Phosphorylation-dependent Regulation of the Interaction of Amyloid Precursor Protein with Fe65 Affects the Production of β-Amyloid*

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Neuronal Fe65 is an adapter protein that interacts with the cytoplasmic domain of the β-amyloid precursor protein (APP). Although the interaction has been reported to occur between the second phosphorytrosine interaction domain of Fe65 and the YENPTY motif in the cytoplasmic domain of APP, the regulatory mechanism and biological function of this interaction remain unknown. We report here that (i) a single amino acid mutation at the Thr-668 residue of APP695, located 14 amino acids toward the amino-terminal end from the 682YENPTY687 motif, reduced the interaction between members of the Fe65 family of proteins and APP, whereas interaction of APP with the phosphorytrosine interaction domain of other APP binders such as XI1-like and mammalian disabled-1 was not influenced by this mutation; (ii) the phosphorylation of APP at Thr-668 diminished the interaction of APP with Fe65 by causing a conformational change in the cytoplasmic domain that contains the Fe65-binding motif, YENPTY; and (iii) the expression of Fe65 slightly suppressed maturation of APP and decreased production of β-amyloid (Aβ). Mutation at Thr-668 of APP abolished the effect of Fe65 on APP maturation. This mutation blocked the Fe65-dependent suppression of Aβ production and resulted in the release of increased levels of Aβ in the presence of Fe65. We previously reported that during maturation of APP in neurons, the protein is specifically phosphorylated at Thr-668 and undergoes O-glycosylation. The present results suggest that the phosphorylation of O-glycosylated mature APP at Thr-668 causes a conformational change in its cytoplasmic domain that prevents binding of Fe65 in neurons and may lead to an alteration in the production of Aβ.

β-Amyloid precursor protein (APP)1 is the precursor of β-amyloid (Aβ), which is associated with the pathogenesis of Alzheimer’s disease (AD) (reviewed in Ref. 1). APP belongs to a member of a family of proteins that includes APP-like proteins (APLPs) 1 and 2. This family of proteins has a membrane-associated receptor-like structure composed of a large extracellular, a single transmembrane, and a short cytoplasmic domain (2–6). APP is ubiquitously expressed as different isoforms in neuronal and nonneuronal tissues. The protein is cleaved proteolytically to generate Aβ during its association with protein secretory and/or endocytotic pathways (reviewed in Ref. 7). Three functional motifs in the cytoplasmic domain of APP are thought to regulate its rate of secretion, endocytosis, and Aβ production. The amino acid sequence 653YTSI656 (human APP695 isoform numbering) forms a characteristic internalization and/or basolateral sorting signal, XXXI (8, 9). The motif 667VTPEER672 contains Thr-668, the amino acid phosphorylated by neuronal cyclin-dependent kinase (cyclin-dependent kinase 5) in neurons (10), cdc2 kinase in dividing cells (11, 12), and glycogen synthase kinase 3β and stress-activated protein kinase 1b in vitro (13, 14). The phosphorylation of APP regulates neurite extension in differentiating PC12 cells (15). The motif also contains Arg-672, a necessary residue for the metabolism of APP by the nonamyloidogenic pathway (16). The amino acid sequence, 682YENPTY687, contains an NPXY element, which is a typical internalization signal for membrane-associated receptor protein (8, 17, 18); however, in APP, Tyr668 is not phosphorylated (17, 19). These motifs are thought to function through interaction with cytoplasmic proteins termed APP binders.

Several APP binders have been identified. A microtubule-interacting protein, PAT1, interacts with the 667VTPEER672 motif and regulates the basolateral sorting of APP (9). The phosphorytrosine interaction (PI) domains of the proteins Fe65, Fe65-like (Fe65L) 1, Fe65L2, X11, X11-like (X11L), X11L2, and mammalian disabled-1 (mDab1) interact with the 653YTSI656 motif (20–27). The UV-damaged DNA-binding protein, which does not contain a PI domain, also interacts with the 682YENPTY687 motif (28). However, the role of the 667VTPEER672 motif in the binding of APP to APP binders remains to be determined. Recent analysis of this motif by multidimen-

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¶¶¶¶§§§§*** The abbreviations used are: APP, β-amyloid precursor protein; Aβ, β-amyloid; AD, Alzheimer’s disease; APLP, amyloid precursor-like protein; Fe65, phosphotyrosine interaction; PI1 and PI2, the first and second PI, respectively; Fe65L, Fe65-like protein; XI1L, XI1-like protein; mDab1, mammalian disabled-1 protein; CD, circular dichroism; GST, glutathione S-transferase; HEK, human embryonic kidney; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonic acid; TRIS, Tris-buffered saline including Tween 20; TFE, trifluoroethanol; sAPP, large extracellular amino-terminal domain truncated at α- and/or β-sites.

