FE65 serine-610 phosphorylation and its functional implications in Alzheimer disease amyloid precursor protein processing

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KEY MESSAGES

1. FE65 Serine610 (S610) phosphorylation regulates amyloid precursor protein (APP)/FE65 interaction and FE65-mediated APP processing.
2. FE65 S610 is phosphorylated by serum- and glucocorticoid-induced kinase 1.
3. The effect of FE65 S610 phosphorylation on APP processing is associated with the role of FE65 in metabolic turnover of APP via the proteasome.

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Introduction

Aggregation of amyloid-β peptide (Aβ), derived from the aberrant processing of amyloid precursor protein (APP), is crucial in the pathogenesis of Alzheimer disease. FE65 is a neural enriched adaptor protein that interacts with APP via its PTB1 domain. FE65/APP interaction can stimulate APP processing. Therefore, it is essential to understand the mechanism(s) by which FE65/APP interaction is regulated. FE65 is a phospho-protein with several reported phosphorylation sites. However, the biological significance of FE65 phosphorylation is largely unknown. FE65 Serine-610 (S610) is an uncharacterised phospho-residue located in the interaction interface of APP/FE65. Phosphorylation of FE65 S610 may play a role in regulating APP/FE65 interaction and altering APP processing.

Methods

Phosphomimetic mutants in GST pull down and co-immunoprecipitation assays were used to determine the effect of FE65 S610 phosphorylation on APP/FE65 interaction. APP processing was determined by APP-GAL4 luciferase reporter assay, APP C-terminal fragment analysis, and Aβ ELISA. FE65 S610 kinase was identified by Kinase Finder radiometric protein kinase assays and then validated by in vitro and in vivo kinase assays. The effect of FE65 on APP protein turnover was analysed by cycloheximide chase assay.

Results

Phosphorylation of FE65 S610 interferes with APP-FE65 interaction

According to the crystal structure of AICD/FE65-PTB2 complex, FE65 S610 is found to lie in their interaction interface. Such residue is phosphorylated in an FE65 isoform from mass spectrometric analysis. To test whether FE65 S610 phosphorylation alters APP/FE65 interaction, co-immunoprecipitation was performed by co-transfecting APP with either myc-tagged FE65 S610A or S610D to CHO cells. Western blotting revealed that greater amount of APP co-immunoprecipitated with FE65 S610A than with FE65 S610D (Fig 1). This evidence suggested that phosphorylation of FE65 S610 attenuates APP-FE65 interaction.

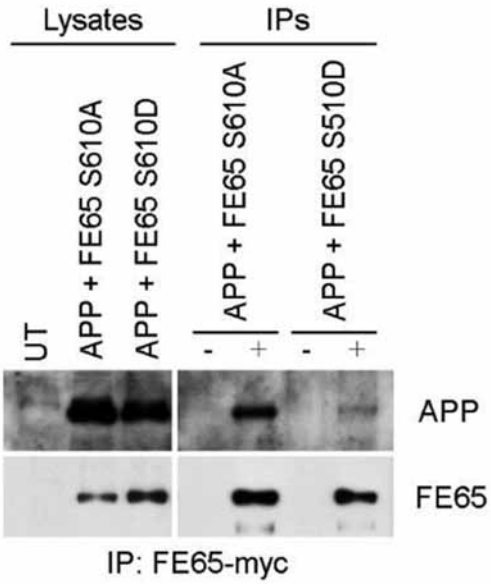

FIG 1. FE65 S610 phosphomimetic mutation reduces amyloid precursor protein (APP)/FE65 interaction. Bacterially expressed GST-APPc was used as bait for GST pull-down assay from FE65 S610A- or S610D-transfected cell lysate. FE65 S610A and S610D were detected by 9B11 anti-myc antibody against the C-terminal myc tag.

SGK1 phosphorylates FE65 S610

To identify FE65 S610 kinases, we used commercial Kinase Finder radiometric protein kinase assay (ProQinase, Germany), which is a radiometric kinase assay for a panel of 190 serine/threonine kinases. The results suggested that SGK1 is a putative FE65 S610 kinase (data not shown). Intriguingly, the amino acid sequence around FE65 S610 is found to be SGK1 targeting consensus (R-X-R-X-X-S/T-φ; φ = hydrophobic residue).[1,2] In order to validate FE65 S610 as the target residue of SGK1, bacterially expressed GST-FE65598-619 WT and GST-FE65598-619 S610A were incubated with SGK1-CA immunoprecipitated from transfected cell lysate together with [γ-^{32}P]-ATP for 30 minutes at 30°C. The reaction mixture was resolved on SDS-PAGE and exposed to an autoradiogram. SGK1 induced phosphorylation on wildtype but not on S610A mutant (Fig 2a).

