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Amyloid Precursor Protein Binding Protein-1 Is Up-regulated in Brains of Tg2576 Mice

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Amyloid precursor protein binding protein-1 (APP-BP1) binds to the carboxyl terminus of amyloid precursor protein and serves as a bipartite activation enzyme for the ubiquitin-like protein, NEDD8. Previously, it has been reported that APP-BP1 rescues the cell cycle S-M checkpoint defect in Ts41 hamster cells, that this rescue is dependent on the interaction of APP-BP1 with hUba3. The exogenous expression of APP-BP1 in neurons has been reported to cause DNA synthesis and apoptosis via a signaling pathway that is dependent on APP-BP1 binding to APP. These results suggest that APP-BP1 overexpression contributes to neurodegeneration. In the present study, we explored whether APP-BP1 expression was altered in the brains of Tg2576 mice, which is an animal model of Alzheimer's disease. APP-BP1 was found to be up-regulated in the hippocampus and cortex of 12 month-old Tg2576 mice compared to age-matched wild-type mice. In addition, APP-BP1 knockdown by siRNA treatment reduced cullin-1 neddylation in fetal neural stem cells, suggesting that APP-BP1 plays a role in cell cycle progression in the cells. Collectively, these results suggest that increased expression of APP-BP1, which has a role in cell cycle progression in neuronal cells, contributes to the pathogenesis of Alzheimer's disease.

Key Words: Amyloid precursor protein binding protein-1, Amyloid precursor protein, Alzheimer's disease, cell cycle, Tg2576 mice

INTRODUCTION

Amyloid precursor protein binding protein-1 (APP-BP1) interacts with the intracellular carboxyl (C-) terminus of amyloid precursor protein (APP), which is the precursor protein of amyloid beta peptide (Aβ). Aβ is the main component of neuritic plaques in Alzheimer's disease (AD) patients [1-3]. The intracellular C-terminal domain of APP interacts with the following proteins; members of the Fe65 protein family [4], JNK interacting protein (JIP1) [5], X11 [6], APP-BP1 [2], and others.

APP-BP1 acts as one component of the bipartite activating enzyme for the ubiquitin-like small molecule, NEDD 8 [7-9]. NEDD 8 has a highest homology to ubiquitin and is conserved among a wide range of organisms such as yeast, plants, and mammals [10-13]. The process of neddylation is involved in various cellular functions including cell cycle progression [14-16].

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Neddylation targets in mammalian cells have been previously identified and include the cullin (Cul) family members, which are a major constituent of the ubiquitin-ligase, Skp-1-Cul-1-F box (SCF) complex [17,18]. SCF ubiquitin ligase targets the cyclin-dependent kinase (cdk) inhibitor p27 for degradation during the transition of cells from the G_0/G_1 to the S phase of the cell cycle [19]. SCF ligases also regulate other key substrates within the cell division cycle such as PDCD4, Cdc25A, Claspin, Wee1, Emi1, cyclin E, and cyclin D1 [20,21].

It has also been demonstrated that APP-BP1 rescues the cell cycle S-M checkpoint defect in Ts41 hamster cells, that this rescue is dependent on the interaction of APP-BP1 with hUba3 [22]. In addition, overexpression of APP-BP1 in primary neurons induces apoptosis and increases DNA synthesis [23]. Furthermore, up-regulated APP-BP1 expression has been observed in the lipid rafts in the hippocampi of AD brains when compared to age-matched control brains [1].

Recently, overexpression of APP-BP1 in primary cortical neurons has been reported to increase Rab5-mediated endocytosis, in an APP binding dependent manner [24]. Endocy-

ABBREVIATIONS: AD, Alzheimer's disease; APP, amyloid precursor protein; APP-BP1, amyloid precursor protein binding protein-1; cdk, cyclin dependent kinase; Cul, cullin; DAB, 3,3-diaminobenzidine; PBS, phosphate-buffered saline; SCF, Skp1-Cul-1-Fbox; SDS, sodium dodecyl sulfate.

tosis has been linked to the cell cycle [25].

In this study, we investigated whether APP-BP1 expression was altered in the brains of Tg2576 mice, which are an AD animal model. Comparing the brains of AD mice with wild-type brains demonstrated that APP-BP1 was up-regulated in the hippocampus and cortices from 12 month-old Tg2576 mice. In addition, APP-BP1 knock-down reduced the neddylation of Cul-1, which is responsible for SCF complex activation, in fetal neural stem cells, suggesting that APP-BP1 is involved in cell cycle progression in neural precursor cells. Collectively, an up-regulation in the expression of APP-BP1 in the brains of aged Tg2576 mice may indicate that this protein is one of factors contributing to AD pathogenesis.