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sional NMR spectroscopy showed it to consist of a type I β-turn and an amino-terminal helix-capping box structure (29). It was therefore expected that the 667VTPEER672 motif would influence the maintenance of the overall structure of the cytoplasmic domain of APP. Furthermore, it is possible that phosphorylation at Thr-668 of APP, which changes the conformation of the cytoplasmic domain, regulates the interaction between the cytoplasmic motifs of APP and APP binders. Therefore, we explored the effects of phosphorylation of Thr-668 on the interaction between APP and APP binders.

In previous studies it has been shown that the 685YENPTY-687 motif of APP is essential for the basic interaction with Fe65 (30). In this study, we demonstrate that this interaction is regulated by phosphorylation at Thr-668. Analysis by CD spectroscopy showed that the structure of the cytoplasmic peptide phosphorylated at Thr-668 differed from that of the nonphosphorylated peptide. Thus, phosphorylation of APP at Thr-668 is likely to alter the conformation of its cytoplasmic domain.

Overexpression of Fe65 decreased the production of Aβ from APP; however, this effect was diminished when a mutation at Thr-668 was introduced. These observations suggest that APP metabolism is regulated by the neuron-specific phosphorylation of APP at Thr-668, which causes a conformational change in the cytoplasmic domain.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Peptides—** Anti-FLAG (M2) monoclonal antibody was purchased from Sigma. The anti-Myc monoclonal antibody was purchased from Invitrogen. The anti-APP polyclonal antibody, UT-18, was raised against the cytoplasmic peptide [Cys]APP(676–695) (15). A polyclonal phosphorylation state-specific antibody (aPThr668) was raised against the phosphopeptide, APP(665–673)-[Cys]PThr668, as described (15). UT-33 recognizes APP phosphorylated at Thr-668 and does not recognize nonphosphorylated APP (10, 15). The cytoplasmic peptides of APP(648–685), with or without phosphate at the Thr-668 residue, and Ap-1(4–40) and Ap-2(41–42) peptides were synthesized using solid phase N-terti-butyloxycarbonyl chemistry. The peptides were purified by reversed-phase high pressure liquid chromatography to greater than 90% purity, and their expected molecular weights were confirmed by mass spectroscopy. The shorter cytoplasmic peptides Cys-APP(673–675), either phosphorylated or nonphosphorylated, were synthesized as described (15).

**Preparation of Proteins and in Vitro Pull-down Assay—** Human cDNAs encoding Fe65, Fe65L, and Fe65L2 were isolated in a yeast two-hybrid assay using the cytoplasmic domain of APP as bait. Fe65 cDNA was cloned into pcdNA3.1-Fe65myc/HisA (CLONTECH). The cDNAs encoding the second PPI domains derived from Fe65 (amino acids 540–665 of GenBank TM accession number O00213), Fe65L1 (amino acids 548–684 of GenBank TM/Berkeley Databank accession number Q92870), and Fe65L2 (amino acids 287–417 of GenBank TM/Berkeley Databank accession number 905794) and WW domains derived from Fe65 (amino acids 232–288 of GenBank TM/Berkeley Databank accession number O00213) were subcloned into pGEX-4T-1 (Amersham Pharmacia Biotech) to produce GST-fusion proteins. The cDNA encoding the PI domain of Dab1 was cloned by reverse transcription-polymerase chain reaction using human brain total RNA (CLONTECH) isolated from medium by immunoprecipitation with anti-FLAG antibody and separated by SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose membranes, the samples were incubated for 1 h at 4 °C and then centrifuged. The beads were washed with TBST three times, and proteins were eluted by boiling the beads in SDS sample buffer. Proteins were then analyzed by SDS-polyacrylamide gel electrophoresis (6% (w/v) polyacrylamide) and immunoblotted with anti-FLAG antibody. Immunocomplexes were detected using an ECL detection kit (Amersham Pharmacia Biotech).

**CD Spectroscopy—** CD spectra were recorded at 25 °C on a JASCO J-600 spectropolarimeter using a 0.1-cm path length cuvette. The cytoplasmic peptides of APP(648–695) with or without phosphate at the Thr-668 residue were dissolved at a concentration of 0.1 mM in 20 mM sodium phosphate buffer (pH 6.0) containing either 0% (v/v) or 47% (v/v) trifluoroethanol (TFE). Each CD spectrum is an average of five scans with a 1.0-nm bandwidth, a time constant of 2 s, and a step resolution of 0.2 nm. The percentage of α-helicity was calculated as [θ]222/[θ]222max × 100, where [θ]222max is the number of residues in the peptide (32).

