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The intracellular domain of the amyloid precursor protein (AICD) enhances the p53-mediated apoptosis

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Abstract

Amyloid precursor protein (APP)-derived intracellular domain (AICD) has a cytotoxic effect on neuronal cells and also participates in the regulation of gene transactivation. However, the precise molecular mechanisms behind the AICD-mediated apoptosis remain unknown. In this study, we have demonstrated that AICD interacts with p53 and enhances its transcriptional and pro-apoptotic functions. p53 was induced to be accumulated and associated with APP in response to cisplatin. Indeed, APP-C57 was co-immunoprecipitated with the endogenous p53. Enforced expression of APP-C57 or APP-C59 in U2OS cells bearing wild-type p53 led to an increase in number of apoptotic cells, whereas they had undetectable effects on p53-deficient H1299 cells, suggesting that AICD contributes to the activation of the p53-mediated apoptotic pathway. Consistent with this notion, the p53-mediated transcriptional activation and apoptosis were significantly enhanced by co-expression with APP-C57 or APP-C59. Thus, our present results strongly suggest that AICD triggers apoptosis through the p53-dependent mechanisms.

Keywords: AICD; Apoptosis; APP; Fe65; γ-Secretase; p53

Amyloid precursor protein (APP) is a type I transmembrane glycoprotein with a large extracellular domain, a single hydrophobic transmembrane region, and a short cytoplasmic tail [1]. APP is cleaved sequentially by α-, β-, and γ-secretases, which results in the generation of the large soluble NH_2-terminal ectodomain, small hydrophobic extracellular amyloid-β (Aβ, 40- and 42-residues) peptide, and APP intracellular domain (AICD, 57- and 59-residue-long COOH-terminal fragments) [2]. Among them, Aβ has been believed to be one of the major neurodegenerative agents in Alzheimer's disease (AD) [3]. Indeed, Aβ rapidly aggregates into fibrils and the extracellular fibrillar Aβ can promote apoptosis in cultured neurons [4]. Alternatively, AICD has been initially identified in brains of AD patients [5] and AICD itself induced apoptosis in human H4 neuroglioma cells [6]. However, the precise molecular mechanisms by which AICD exerts its pro-apoptotic activity remain to be determined. It has been shown that AICD is stabilized and translocated into the nucleus by collaboration with the adaptor protein Fe65 [6–8], raising a possibility that APP transduces signal through the release and translocation of AICD into nucleus. Intriguingly, Cao and Sudhof found that AICD interacts with Fe65 as well as Tip60 thereby regulating the transcription [9]. In support with this notion, Baek et al. [10] described that the nuclear AICD/Fe65/Tip60 complex can displace N-CoR co-repressor complex and activate the transcription of KAI1 gene. Telese et al. [11] demonstrated that the nucleosome assembly factor SET is required for the nuclear AICD/Fe65/Tip60 complex-mediated transactivation of KAI1 gene. Although these observations suggest that AICD participates in the transcriptional

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regulation in combination with Fe65 and Tip60, the physiological target(s) that is activated by this nuclear complex remains to be identified. As described [12–14], the activation of tumor suppressor p53 might contribute to the genesis of AD and other neurodegenerative diseases of the adult central nervous system, however, the signal(s) responsible for the activation of p53 during this process is not known. Of note, mutant form of APP derived from familial AD (FAD) enhances the p53-dependent transactivation [15,16]. In addition, Legube et al. [17] found that Tip60 associates with p53 and functions as a p53 co-activator. Thus, it is likely that there could exist a functional interaction between APP and p53. In the present study, we found that AICD interacts with p53 and enhances its transcriptional and pro-apoptotic functions, suggesting that AICD-mediated activation of p53 might be one of the cytotoxic mechanisms exerted by AICD in neuronal cells.

Materials and methods

Cell culture and transfection. U2OS and H4 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotic mixture. H1299 and SH-SY5Y cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FBS and antibiotic mixture. Transfection was performed using LipofectAMINE 2000 (Invitrogen).

Immunofluorescence. Cells were fixed in 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. Cells were then incubated with anti-FLAG antibody (M2, Sigma) followed by an incubation with FITC-conjugated secondary antibody (Invitrogen) and observed under Fluoview laser scanning confocal microscope (Olympus).

Immunoblotting. Lysates were subjected to SDS–PAGE and transferred onto Immobilon-P membranes (Millipore). Membranes were incubated with anti-p53 (DO-1, Oncogene Research Products), anti-$p21^{WAF1}$ (Ab-1, Oncogene Research Products), anti-phosphorylated from of p53 at Ser-15 (Cell Signaling), anti-APP, or anti-actin antibody (20–33, Sigma), and developed with an ECL system (Amersham Biosciences).

Immunoprecipitation. Precleared lysates were incubated with the indicated antibodies followed by incubation with protein G–Sepharose beads. Immune complexes were washed with lysis buffer, eluted in 2× SDS-sample buffer, and separated by SDS–PAGE. Gels were transferred onto Immobilon-P membranes, and immunoblotted.