METHODS

Reagents and antibodies

Anti-APP-BP1 purified mouse monoclonal antibody was obtained from Abcam (CA, USA); anti-Cul-1 and anti-NEDD8 antibodies were from Santa Cruz (CA, USA). The source of siRNA for APP-BP1 (SMARTpool® reagent) was Dharmacon (CO, USA).

Fetal neural stem cell culture

Fetal neural stem cell cultures were performed as previously described [26]. Embryonic CNS tissues were dissected from C57BL6 embryonic mouse cortex at gestation day 13. Timed pregnant 13 (TP13) females were from Japan SLC., Inc. (Haruno, Japan). Cells were isolated by mechanical dissociation in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution. The generation of neural spheres derived from fetal neural stem cells was performed as previously described [27]. Briefly, fetal neural stem cells were aggregated into a neurosphere and were then allowed to proliferate in a proliferating media composed of Dulbecco's modified Eagle's medium/F-12 (1 : 1) (Gibco, NY, USA) medium supplemented with 2 mmol/l L-glutamine (Gibco), 0.6% glucose, 5 μmol/l HEPES, 25 μg/ml insulin, 100 μg/ml apo-transferrin, 30 nmol/l sodium selenite, 100 nmol/l putresine, and 20 nmol/l progesterone (all supplements purchased from Sigma, MO, USA) with 10 ng/ml recombinant basic fibroblast growth factor (bFGF; Roche, Mannheim, Germany) and 20 ng/ml epidermal growth factor (EGF; BD sciences, MA, USA) for 4 days. We treated non-targeting siRNA and siRNA for APP-BP1 at 10 nM for 72 h to suppress endogenous APP-BP 1 in fetal neural stem cells.

Transgenic animals

All experiments were performed in accordance with 'the Guidelines for Animal Experiments from the Ethics Committee at Seoul National University'. They had free access to food and water. Tg2576 mice harboring the Swedish double mutated human APP695 gene were obtained from Taconic (German Town, NY, USA). The production genotyping, and background strain (C57BL/6_SJL) of Tg2576 mice used in this study were described previously [28].

Western blotting

Proteins were resolved in 12% sodium dodecyl sulfate (SDS) polyacrylamide gels, electrophoresed with 30~50 μg protein per lane transferred onto a nitrocellulose membrane (GE Healthcare AB, Sweden) according to the method of Laemili [29]. The protein blot was confirmed with appropriate antibodies and detected with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare AB, Sweden). Immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL; GE Healthcare AB, Sweden).

Immunoprecipitation

A total of 100 μg of each sample was rotated with Protein G Plus/Protein A agarose (Oncogene, Darmstadt, Germany) in 1 ml lysis buffer for 1 h at 4°C and then centrifuged at 14,000 r.p.m. for 20 min. Supernatants were incubated with 1 μg of primary antibody with rotation for 1 h at 4°C, treated with Protein G Plus/Protein A agarose for 1 h at 4°C, and centrifuged at 14,000 r.p.m. for 20 min. The pellet was boiled for 7 min in sample buffer (0.2 M Tris-HCl pH 6.8, 10% SDS, 25% glycerol and 0.01% bromophenol blue) and then centrifuged at 13,000 r.p.m. for 15 min. The supernatant was then subjected to SDS polyacrylamide gel electrophoresis.

Immunohistochemistry

Mice brains in 10% neutral buffered formalin for 48 h were dehydrated and embedded in paraffin. Before immunostaining, slides were deparaffinized in xylene and then dehydrated through graded ratios of alcohol to water. Fluorescent immunohistochemistry (IHC) was performed with appropriate primary antibodies at 4°C overnight and visualized using Cy3-conjugated secondary antibody (Jackson Laboratory, CA, USA). 4',6'-Diamidino-2-phenylindole (DAPI) counter staining was performed. Images were collected using the LSM 510 program on a Zeiss confocal microscope (Carl Zeiss MicroImaging, Inc., Germany).

For the non-fluorescence labeling, IHC was performed using a Vectastain avidin-biotin complex elite kit (Vector, Burlingame, CA). Reaction product was detected using 3, 3-diaminobenzidine (DAB) as a chromogen (0.05% diaminobenzidine, 0.01% H_2O_2 in PBS). Peroxidase stained sections were examined under a light microscope (BX51TF; Olympus Optical Co., Japan) equipped with an exposure control unit (PM-CB20) and a camera (CPM-C35DX).