**Pulse-Chase Study—** Pulse-chase labeling of cells was carried out with [35S]methionine (1 μCi, Amersham Pharmacia Biotech/AAQ0087). HEK293 cells were cotransfected with a combination of pcdNA3-FLAGAPPP695 and pcdNA3.1-Fe65myc/HisA or pcdNA3-FLAGAPPP695 and pcdNA3.1(−)myc/HisA. At 48 h after transfection, the cells were labeled metabolically in Dulbecco’s modified Eagle’s medium with [35S]methionine (1 μCi/ml, Amersham Pharmacia Biotech) for 15 min. The chase was initiated by replacing the culture medium with fresh medium excluding cold methionine for 5 min followed by a chase period as indicated. The chase was initiated by replacing the labeled medium with medium containing excess unlabeled methionine. APP was immunoprecipitated from cell lysates using the anti-FLAG antibody M2 (Sigma) and separated by SDS-polyacrylamide gel electrophoresis (6% (w/v) polyacrylamide). Radioimmuneprecipitation buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS) containing 25 μg/ml radiolabeled [35S]methionine was used for cell lysis. αAPP, a large extracellular amino-terminal domain cleaved at α- and β-sites, was isolated from medium by immunoprecipitation with anti-FLAG antibody and separated by SDS-polyacrylamide gel electrophoresis (7.5% (w/v) polyacrylamide). Radioactivity in APP and sAPP was analyzed using a Fujifilm BAS 2000 imaging analyzer and autoradiography.

**Quantification of β-Amyloid—** HEK293 cells were transiently transfected with a combination of pcdNA3-FLAGAPPP695 and either pcdNA3.1-Fe65myc/HisA or pcdNA3.1(−)myc/HisA or a combination of pcdNA3-FLAGAPPP695 and pcdNA3.1-Fe65myc/HisA or pcdNA3.1(−)myc/HisA. The cells were supplied with fresh growth medium 5 h after the start of transfection, and conditioned medium from cells (2 × 106 cells) was collected 48 h after the medium change. Ap40 and Ap42 were quantified with sandwich enzyme-linked immunosorbent assay using three types of Ap-specific monoclonal antibodies (33).

**RESULTS**

**Role of Thr-668 of APP695 in Interaction with Fe65—** The motif, 667VTPEER672 (numbering for APP695 isoform), of APP is thought to be important for the regulation of function and
Family of APP, which consists also of APLP1 and APLP2 (2–5). We have demonstrated previously that the phosphorylation site corresponding to Thr-668 of APP695 is conserved in the cytoplasmic domain of APLP2 as Thr-736 (numbering for APLP2–736 isoform) but not in the cytoplasmic domain of APLP1 (31). In neurons, Thr-736 of APLP2 is phosphorylated in an identical manner to that of Thr-668 in APP.2

In addition, the YENPTY motif is also conserved in APLP2. Therefore, we examined the influence of Thr-736 on the binding of Fe65 to APP regardless of the presence of a mutation at Thr-668.

The importance of the Thr-668 residue of APP on the interaction with Fe65 was confirmed in vitro using a co-immunoprecipitation assay. Full-length Fe65 protein tagged with Myc/His sequences (Fe65-myc/His) and APP695 tagged with a FLAG sequence (FLAG-APP695) were expressed transiently in HEK293 cells. The detergent-soluble fraction of these cell lysates was subjected to immunoprecipitation using anti-Myc antibody, and the immunoprecipitates were analyzed by Western blot using anti-FLAG (Fig. 2) or anti-Myc (data not shown) antibodies. A large quantity of wild-type APP (T) was recovered in association with Fe65. However, comparatively small quantities of APP carrying Ala (A) and Glu (E) substitutions for Thr-668 were co-immunoprecipitated with Fe65 even though the amount of APP expressed was identical between experiments. Furthermore, APP was not co-immunoprecipitated with anti-Myc antibody when only APP (− in Fe65-myc) or Fe65/myc/His (− in APP) were expressed. These results demonstrate that the stable binding of Fe65 family members to APP requires the presence of Thr-668 of APP695. Other APP binders such as X11L and mDab1 did not need Thr-668 to be present for stable binding to APP.

Interaction of APLP2 with Fe65—APP is a member of the APP family of proteins, which consists also of APLP1 and APLP2 (2–5). We have demonstrated previously that the phosphorylation site corresponding to Thr-668 of APP695 is conserved in the cytoplasmic domain of APLP2 as Thr-736 (numbering for APLP2–736 isoform) but not in the cytoplasmic domain of APLP1 (31). In neurons, Thr-736 of APLP2 is phosphorylated in an identical manner to that of Thr-668 in APP.2

In addition, the YENPTY motif is also conserved in APLP2. Therefore, we examined the influence of Thr-736 on the binding of Fe65 to APLP2 (Fig. 3). Glutathione-Sepharose beads bearing GST-fusion proteins consisting of Fe65PI2 were incubated with lysate from cells transiently expressing wild-type APLP2

