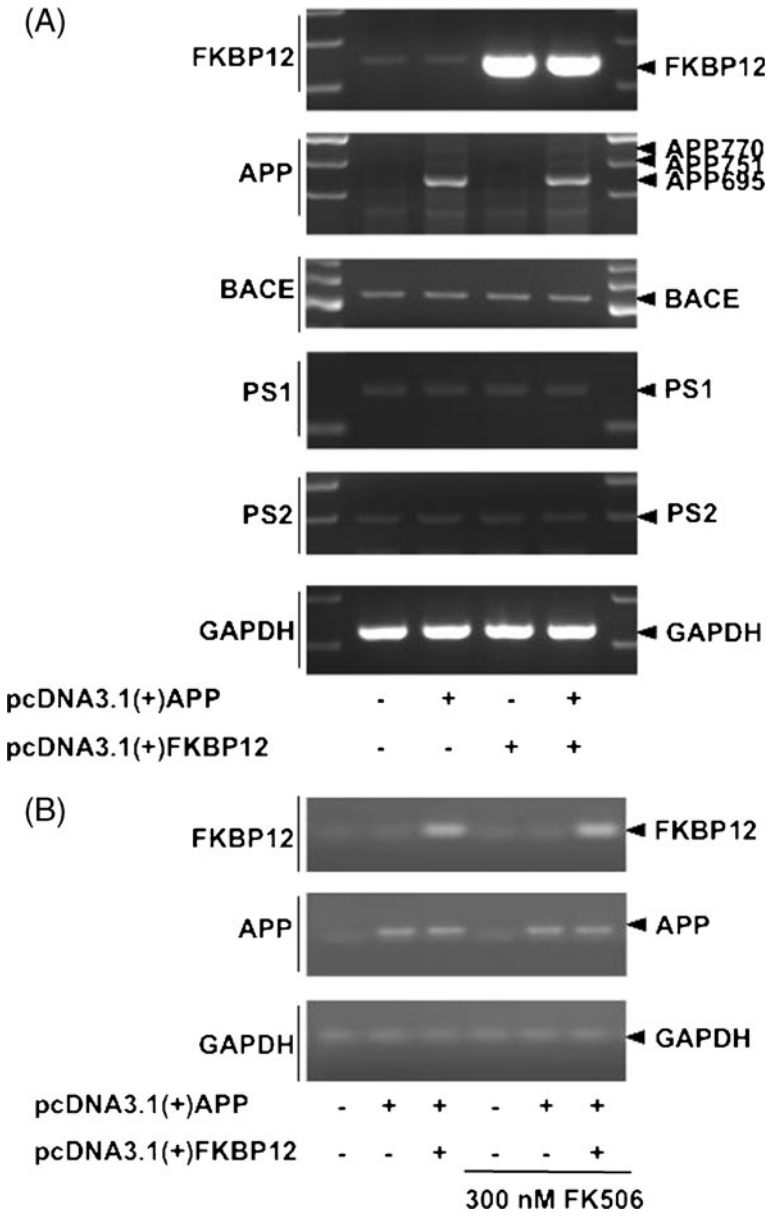


Figure 2. Overexpression of FKBP12 has no effect on the mRNA levels of BACE and presenilins. (**A**) Agarose gel electrophoresis of reverse transcription-PCR products of BACE (511 bp), PS1 (140 bp), and PS2 (296 bp) mRNAs isolated from HEK293T cells transfected with indicated expression vectors 40–48 h post transfection. GAPDH (PCR product: 226 bp) was used as a positive control. No changes in the mRNA expression levels of BACE and presenilins were observed. (**B**) HEK293T cells transfected with combinations of expression vectors as indicated were treated with 300 nM FK506 16 h post-transfection and harvested 48 h post-transfection. Reverse transcription-PCR products of APP and FKBP12 mRNAs isolated were analysed by agarose gel electrophoresis. GAPDH was used as a positive control. No changes in the mRNA expression levels of APP and FKBP12 were observed upon FK506 treatment.