Statistical analysis

Data are expressed as mean±SEM values. One-way ANOVA and Student's *t*-test (SPSS, Chicago, IL) were used for determining statistical significance. Results were considered statistically significantly for $p < 0.05$.

RESULTS

APP-BP1 was up-regulated in Tg2576 mice brains compared to wild-types.

Here, we investigated whether APP-BP1 expression was altered in the brains of Tg2576 mice. We found that according to the increase in age, the protein level of APP-BP1 was increased in wild type and Tg2576 mice. APP-BP1 expression was significantly up-regulated in the hippocampus

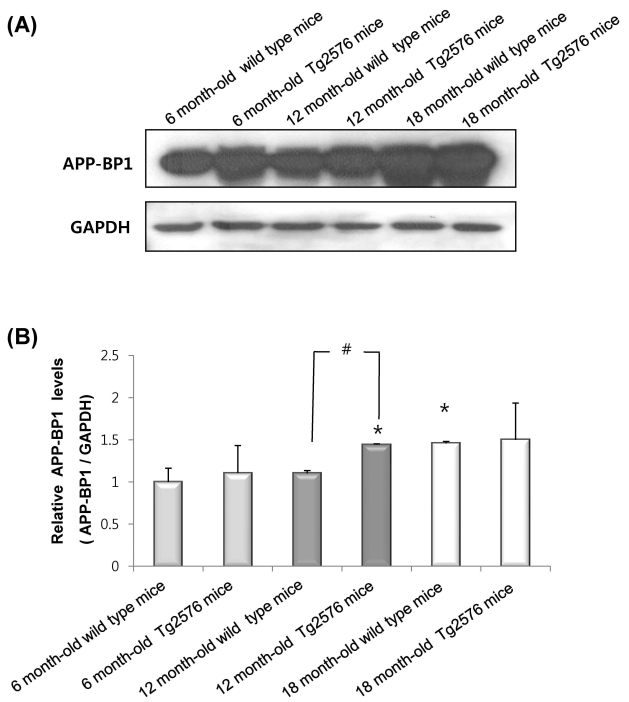

Fig. 1. APP-BP1 protein level was up-regulated in the hippocampus from 12 month-old Tg2576 mice. (A) APP-BP1 protein level was examined by western blotting in the hippocampus from 6-, 12- and 18-month- old Tg 2576 and wild-type mice. This blot is a representative of 4 independent experiments. (B) Densitometric analysis was also performed. Data represent mean ± SEM. $*p < 0.05$ compared with 6 month-old wild type mice (n=4) and $^{\#}p < 0.05$ compared with 12 month-old wild type mice, by one-way ANOVA followed by Duncan's post hoc test.