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Western blot analysis with anti-Myc antibody. Cell lysates (Lysate) glutathione beads. The amount of bound APLP2 was quantified by A contained equal amounts of FLAG-APLP2 (T724A) or Lysates derived from COS7 cells expressing wild-type APLP2-myc/His 40356 751, on the interaction with the PI2 domain from Fe65 and PI domains of APP binders. Role of Thr-724, a phosphorylation site in APLP2–751, on the interaction with the PI2 domain from Fe65 and PI domain from mDab1 was analyzed with pull-down assays in vitro. Lysates derived from COS7 cells expressing wild-type APLP2-myc/His (T) or APLP2-myc/His containing an alanine (A) substitution for Thr-724 (A) were incubated with the GST-PI2 domain of Fe65 (Fe65PI2) or GST-PI domain of mDab1 (mDab1PI) or GST alone (GST), coupled to glutathione beads. The amount of bound APLP2 was quantified by Western blot analysis with anti-Myc antibody. Cell lysates (Lysate) contained equal amounts of FLAG-APLP2 (T) and FLAG-APLP2T724A (A). Higher molecular weight forms are sulfated APLP2 (5, 31).

Phosphorylation at Thr-724 Interferes with the Interaction of Fe65 with the 682–YENPTY687 Motif of APP—Phosphorylation-dependent Interaction of APP with Fe65

Phosphorylation at Thr-668 with or without phosphate at the Thr-668 residue as competitors (Fig. 4). Glutathione-Sepharose beads bearing GST-fusion proteins consisting of Fe65PI2 were incubated with the APP cytoplasmic domain peptide, APP-(648–695), or the phospho-Thr-668 peptide, [pThr-668]APP-(648–695), prior to incubation with lysate derived from COS7 cells, which expressed FLAG-APPP695 transiently. After washing the beads, the amount of APP that had bound to the beads was determined by Western blot analysis using an anti-FLAG antibody (Fig. 4a), and the interaction of APP with Fe65PI2 was quantified (Fig. 4c). The APP cytoplasmic peptide containing nonphosphorylated Thr-668 interfered with the interaction of Fe65 with APP695 more strongly than that containing phospho-Thr-668, although both peptides, which contain the 682–YENPTY687 motif, involved in recognition of Fe65, had the ability to compete with APP695 for the binding of Fe65PI2. Shorter peptides that consisted of nonphosphorylated Thr-668 (APPP663–676) or phosphorylated Thr-668 (APPP663–676) without the 682–YENPTY687 motif did not interfere with the interaction of Fe65 with APP (Fig. 4b). These results indicate that the cytoplasmic domain peptide of APP that contains both the 682–YENPTY687 motif and a nonphosphorylated Thr-668 is highly competitive with APP for the binding of Fe65. The amino acid sequence around Thr-668 did not seem essential for recognition by Fe65.

We previously found that the phosphorylation of APP at Thr-668 is specific to neuronal tissue (10). In neurons, ~10% of mature APP695 (N- and O-glycosylated) exists in the phosphorylated form and is distributed in the plasma membrane and in neurites. Immature APP695 (N-glycosylated) is localized to the endosomal reticulum and early Golgi and exists in the completely nonphosphorylated form. Therefore, we examined the interactions of the different forms of endogenous APP derived from brain with Fe65PI2 (Fig. 5). APP was recovered from the brain lysate of adult rats by immunoprecipitation using the anti-APP antibody, UT-18 (AbAPP). Quantification of the content of APP and APP phosphorylated at Thr-668 (pAPP) was performed by Western blot analysis using UT-18 and the anti-phospho-Thr-668 APP antibody, UT-33 (Fig. 5a). As described previously, the phosphorylated form of APP (pAPP) consists of two types of mature APP695 modified with differential O-glycosylation, whereas the nonphosphorylated form contains immature APP695 (10). Aliquots of the same brain extract containing these endogenously phosphorylated and nonphosphorylated forms of APP were incubated with glutathione-Sepharose beads coupled with GST-fusion proteins consisting of Fe65PI2 or X11LPI. The APP that attached to the beads was eluted and analyzed by Western blot using UT-18 and UT-33 (Fig. 5b). Two mature APP and one immature APP species bound to Fe65PI2 and X11LPI but not to GST alone. The immature APP preferentially bound to Fe65PI2, but both mature APP and immature APP bound to X11LPI equally with respect to the physiological ratio of mature APP to immature APP in the brain (compare the left panels in Fig. 5, a and b). Although mature APP did bind to Fe65PI2, the level of association of the phosphorylated form of mature APP with Fe65PI2 was significantly lower compared with that of the nonphosphorylated form. Phosphorylated mature APP showed a stronger association with X11LPI compared with its association with Fe65PI2 (compare right panel with left panel in Fig. 5b). This result indicates that Fe65 interacts preferentially with APP nonphosphorylated at Thr-668; however, the phosphorylated form of APP can still interact with X11L.