To determine whether SGK1 interferes with APP-FE65 interaction, co-immunoprecipitation was performed from cells co-transfected with APP + FE65 or APP + FE65 + SGK1-CA. FE65 was immunoprecipitated by an anti-myc antibody and the immunocomplex was detected for APP. Less APP co- immunoprecipitated with FE65 in the presence of SGK1-CA, indicating that SGK1-CA weakens the interaction between APP and FE65 (Fig 2b).

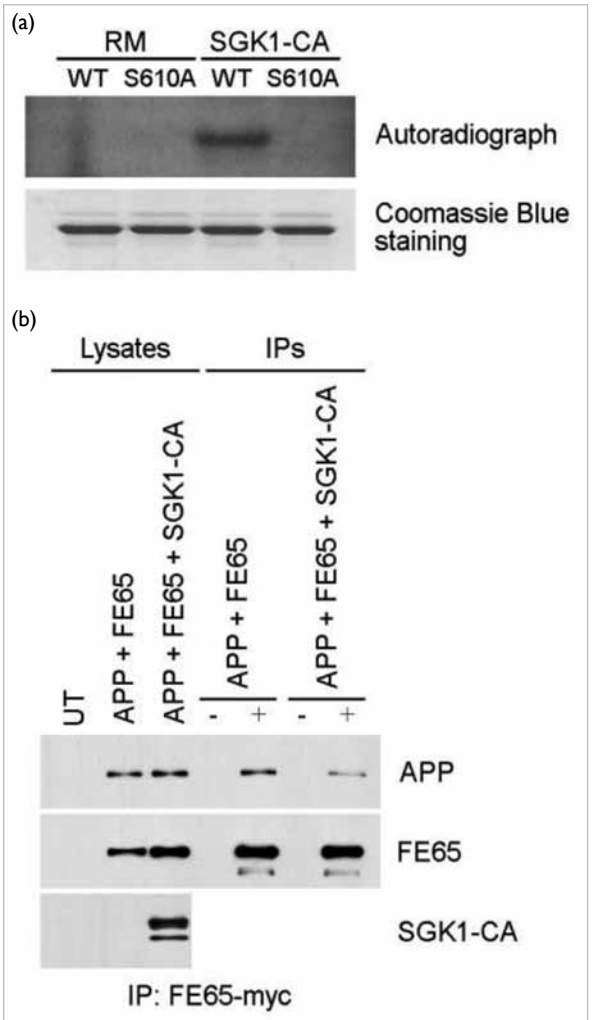

FIG 2. SGK1 phosphorylates FE65 S610 and to reduces amyloid precursor protein (APP)/FE65 interaction. (a) Bacterially expressed GST-FE65598-619 WT or S610A were incubated with SGK1-CA immunoprecipitated from transfected cell lysate together with [γ-32P]-ATP for 30 minutes at 30°C. RM is the reaction mix only without kinase. (b) Co-immunoprecipitation was performed from CHO cells transfected with APP + FE65 or APP + FE65 + SGK1-CA using anti-myc antibody. APP was detected with anti-APP antibody while FE65 was detected by an anti-myc antibody. SGK1 interferes with APP/FE65 interaction.

Phosphorylation of FE65 S610 abolishes the effect of FE65 on Aβ generation by enhancing APP degradation via proteasome

Aberrant processing of APP results in excessive Aβ generation, and the process is known to be modulated by a number of APP-interacting proteins including FE65. As FE65 S610 phosphorylation prevents APP-FE65 interaction, we speculated that this phosphorylation event might be involved in the regulation of FE65-mediated APP processing. APP + FE65/FE65 S610A/FE65 S610D were co-transfected into CHO cells, and the amount of secreted Aβ was measured by Aβ ELISA kit. The amount of Aβ was elevated in cells overexpressing FE65 or FE65 S610A but not in cells overexpressing FE65 S610D (Fig 3a). SGK1-CA suppressed FE65-mediated increase in Aβ generation but had no significant effect on FE65 S610A-promoted Aβ level (Fig 3b). This finding further supports that phosphorylation of FE65 at S610 by SGK1 abolishes the effect of FE65 on APP processing and Aβ generation.

To determine the effect of FE65 S610 phosphorylation on the turnover of APP, APP knockdown resulted in reduction of FE65 and FE65 S610A levels (lane 1 vs lane 2; lane 5 vs lane 6), and the depletion was blocked upon proteasome inhibitor MG132 treatment (lane 3 vs lane 4; lane 7 vs lane 8) [Fig 3c]. This indicates that APP stabilises FE65 and FE65 S610A by preventing their degradation through Ubiquitin-Proteasome System (UPS). Nonetheless, the protein level of FE65 S610D, which is unable to bind to APP, was not affected by endogenous APP level (lane 9 vs lane 10). Taken together, the current findings suggest that the interaction between APP and FE65 protects both APP and FE65 from being degraded through UPS, and this process is regulated by the phosphorylation status of FE65 S610.