phosphorylation state of APP has been shown to affect the interaction of AICD (or APP) with its binding partners such as Fe65, G_o, Pin1, etc. (Nishimoto *et al.* 1993, 1997; Giambarella *et al.* 1997; Pastorino *et al.* 2006). To see whether the interaction between AICD and FKBP12 is affected by Thr668 phosphorylation, cell lysates from HEK293T cells cotransfected with expression vectors of wild-type or mutant (Thr668Glu (T668E) or Thr668Val (T668V)) APP together with EGFP-FKBP12 fusion protein were immunoprecipitated with anti-GFP antibody and analysed by immunoblotting (figure 3). Interestingly, our results indicated that APPT668E, which mimics the

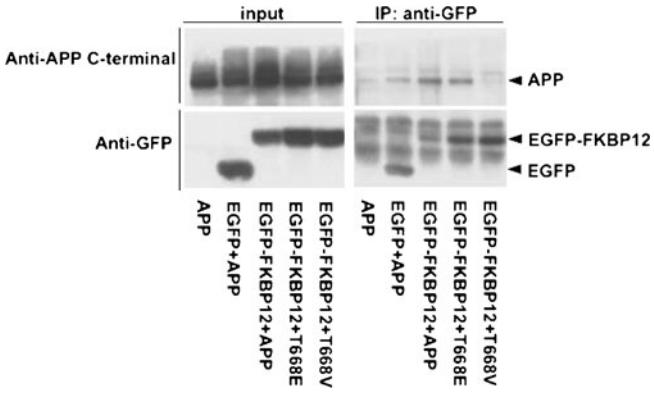


Figure 3. FKBP12 is preferentially associated with Thr668 phosphorylated APP. Lysates from HEK293T cells transfected with pcDNA3.1(+)APP (wild-type or Thr668 mutant) and pEGFP-C1-FKBP12 (or mock) were immunoprecipitated with an antibody against GFP (IP). Protein-antibody complexes bound to protein A Sepharose CL-4B beads (GE Healthcare) were separated by 10% SDS-PAGE and analysed by Western blot. Aliquots of cell lysate (about 10% of input) were also loaded directly onto the gels and analysed by Western blot (input).

constitutively Thr668 phosphorylated APP, had a stronger interaction with FKBP12 compared with that of APPT668V, suggesting that Thr668 phosphorylation state of APP does indeed influence its interaction with FKBP12.

3.5 *FKBP12 overexpression alters subcellular distribution of APP*

As Thr668 phosphorylation has been shown to affect the subcellular localization of APP, which is believed to be involved in the regulation of APP processing (Lee *et al.* 2003), we were prompted to examine whether the observed shift in APP processing was correlated with a differential distribution of APP to detergent-resistant membranes (DRMs), presumably due to elevated levels of Thr668 phosphorylation. Lysates from mock- or pcDNA3.1(+)FKBP12-transfected SH-SY5YAPP cells (i.e. APP695-expressing SH-SY5Y cells) were fractionated by sucrose gradient ultracentrifugation as described. Not surprisingly, the distribution of full-length APP shifted to the lighter fractions when FKBP12 was overexpressed, resulting in an increase in the amounts of APP colocalized with the lipid raft marker flotillin-1 (figure 4). No change in the subcellular distribution of APP was observed in DAPT-treated cells (figure 4), indicating that modulation of γ-secretase activity alone would not affect the subcellular distribution of APP. Neither DAPT treatment nor FKBP12 overexpression affects the distribution of organelle markers such as flotillin-1 (raft marker), CD71 (transferrin receptor, a marker for endosome and non-raft plasma membrane), and GRP78 (endoplasmic reticulum marker) (figure 4). We also found that the alteration of APP processing caused by FKBP12 overexpression was abrogated when rafts were disrupted by cholesterol-complexing agent methyl-β-cyclodextrin (MβCD) (Liu TY, Lo YY and Kung FL, unpublished work). These findings further highlight the importance of Thr668 phosphorylation on APP distribution and APP processing.