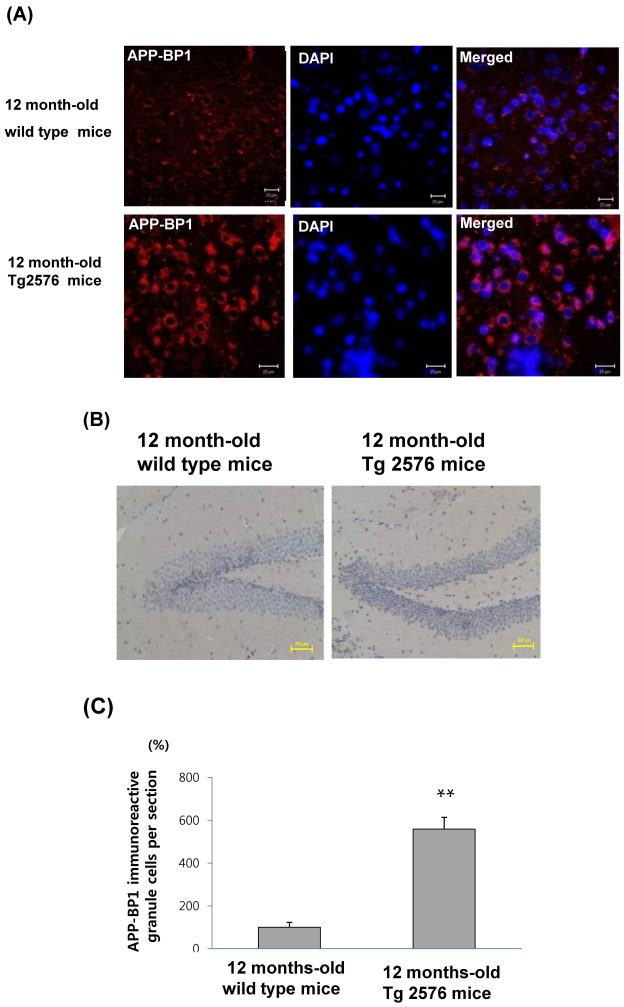

Fig. 2. APP-BP1 immunoreactivities are increased in the cortex and hippocampus from 12-month-old wild-type and Tg2576 mice. The fixed brains from 12-month-old wild type and Tg2576 mice (n=5) in 10% neutral buffered formalin for 48 h were dehydrated and embedded in paraffin. (A) The fluorescent immunohistochemistry was performed in the cortex with the anti-APP-BP1 antibody for 2 h, which was then visualized using a Cy3-conjugated secondary antibody. DAPI counter staining was performed. Images were collected using the LSM 510 program on a Zeiss confocal microscope. This figure is a representative of 5 independent experiments. Scale bars=25 μm. (B) Immunoreactivities of APP-BP1 was examined in the dentate gyrus of 12 month-old wild type and Tg2576 mice. IHC was performed using a Vectastain avidin-biotin complex elite kit. Peroxidase stained sections were examined under a light microscope (BX51TF; Olympus Optical Co., Japan) equipped with an exposure control unit (PM-CB20) and a camera (CPM-C35DX). Scale bars=50μm. (C) Quantative analysis for (B) was performed by calculating APP-BP1 immunoreactive cells per section from 5 independent samples ($**p < 0.01$ by Student's *t*-test).

of 12 month-old Tg 2576 mice compared to that of wild-type mice, whereas there was no difference between wild type and Tg2576 mice at the age of 6 and 18 months (Fig. 1). Further research is required on this more in detail.

Additionally, we confirmed that APP-BP1 levels were increased in the cortex of the 12 month-old Tg 2576 mouse brains compared to the same aged wild-type mice brains by immunohistochemical experiments using fluorescent signals (Fig. 2A).

IHC using DAB staining was employed to examine the expression of APP-BP1 in the hippocampus (dentate gyrus) from 12 month-old wild-type and Tg2576 mice brains. The APP-BP1 immunoreactive granule cells were increased by about 5.5 fold in the brains of Tg2576 mice compared to wild-type brains (Fig. 2B and 2C).

APP-BP1 knockdown reduces Cul-1 neddylation in fetal neural stem cells.

Previously, we found that APP-BP1 modulates cell cycle progression in fetal neural stem cells (*Unpublished finding*). Here, we aimed to investigate whether APP-BP1 is involved in cell cycle progression by inducing SCF complex activation in fetal neural stem cells.

We first tested the knockdown effect of siRNA for APP-BP1 (APP-BP1 siRNA) by comparing them with a non-targeting control siRNA. We found that treatment with the targeted siRNA for 72 h reduced the protein levels of APP-BP1 by about 50% (Fig. 3A). Next, neddylated Cul-1 was examined by immunoprecipitation of Cul-1 followed by western blotting for NEDD8. APP-BP1 knockdown by siRNA treatment was found to reduce neddylation of Cul-1 (Fig. 3B).