Phosphorylation of APP at Thr-668 Causes a Structural Change in the Cytoplasmic Domain of APP—Phosphorylation at Thr-668 alters the interaction of APP with Fe65. This chapter reviews the current understanding of the role of Thr-668 phosphorylation in APP–Fe65 interactions and highlights the potential clinical implications of these findings.
change in the cytoplasmic domain of APP, we recorded CD spectra of the APP cytoplasmic domain peptide, APP-(648–695), with or without phosphate at the Thr-668 residue. As previously reported for experiments performed at 4 °C (36), we found that the spectrum of the nonphospho-peptide (Thr) indicated the presence of a random coil structure under conditions of 0% (v/v) TFE at 25 °C (Fig. 6a). The phospho-peptide (pThr) also showed an identical spectrum indicating a random coil structure (Fig. 6a). Under different conditions consisting of 47% (v/v) TFE, the CD spectrum of the nonphospho-peptide indicated the presence of an α-helix structure (Fig. 6b). The spectrum of the phospho-peptide also indicated an α-helix pattern; however, the decrease in ellipticity was less than that for the nonphospho-peptide (Fig. 6b). The percentage of the α-helix of the phospho-peptide was 18.76% and that of the nonphospho-peptide was 33.49% under these conditions. This result indicates that, in a hydrophobic environment, the structure of the cytoplasmic domain of APP phosphorylated at Thr-668 is different from the unphosphorylated form.

Effect of the Thr-668 Residue of APP, in the Presence of Fe65, on APP Metabolism and Aβ Release—The interaction of some APP binders with APP is known to modify intracellular APP metabolism and Aβ production (20, 24, 33, 37, 38). The effect of the phosphorylation site, Thr-668 of APP, on Fe65-dependent modulation of APP metabolism was analyzed using APP carrying a Glu substitution for Thr-668. We first examined the effect of Fe65 on APP metabolism in HEK293 cells using a pulse-chase study. In HEK293 cells, expression of endogenous Fe65 is under detection level (39). The phosphorylation of immature APP and mature APP was not observed by Western blot analysis (data not shown). When the cells were subject to synchronization at G2/M phase of the cell cycle, phosphorylation-dependent Interaction of APP with Fe65 was studied.
tion of immature APP but not mature APP was detected as reported (11, 12). Wild type (T) or the Thr-668Glu (E) mutant of APP was expressed transiently with or without Fe65 in HEK293 cells. The expression of Fe65 does not affect the level of APP expression. The cells were labeled metabolically and followed by a chase period as indicated. Immature APP and mature APP in cell lysates and secreted APP in medium were isolated by immunoprecipitation and quantified, and their relative ratios with respect to total APP levels were calculated (Fig. 7). At 0 h of chase time, almost all the APP was in the immature APP form, and mature APP was almost undetectable (indicated as 1.0 for immature APP plus mature APP at 0 h). The levels of immature APP decreased gradually with time, whereas mature APP levels increased because of secretion during the chase period. APP mutated at Thr-668 (Fig. 7, a and b) behaved similarly. This indicated that maturation and secretion were not influenced by the mutation of Thr-668 in the absence of Fe65.

Expression of Fe65 slightly delayed the decrease in levels of wild-type immature APP, which indicates that Fe65 tends to slow the maturation of wild-type APP (Fig. 7d). The slight suppression of APP maturation by expression of Fe65 resulted in a slight decrease in the relative levels of mature APP (Fig. 7e). These effects were weakened if Thr-668 was changed to Glu-668 (Fig. 7, g and h). These results suggest that Fe65 delays the maturation of APP by binding to the cytoplasmic domain of immature APP. The mutation of Thr-668 does not affect the secretion of secreted APP in the absence of Fe65 (Fig. 7c). Secreted APP from wild-type APP (T) was suppressed by the expression of Fe65 (Fig. 7f). The suppression of secreted APP secretion by Fe65 expression was also cancelled partially in T668E mutation of APP (Fig. 7i). The mean with S.D. from duplicate assays is shown (n = 2), and consistent results were reproduced in two independent experiments. The autoradiograms of cellular APP are shown (Fig. 7, j and k). It is possible that the mutation of APP at Thr-668 weakened the association of Fe65 with APP and partially cancelled the effect of Fe65 on APP metabolism. The mutation at Thr-668 does not completely inhibit the association with Fe65 (Figs. 1 and 2). This may be a reason why the mutation did not cancel the Fe65-dependent modification completely (Fig. 7, g–i).

Fe65 regulates mature APP metabolism, which influences the production and secretion of Aβ. Wild-type APP695 or a mutant APP695 carrying a substitution of Glu for Thr-668 (T668E) was expressed transiently in HEK293 cells with or without Fe65. The amount of Aβ in the medium was quantified using sandwich enzyme-linked immunosorbent assay (Fig. 8). The secretion of Aβ40 and Aβ42 from cells expressing wild-type APP was decreased by the presence of Fe65 (Fig. 8a). The suppression of Aβ40 and Aβ42 production by Fe65 was prevented in part by the T668E mutation of APP (Fig. 8b). Secretion of Aβ40 and Aβ42 from cells expressing wild-type and mutant APP is identical in the absence of Fe65 (Fig. 8c). These results indicate that Fe65 suppresses the production of Aβ40 and Aβ42 in HEK293 cells and that the mutation of APP at Thr-668 prevents this effect by interfering with the interaction of Fe65 with the cytoplasmic domain of APP.