4. Discussion

Here, we demonstrate that FKBP12 overexpression results in a higher C99/C83 ratio, and therefore shifts APP processing toward a more amyloidogenic pathway (figure 1). AICD has been previously shown to interact with FKBP12 (Liu *et al.* 2006), probably via the Thr668-Pro669 dipeptide since it is structurally mimicking the functional moieties of FK506 important for FKBP12 binding. Our modeling results also support such a possibility (Liu FL and Kung FL, unpublished work). This dipeptide region sits within the G_o binding site and is close to the PTB domain-interacting site of AICD (His657-Lys676 and Tyr682-Tyr687, respectively) (Nishimoto *et al.* 1993), and harbors an important neuron-specific phorphorylation site, Thr668 (Oishi *et al.* 1997; Iijima *et al.* 2000; Lee *et al.* 2003). It has been demonstrated that Thr668 phosphorylation is able to induce a conformational change of AICD, which may then affect the interaction of AICD (or APP) with its binding partners, as indicated in the cases of Fe65, G_o, and Pin1, etc. (Nishimoto *et al.* 1993, 1997; Giambarella *et al.* 1997; Pastorino *et al.* 2006). This interaction-modulating effect may cause a change in Aβ generation as well (Ando *et al.* 2001; Kimberly *et al.* 2005; Chang *et al.* 2006; Nakaya and Suzuki 2006; Pastorino *et al.* 2006). Our co-IP results reveal that Thr668 phosphorylated APP has a stronger interaction with FKBP12 compared with

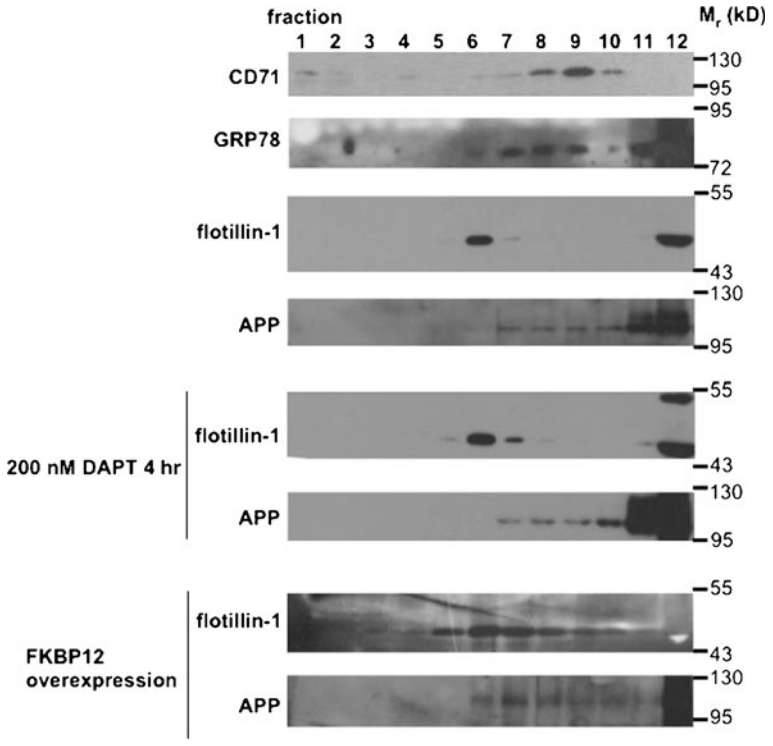


Figure 4. FKBP12 overexpression alters subcellular distribution of APP in SH-SY5YAPP cells. Lysates from mock- or pcDNA3.1(+)FKBP12-transfected SH-SY5YAPP cells were subjected to sucrose gradient ultracentrifugation. Fractions were collected from top (Fraction 1) to bottom (Fraction 12). Proteins from each fraction were separated by 8% SDS-PAGE and analysed by Western blot. CD71 (transferrin receptor) and GRP78 were used as markers for endosome/non-raft plasma membrane and ER, respectively.

unphosphorylated ones (figure 3). This, in conjunction with the observation that overexpression of FKBP12 significantly increases C99 production and the extent of Thr668 phosphorylation (figure 1A), suggests that while Thr668 phosphorylation makes it easier for the Thr668-Pro669 peptide bond to adopt a *cis* conformation, the presence of FKBP12 may further facilitate this conformational change and help stabilize Thr668 phosphorylated form of APP. In supported of this, several PPIases have been shown to preferentially catalyse phosphorylated substrates (Yaffe *et al.* 1997; Weiwad *et al.* 2000).