With these results, we envision that an overexpression

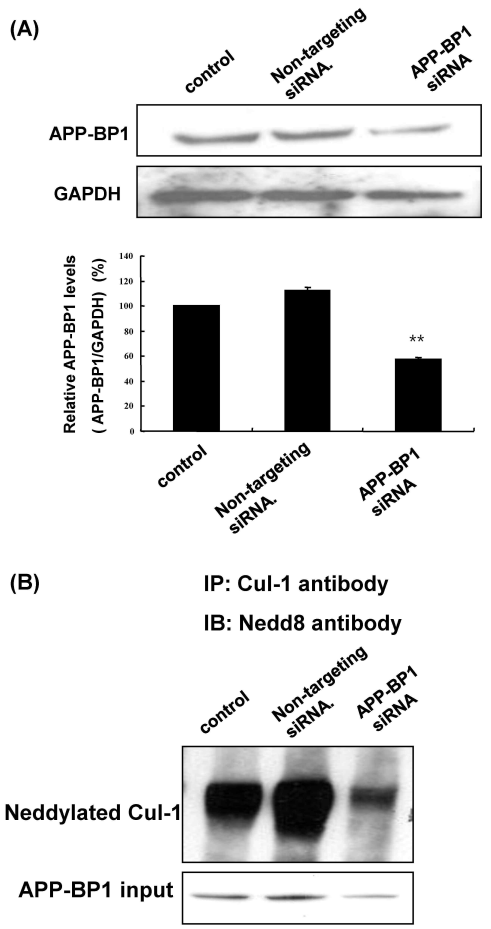

Fig. 3. APP-BP1 knockdown reduced Cul-1 neddylation in fetal neural stem cells. (A) After treatment of fetal neural stem cells with 10 nM non-targeting and APP-BP1 siRNAs for 72 h, APP-BP1 protein levels were examined by western blotting. Densitometric analysis was also performed. Data represent mean±SEM. *$p<0.05$ compared with non-treated cells, by one-way ANOVA followed by Duncan's post hoc test. (B) After treatment with siRNAs in fetal neural stem cells (passage 8) for 72 h, neddylated Cul-1 was examined by immunoprecipitation of Cul-1 followed by western blotting for NEDD8.

of APP-BP1, which is required for cell progression in neural stem cells, in mature neurons could lead post-mitotic neurons to induce apoptosis and contribute to AD pathogenesis.

DISCUSSION

AD is a complex, progressive and irreversible neurodegenerative disease of the brain [30]. It is the leading cause of senile dementia in the U.S.A., where it affects 15% of people over 65 and almost 50% of those over 85 [31]. However, the exact pathogenic mechanism for AD still has not been fully elucidated.

In this study, we investigated the changes in APP-BP1 expression in the brains from Tg 2576 mice.

APP-BP1 is one of several proteins that interact with APP (the precursor protein of Aβ), which is the main component of neuritic plaques formed in the brains of AD patients [3,32]. APP-BP1 was first cloned as an APP-interacting protein in 1996 and has been shown to play a role in activating the neddylation pathway through its binding with Uba3 [22,33,34]. However, APP-BP1 was recently shown to enhance receptor-mediated endocytosis in neurons in a neddylation-independent manner [24].

In this study, we show that APP-BP1 knockdown reduces neddylation of Cul-1 in fetal neural stem cells, suggesting that APP-BP1 is involved in SCF complex activation in fetal neural stem cells (Fig. 3B). The SCF complex serves as an ubiquitin ligase that induces the degradation of several substrates involved in cell cycle progression, which include Wee1, Emi1, p27, cyclin D1, cyclin E, and p27 [19].

Previously, it was demonstrated that APP-BP1 expression was up-regulated in lipid rafts isolated from the hippocampus of AD patients, but not in less affected brain regions [1]. Notably, this result is consistent with our results from Tg2576 mice (Fig. 1 and 2). It is thought that the up-regulation of APP-BP1 which has a role in cell cycle progression, in the brains of Tg2576 mice or AD patients could induce neurons to re-enter into cell cycle. Consequently, it could cause neurodegeneration, contributing to AD pathogenesis.

Over the last decade, cell cycle abnormalities have been suggested as a major neuropathological feature of AD. Several studies have reported increased levels and mislocalization of numerous cell cycle proteins in post-mitotic neurons located in the hippocampal region of postmortem AD brains [32]. The G1, S, G2, and M phases of the cell cycle consist of elaborate feedback mechanisms and regulatory checkpoints that ensure competency [35]. The transition through these phases is controlled by an array of cell cycle proteins, which include the cyclins and the cdks [35,36].

Quiescent cells (i.e., neurons in the adult hippocampus) exist in a nondividing, silent G0 phase. Such cells are terminally differentiated and are generally thought to be incapable of re-entering the cell cycle [35,37].

It has been largely accepted that abnormal cell cycle re-entry of post-mitotic neurons, which do not have the machinery for cell cycle progression, causes apoptosis and ultimately results in progressive neurodegeneration [38,39]. Several findings published recently highlight the similarities between developmental neurogenesis and neurodegeneration in AD. Neuronal populations degenerating in AD (despite their postmitotic status) exhibit phenotypic changes characteristic of cells re-entering the cell division cycle [40-42].

In the present study, we found out that APP-BP1 expression was up-regulated in the hippocampus and cortex from Tg2576 mice brains compared to wild type mice. In addition, it was found that APP-BP1 knockdown significantly reduced Cul-1 neddylation in feral neural stem cells.

APP-BP1 is involved in cell cycle regulation by serving as a bipartite enzyme, which is responsible for SCF complex activation. These findings support the hypothesis that APP-BP1 up-regulation in the brains of Tg2576 mice or AD patients could induce neurons to re-enter the cell cycle.

Based on these results, it was suggested that the up-regulation of APP-BP1 (a protein with a role in cell cycle progression) in AD brains could lead to pathological effects in post-mitotic neurons, thereby contributing to the development of AD.

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