**DISCUSSION**

APP and presenilin are suspected causative factors in the pathogenesis of familial AD. Mutations of these genes have been detected in patients with familial AD; however, familial AD makes up only a minority of all cases of AD. These mutations are known to increase production of Aβ, which is thought to be the first step in the pathogenesis of AD (reviewed in Refs. 1 and 7). The majority of AD cases are of the sporadic type, and these patients do not carry mutations of these causative genes. Other mechanisms such as prevention of Aβ degradation and/or acceleration of Aβ aggregation, as well as the stimulation of Aβ production, are also believed to be involved in the pathogenesis of sporadic AD.

The cytoplasmic domain of APP contains several motifs and amino acid signals associated with the metabolism and function of APP (8, 10, 16, 17, 19). One of these is the Thr-668

**FIG. 6.** CD spectra of APP cytoplasmic peptide with or without phosphate at the Thr-668 residue. Spectra of the APP cytoplasmic peptide, APP-(648–695) phosphorylated at Thr-668 (pThr, solid line) and nonphosphorylated peptide APP-(648–695) (Thr, dashed line) in 0% (a) and 47% (b) TFE.
residue of APP695. Phosphorylation of this residue in mature APP occurs only in the brain, even though APP is expressed in many tissues, and is believed to be mediated by neuronal protein kinase cyclin-dependent kinase 5 (10), glycogen synthase kinase 3 (11), and/or stress-activated protein kinase (14). Therefore, the neuron-specific phosphorylation of APP is thought to play an important role in its function and/or metabolism. It is thought that an intracellular signal may regulate the phosphorylation of APP at Thr-668, because phosphorylation occurs independently from its extracellular domain.3 We have found that the phosphorylation of APP at Thr-668 is important for the extension of neurites of differentiating PC12 cells after nerve growth factor stimuli (15). The phosphorylation of APP at Thr-668 is also observed in the immature APP of dividing cells, but the detection by Western blot with the phosphorylation state-specific antibody is difficult if cells are not synchronized at the G2/M phase of the cell cycle or in nonneuronal tissues, because population of cells in the G2/M phase is very minor, and the term of the G2/M phase in the cell cycle is extremely limited (11, 12). The role of this transient phosphorylation has not been elucidated. In neurons, it was not clear whether the phosphorylation of mature APP affects the regulation of APP metabolism including Aβ production.

It is known that cytoplasmic proteins interact with motifs in the cytoplasmic domain of APP (20–27). Many of these APP binders such as protein belonging to the Fe65 family, X11, X11L, and mDab1 recognize amino acid sequences of APP containing the 652YENPTY657 motif through their PI domain. Proteins in the Fe65 family, X11, and X11L are thought to regulate APP metabolism by interacting with APP (24, 26, 37, 38)
overexpression.

secretion of APP slightly, which resulted in lower levels of mature APP and suppressed secretion of secreted APP. This Fe65-dependent effect on APP maturation was prevented by the introduction of a mutation at Thr-668. Immature APP is phosphorylated transiently at the G2/M phase of the cell cycle in nonneuronal dividing cells (11, 12), whereas immature APP is not phosphorylated in post-mitotic neuronal cells (10). Thus, it is possible to postulate that the phosphorylation of APP at Thr-668 regulates the interaction of Fe65 with mature APP rather than that with immature APP to control APP metabolism in neurons.

We further found that secretion of Aβ40 and Aβ42 was suppressed by overexpression of Fe65 and that this effect was partially cancelled by mutation of APP at Thr-668. The ability of Fe65 to suppress Aβ generation in HEK293 cells contradicts previous reports in which Fe65 and/or Fe65L stimulated APP maturation, sAPP production, and Aβ secretion in Madine-Darby canine kidney and H4 neuroglioma cells (37, 38). The difference may depend on the cell types used. Despite these contradictions, our results obtained with HEK293 cells are consistent with our preliminary observation that, in primary cultured neurons from rodents that expresses human APP, inhibition of phosphorylation of APP suppressed the release of Aβ derived from wild-type APP but not from APP that carried an Ala substitution for Thr-668. This suggests that in neurons, Fe65 can suppress the release of Aβ through its interaction with the cytoplasmic domain of APP. Therefore, we strongly believe that Fe65 regulates the generation of Aβ in an APP phosphorylation-dependent manner in neurons.