The phosphorylation state of APP at Thr668 has been correlated with its subcellular localization of APP, which, in turn, might modulate APP processing (Lee *et al.* 2003). As shown in figure 4, the distribution of full-length APP shifted to the lighter fractions when FKBP12 was overexpressed, resulting in an increase in the amounts of APP colocalized with the lipid raft marker flotillin-1. These findings further support our hypothesis that FKBP12 may participate in the regulation of APP processing in a Pin1-like manner. FKBP12 overexpression may, as stated earlier, lead to the stabilization of a certain isomer (presumably the *cis* form) of the Thr668-Pro669 peptide bond in AICD, and therefore change its affinity to flotillin-1 or other raft-associated proteins (e.g., caveolins in HEK293T cells), and eventually change the localization pattern of APP, distributing it to the sites where the amyloidogenic processing of APP seems to occur. These studies will be conducted in primary cortical of hippocampal cells to further validate our hypothesis.

This FKBP12-overexpression-induced effect was reverted by FK506, a clinically used immunosuppressant known to inhibit PPIase activity of FKBPs (figure 1E). FK506 and other FKBP-binding macrocyclic molecules all consist of two domains: a binding domain and an exposed domain. The former, including the pipecolinyl and pyranose rings and the dicarbonyl region between them in the case of FK506, is responsible for direct contact with FKBP12 and may interfere with FKBP12 isomerase activity, whereas the latter is mainly for other intermolecular interactions and is likely to be responsible for the different pharmacological effects elicited by different ligands (Bierer *et al.* 1990; Becker *et al.* 1993). For

example, it has been proposed that the binding of FKBP12-FK506 complex to calcineurin may generate subtle alternation in the geometry of calcineurin active site and therefore a non-competitive inhibitory effect. Such a calcineurin inhibition activity contributes to the immunosuppressive effect of FK506 (Jain *et al.* 1993; McCaffrey *et al.* 1993; O'Keefe *et al.* 1992).

The recently discovered neurotrophic/neuroprotective activity of FK506, on the other hand, has been shown to be correlated with the inhibitory effect of FK506 to FKBP12 PPIase (Gold 1997; Steiner *et al.* 1997; Snyder *et al.* 1998; Brecht *et al.* 2003) and is likely to be separated from the calcineurin-mediated immunosuppressive effect (Liu *et al.* 1991; Griffith *et al.* 1995). Actually, a decrease in calcineurin activity has been shown to be correlated with hyperphosphorylation of tau protein, the major constituent of the neurofibrillary tangles observed in the brains of AD patients (Lian *et al.* 2001). Although recent *in vivo* results have implicated a role of calcineurin in Aβ-induced neurotoxicity in AD mouse models (Sun *et al.* 2009; Wu *et al.* 2010; Rozkalne *et al.* 2011; Spires-Jones *et al.* 2011), current evidence does not rule out the possibility that the neuroprotective effect of FK506 is exerted through a clacineurin-independent mechanism. The observation that the presence of FK506 is able to reverse the C99/C83 ratio change caused by FKBP12 overexpression, however, does suggest that the FK506-reversible regulatory effect of FKBP12 on APP processing is not likely to be mediated via the mTOR pathway (particularly the mTORC1 pathway), as FK506 is known not to bind to the FKBP12-rapamycin binding (FRB) domain of mTOR.

The exact neuroprotective mechanism(s) of FK506 still remains unclear. Due to the structure similarity between the Thr668-Pro669 dipeptide of AICD and the FKBP12 binding moieties of FK506, it is possible that FK506 can compete with AICD for the same binding site on FKBP12, or less likely but not impossible, AICD may be forced to dissociate from FKBP12 as a result of the steric hindrance caused by FK506 binding, or the conformational change of FKBP12 upon FK506 binding. Either way, the PPIase activity is very likely to be affected. Our result suggests the importance of FKBP12-AICD interaction in modulating APP processing. The current findings, however, are unable to answer the question of whether PPIase activity is involved in the regulatory mechanism. We are currently addressing this question using FKBP12 mutants deficient in PPIase activity, or FK506 binding activity, or both.

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