Discussion

We demonstrated that phosphorylation of FE65 S610 by SGK1 attenuates the interaction between

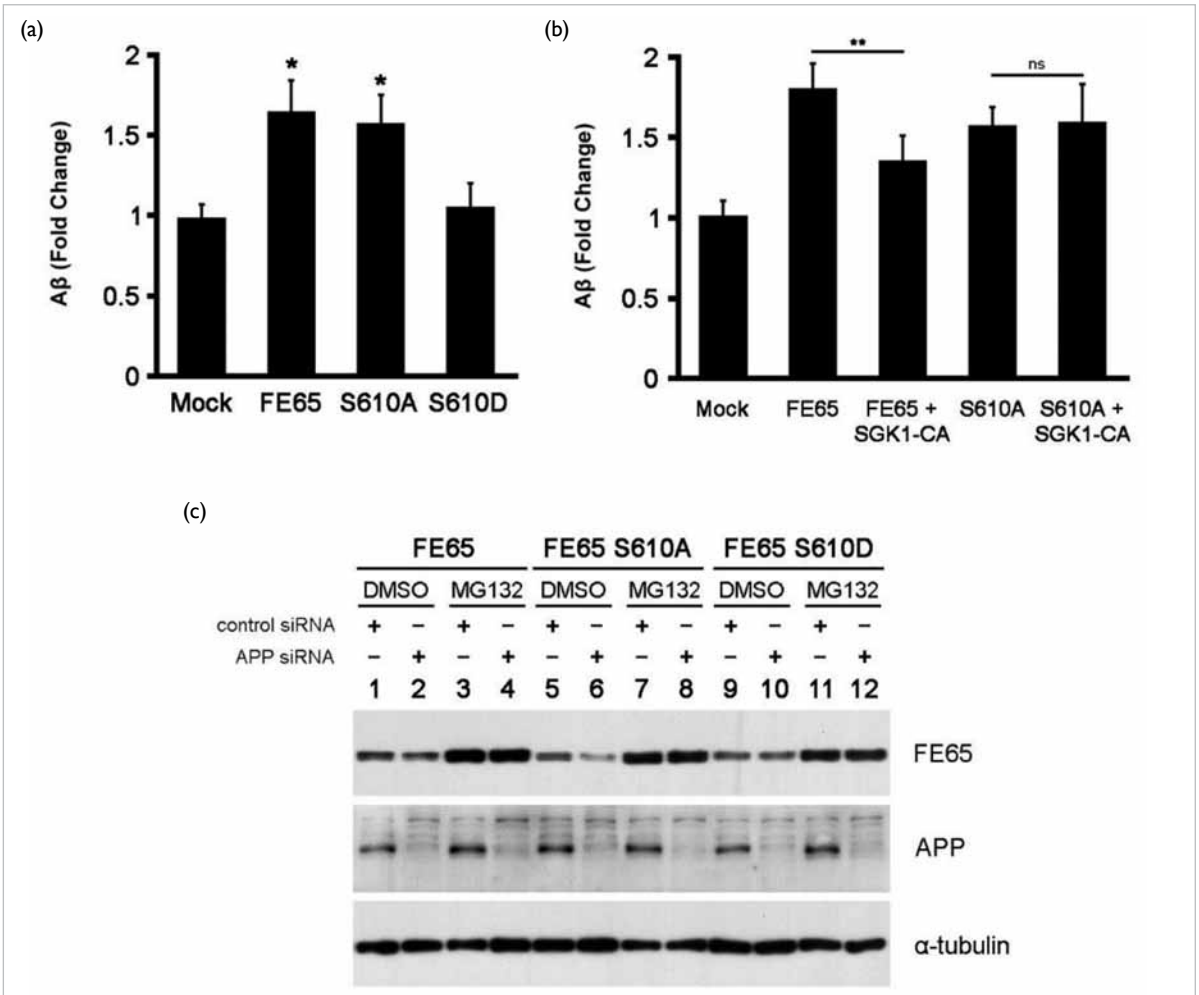

FIG 3. Phosphorylation of FE65 S610 and abolishes the effect of FE65 on Aβ generation by enhancing amyloid precursor protein (APP) degradation via proteasome. (a) CHO cells were co-transfected with APP + mock, FE65, FE65 S610A or FE65 S610D. At 48 hours post-transfection, cell culture medium were aspirated and changed to fresh medium. The level of secreted **Aβ** was assayed using ELISA kit 7 hours after change of medium. FE65 S610D does not enhance **Aβ** liberation. (b) CHO cells were co-transfected with APP + the indicated constructs, and the level of secreted **Aβ** was assayed. SGK1 suppresses FE65-enhanced **Aβ** liberation through phosphorylation of S610. (c) Cells co-transfected with FE65, FE65 S610A, or FE65 S610D + control or APP siRNA were incubated with 2.5 μM MG132 or DMSO vehicle for 16 hours. The protein levels of FE65 in transfected cell lysates were compared by western blotting. APP knockdown-mediated reduction of FE65 and FE65 S610A levels is rescued by MG132. Protein level of FE65 S610D is unaffected by APP knockdown.