Fe65 requires the last 30 amino acids of the cytoplasmic domain of APP that includes a recognition motif, 682YENPTY687, for interaction (35). The Thr-668 residue is located 14 amino acids toward the amino-terminal end from the recognition motif, 682YENPTY687. It is important to elucidate how phosphorylation of Thr-668 influences the binding of Fe65 to the 682YENPTY687 motif to understand how Fe65 regulates Aβ generation. Recent analysis with multidimensional NMR spectroscopy demonstrated that the amino acid sequence around Thr-668 consists of a type I β-turn and an amino-terminal helix-capping box structure (29). Phosphorylation of Thr-668 may disturb this conformation and alter the structure of its carboxyl-terminal sequence including the recognition motif of Fe65. In the present study, the peptides 667VTPEER672 containing Thr-668 or phospho-Thr-668 but missing the 682YENPTY687 motif did not compete with the association of Fe65 and APP. This result supports the idea described above that the phosphorylation site Thr-668 is a regulatory site but not a binding site for Fe65.

The amino-terminal helix-capping box is reported to influence the stability of following α-helix structures in proteins (40). The 667VTPEER672 motif of APP is followed by a nascent helical region (29), and it is possible that phosphorylation at Thr-668 causes some structural change not only in the 667VTPEER672 motif but also in the following nascent helical region. In the hydrophobic environment provided by 47% (v/v) TFE, there was a clear difference in the molar ellipticity of the CD spectrum between the phospho- and nonphospho-cytoplasmic peptides of APP. The percentage of α-helix state of the phosphopeptide was less than that of nonphospho-peptide. This result indicates that the phosphorylation state of Thr-668 influences the structure of the cytoplasmic domain of APP in response to the environment. Recent NMR analysis suggest that, in general, regions such as the helix-capping box and the nascent helix region are relatively unstable structures common to early states in the protein folding process (29). Changes to the conformational structure of the cytoplasmic domain of APP in response to the solute environment suggest that the interaction
of APP binders with the cytoplasmic domain of APP may provide a hydrophobic environment. Other APP binders such as X11L and mDab1, which bind to APP regardless of the phosphorylation state of Thr-668, may also contribute to such a hydrophobic environment. Furthermore, while our manuscript was being reviewed, a report showing that phosphorylation of Thr-668 in the cytoplasmic domain of APP changes its structure was published (41). This observation using NMR analysis strongly supports our idea.

In neurons, mature APP is phosphorylated specifically at Thr-668 (10). We propose that phosphorylation of mature APP regulates the interaction of Fe65 with the cytoplasmic domain of APP and control the metabolism of mature APP in neurons. The production of Aβ40 and Aβ42 is believed to occur in association with a protein secretory pathway either within or following the trans-Golgi network (reviewed in Ref. 1). Within these compartments, APP is phosphorylated, and O-glycan residues are added (10). The identification of the intracellular location at which APP is phosphorylated and Aβ is generated may support our idea that the generation of Aβ is regulated by controlling the association of Fe65 with the cytoplasmic domain of APP through the phosphorylation of APP. If the phosphorylation level of APP at Thr-668 increases in the brains of patients with AD, less Fe65 will associate with the cytoplasmic domain of APP, which will result in changes to the rate of Aβ production. The potential to control the phosphorylation of APP at Thr-668, with its subsequent influence on the interaction of Fe65 with the cytoplasmic domain of APP may provide a useful treatment to suppress Aβ production in AD.

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REFERENCES

1. Price, D., Sisodia, S. S., and Borchelt, D. R. (1998) Science 282, 1079–1083
2. Wasco, W., Bupp, K., Magendanz, M., Gasella, J. F., Tanzi, R. E., and Solomon, F. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 10758–10762
3. Wasco, W., Gurubhagavatula, S., Paradis, M. D., Romano, D. M., Sisodia, S. S., Hyman, B. T., Neve, R. L., and Tanzi, R. E. (1993) Nat. Genet. 5, 95–100
4. Sprecher, C. A., Grant, F. J., Grimm, G., O’Hara, P. J., Norris, F., and Foster, D. C. (1993) Biochemistry 32, 4481–4486
5. Slunt, H. H., Thinakaran, G., Von Koch, C., Lo, A. C., Tanzi, R. E., and Sisodia, S. S. (1994) J. Biol. Chem. 269, 2637–2644
6. Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, R. H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987) Nature 325, 733–736
7. Selkoe, D. J. (2001) Physiol. Rev. 81, 741–766
8. Lai, A., Sisodia, S. S., and Trowbridge, I. S. (1995) J. Biol. Chem. 270, 3565–3673
9. Zheng, P., Eastman, J., Vande Pol, S., and Pimplikar, S. W. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 14745–14750
10. Iijima, K., Ando, K., Takeda, S., Sato, Y., Itohara, S., Greengard, P., Kirino, Y., Nairn, A. C., and Suzuki, T. (2000) J. Neurochem. 75, 1085–1091
11. Suzuki, T., Oishi, M., Marshak, D. R., Czernik, A. J., Nairn, A. C., and Greengard, P. (1994) EMBO J. 13, 1114–1122
12. Oishi, M., Nairn, A. C., Czernik, A. J., Lim, G. S., Itohara, T., Gandy, S. E., Greengard, P., and Suzuki, T. (1999) J. Neurosci. 19, 4421–4427
13. Tomita, S., Kirino, Y., and Suzuki, T. (1998) J. Biol. Chem. 273, 19304–19310
14. Koo, E. H., and Squazzo, S. L. (1994) J. Biol. Chem. 269, 17386–17389
15. Chen, W. J., Goldstein, J. L., and Brown, M. S. (1990) J. Biol. Chem. 265, 3116–3123
16. Haas, C., Hung, A. Y., Schlossmacher, M. G., Teplow, D. B., and Selkoe, D. J. (1993) J. Biol. Chem. 268, 3021–3024
17. Borg, J. P., Yang, Y., De Taddeo-Borg, M., Margolis, B., and Turner, R. S. (1998) J. Biol. Chem. 273, 14761–14766
18. Duilio, A., Faraonio, R., Minopoli, G., Zambrano, N., and Russo, T. (1998) Biochem. J. 330, 513–519
19. Fiore, F., Zambrano, N., Minopoli, G., Donini, V., Duilio, A., and Russo, T. (1995) J. Biol. Chem. 270, 30853–30856
20. Guenette, S. Y., Chen, J., Joudro, P. D., and Tanzi, R. E. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 10832–10837
21. Sastre, M., Turner, R. S., and Levy, E. (1998) J. Biol. Chem. 273, 22351–22357
22. Tanahashi, H., and Tabira, T. (1999) Biochem. Biophys. Res. Commun. 255, 663–667
23. Tomita, S., Ozaki, T., Taru, H., Oguchi, S., Takeda, S., Yagi, Y., Sakiyama, S., Kirino, Y., and Suzuki, T. (1997) J. Biol. Chem. 272, 2243–2254
24. Tronnierhoff, M., Borg, J. P., Margolis, B., and Herz, J. (1998) J. Biol. Chem. 273, 33556–33560
25. Watanabe, T., Sukegawa, J., Sukegawa, I., Tomita, S., Iijima, K., Oguchi, S., Suzuki, T., Nairn, A. C., and Greengard, F. (1999) J. Neurochem. 72, 549–556
26. Ramelot, T. A., Gentile, L. N., and Richardson, L. K. (2000) Biochemistry 39, 2714–2725
27. Borg, J. P., Osi, J., Levy, E., and Margolis, B. (1996) Mol. Cell. Biol. 16, 6229–6241
28. Suzuki, T., Ando, K., Itohara, T., Oishi, M., Lim, G. S., Satoh, Y., Wescoe, R., Tanzi, R. E., Nairn, A. C., Greengard, P., Gandy, E. D., and Kirino, Y. (1997) Biochemistry 36, 4645–4649
29. Chen, Y. H., Yang, J. T., and Chau, K. H. (1974) Biochemistry 13, 3530–3535
30. Tomita, S., Fujita, T., Kirino, Y., and Suzuki, T. (2000) J. Biol. Chem. 275, 13056–13060
31. Tomita, S., Kirino, Y., and Suzuki, T. (1998) J. Biol. Chem. 273, 6277–6284
32. Zambrano, N., Buxbaum, J. D., Minopoli, G., Fiore, F., De Candia, P., De Renzi, S., Faraonio, R., Sabo, S., Cheetham, J., Suboli, M., and Russo, T. (1997) J. Biol. Chem. 272, 6399–6405
33. Kroenke, C. D., Ziemnicka-Kotula, D., Xu, J., Kotula, L., and Palmer, A. G., III (1999) Biochemistry 38, 8145–8152
34. Guenette, S. Y., Chen, J., Jerland, A., Haass, C., Capell, A., and Tanzi, R. E. (1999) J. Neurochem. 75, 985–993
35. Sabo, S. L., Lanier, L. M., Ikin, A. F., Khorkova, O., Sahasrabudhe, S., Greengard, P., and Buxbaum, J. D. (1999) J. Biol. Chem. 274, 7952–7957
36. Bresader, S. L., Gray, M. D., Sopher, B. L., Hu, Q., Heem, M. G., Pharr, D. G., Dinolos, M. B., Fukuchi, K., Sisodis, S. S., Miller, M. A., Disteche, C. M., and Martin, G. M. (1996) Hum. Mol. Gen. 5, 1589–1598
37. Petukhov, M., Uyono, N., Maruse, O., Nommura, R., and Yoshikawa, S. (1996) Biochemistry 35, 387–397
38. Ramelot, T. A., and Nicholson, L. K. (2001) J. Mol. Biol. 307, 871–884
Phosphorylation-dependent Regulation of the Interaction of Amyloid Precursor Protein with Fe65 Affects the Production of β-Amyloid

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