FE65 and APP. Our findings are similar to those of others who provided evidence that SGK1 phosphorylates a rat variant of FE65.[3] FE65 S610 is located within the PTB2 domain of FE65, which is the domain that is responsible for APP binding. In fact, structural analysis of FE65 PTB2 domain in complex with AICD revealed that S610 is involved in the interaction interface. Our experimental data and molecular dynamics simulation further support the importance of FE65 S610 as a critical amino acid in APP-FE65 interaction and reveals a novel molecular mechanism that modulates APP-FE65 interaction.

Regulation of APP holoprotein level is important for AD pathogenesis. First, triplication of APP gene on chromosome 21 in patients with Down syndrome is associated with development of early onset AD. On the contrary, in patients with Down syndrome with only partial trisomy 21 that excludes APP gene, AD pathology was not observed even at advanced age. Second, APP mRNA level was reported to be elevated in sporadic AD, accompanied by a marked decrease in miR-106b, a negative regulator of APP expression. Third, degradation of APP through lysosomal pathway and proteasomal pathway has been implicated in reduction of Aβ liberation. We showed that FE65 stabilises APP holoprotein and prolongs its half-life by preventing APP degradation through UPS. Previously, we had demonstrated that FE65 stabilises p53 and huntingtin (Htt) by suppressing their degradation through UPS.[4] The

current finding has two important implications: (1) it reinforces the role of FE65 as a negative regulator in UPS-mediated degradation, and (2) FE65 serves to suppress APP holoprotein turnover through UPS, thereby enhancing Aβ generation, in addition to acting as a bridging molecule between APP and ApoE receptors such as LRP1, ApoER2, and VLDLR which modulate APP processing.

APP can be targeted to proteasome through multiple pathways. For instance, APP was reported to be an ER-associated degradation substrate where it is ubiquitinated by ER-localised E3 ubiquitin ligase HRD1, and the stress-responsive chaperone-protease HtrA2, through binding to the N-terminal region of APP, serves as a shuttling chaperone to assist proteasome targeting. Alternatively, APP can be ubiquitinated by the F-box and leucine rich repeat protein 2, a component of the SCF (Skp1-Cullin1-F-box protein) E3 ubiquitin ligase complex, resulting in enhanced proteasomal degradation. Of note, Aβ generation was reported to decrease by the aforementioned pathways. It is therefore possible that FE65 inhibits APP proteasomal degradation by competing with the E3 ubiquitin ligases HRD1 and F-box and leucine rich repeat protein 2 for APP binding, thereby promoting Aβ generation. Further investigation on the underlying mechanism by which FE65 blocks APP degradation through UPS is warranted.

The phosphorylation status of FE65 S610 was shown to regulate the turnover rate of APP. Consistent with the interaction behaviour, phosphorylation of FE65 S610 abrogates the stabilisation effect on APP, leading to a shorter half-life. This provides a mechanistic explanation to the regulatory effect of FE65 S610 phosphorylation on APP processing. Additionally, we provided evidence that degradation of FE65 and FE65 S610A through UPS is suppressed by APP, a phenomenon which is not observed in FE65 S610D. Our current finding underscores the importance of APP-FE65 interaction in regulating the turnover of both APP and FE65 through UPS, which in turn modulates APP processing and thus Aβ generation.

Furthermore, we demonstrated that SGK1 attenuates APP-FE65 interaction through FE65 S610 phosphorylation and suppresses FE65-enhanced Aβ secretion. SGK1 is a serine/threonine kinase downstream of the phosphatidylinositol 3-kinase (PI3K) cascade and its expression is acutely regulated by serum and glucocorticoids. SGK1 has been shown to phosphorylate the γ-secretase component Nicastrin and promote its degradation. Examination of APP, C-terminal fragment, and AICD protein levels and activity of C99-GAL4/VP16 luciferase reporter revealed that γ-secretase-mediated APP cleavage is suppressed by SGK1. Our work demonstrated that SGK1, through phosphorylating FE65 S610, modulates Aβ liberation and that a novel pathway by which SGK1 regulates APP processing.

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Results from this study have been published in:

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