Cell fractionation. Cells were lysed in lysis buffer containing 10 mM Tris–Cl, pH 7.5, 1 mM EDTA, 0.5% NP-40, and protease inhibitor cocktail. Lysates were centrifuged to separate soluble (cytoplasmic) from insoluble (nuclear) fraction. Nuclear and cytoplasmic fractions were analyzed by immunoblotting with anti-Lamin B (Ab-1, Oncogene Research Products), or anti-tubulin α antibody (Ab-2, NeoMarkers), respectively.

Cell viability assay. Cells were transferred to fresh medium containing cisplatin, incubated for 24 h, and 10 μl MTT solution was added to each well. After 1 h of incubation at 37 °C, absorbance readings for each well were performed at 570 nm using the microplate reader (Model 450, Bio-Rad).

Apoptosis assay. U2OS cells were transfected with GFP expression plasmid together with expression plasmid for APP-C57-FLAG or APP-C59-FLAG. Forty-eight hours after transfection, transfected cells were identified by the presence of green fluorescence. Cell nucleus was stained with DAPI.

Luciferase reporter assay. U2OS cells were transfected with p53-responsive luciferase reporter ($p21^{WAF1}$ or MDM2), pRL-TK *Renilla* luciferase cDNA, and p53 expression plasmid along with or without the increasing amounts of expression plasmid for APP-C57-FLAG or APP-C59-FLAG, and subjected to dual-luciferase assay (Promega).

Colony formation assay. H1299 cells were transfected with empty plasmid, p53 expression plasmid, or with p53 expression plasmid plus expression plasmid for APP-C57-FLAG or APP-C59-FLAG. Forty-eight hours after transfection, cells were transferred to the fresh medium containing G418 (400 μg/ml). After 14 days of selection, the plates were stained with Giemsa's solution.

Results

APP is associated with endogenous p53

To test whether there could exist an interaction between APP and p53 during the neuronal apoptosis, human neuroblastoma SH-SY5Y cells bearing wild-type p53 were exposed to cisplatin (CDDP). In accordance with the previous observations [18], SH-SY5Y cells underwent apoptosis in response to CDDP (data not shown). Next, we examined the protein levels of p53 and APP during the CDDP-mediated apoptosis. As shown in Fig. 1, p53 was induced in cells exposed to CDDP in association with an up-regulation of $p21^{WAF1}$ which is one of the p53-targets. In addition, a remarkable phosphorylation of p53 at Ser-15 was detected in response to CDDP. Protein levels of APP remained unchanged regardless of CDDP treatment. Of note, the immunoprecipitation of cell lysates with anti-APP antibody which recognizes the extreme COOH-terminal region of APP [19] resulted in a co-immunoprecipitation of p53 with APP. These observations suggest that APP and/or APP intracellular domain (AICD) might be associated with endogenous p53.

Interaction between AICD and p53

To examine whether AICD could interact with p53, we generated expression plasmids encoding APP-C57 and APP-C59 tagged with FLAG peptide on their

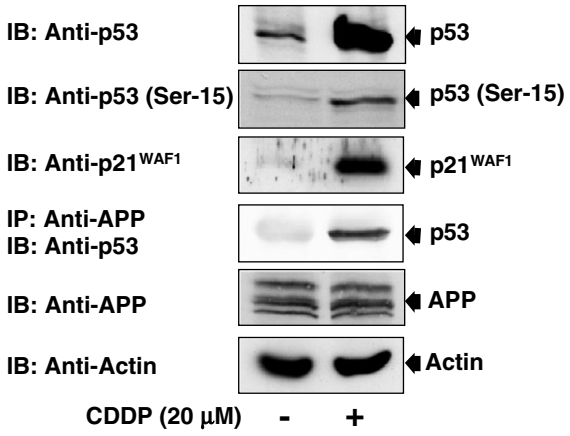

Fig. 1. Interaction between APP and endogenous p53. SH-SY5Y cells were treated with CDDP (20 μM) or left untreated. Twenty-four hours after CDDP treatment, cell lysates were subjected to immunoblotting with the indicated antibodies. Immunoblotting for actin is shown as a control for protein loading. For immunoprecipitation, equal amounts of cell lysates (1 mg of protein) were immunoprecipitated with anti-APP antibody and the immunoprecipitates were processed for immunoblotting with anti-p53 antibody.

COOH-termini (APP-C57-FLAG and APP-C59-FLAG, respectively). Human neuroglioma H4 cells were transfected with APP-C57-FLAG or APP-C59-FLAG expression plasmid and transfected cells were fixed followed by staining with anti-FLAG antibody. Consistent with the previous reports [6,8,20], APP-C57-FLAG and APP-C59-FLAG were detected both in cytoplasm and nucleus (Fig. 2A). Similar results were also obtained in immunoblotting using cytoplasmic and nuclear fractions prepared from H4 cells expressing APP-C57-FLAG or APP-C59-FLAG (Fig. 2B).

To verify the interaction between AICD and p53, cell lysates prepared from H4 cells co-transfected with expression plasmids for APP-C57-FLAG and p53 were immunoprecipitated with the normal mouse serum (NMS) or anti-p53 antibody followed by immunoblotting with anti-APP or anti-p53 antibody. As shown in Fig. 2C, APP-C57-FLAG was efficiently co-immunoprecipitated with p53. Similar to SH-SY5Y cells, H4 cells underwent apoptosis in response to CDDP in association with a significant induction of p53 (data not shown). To confirm the interaction between AICD and the endogenous p53, H4

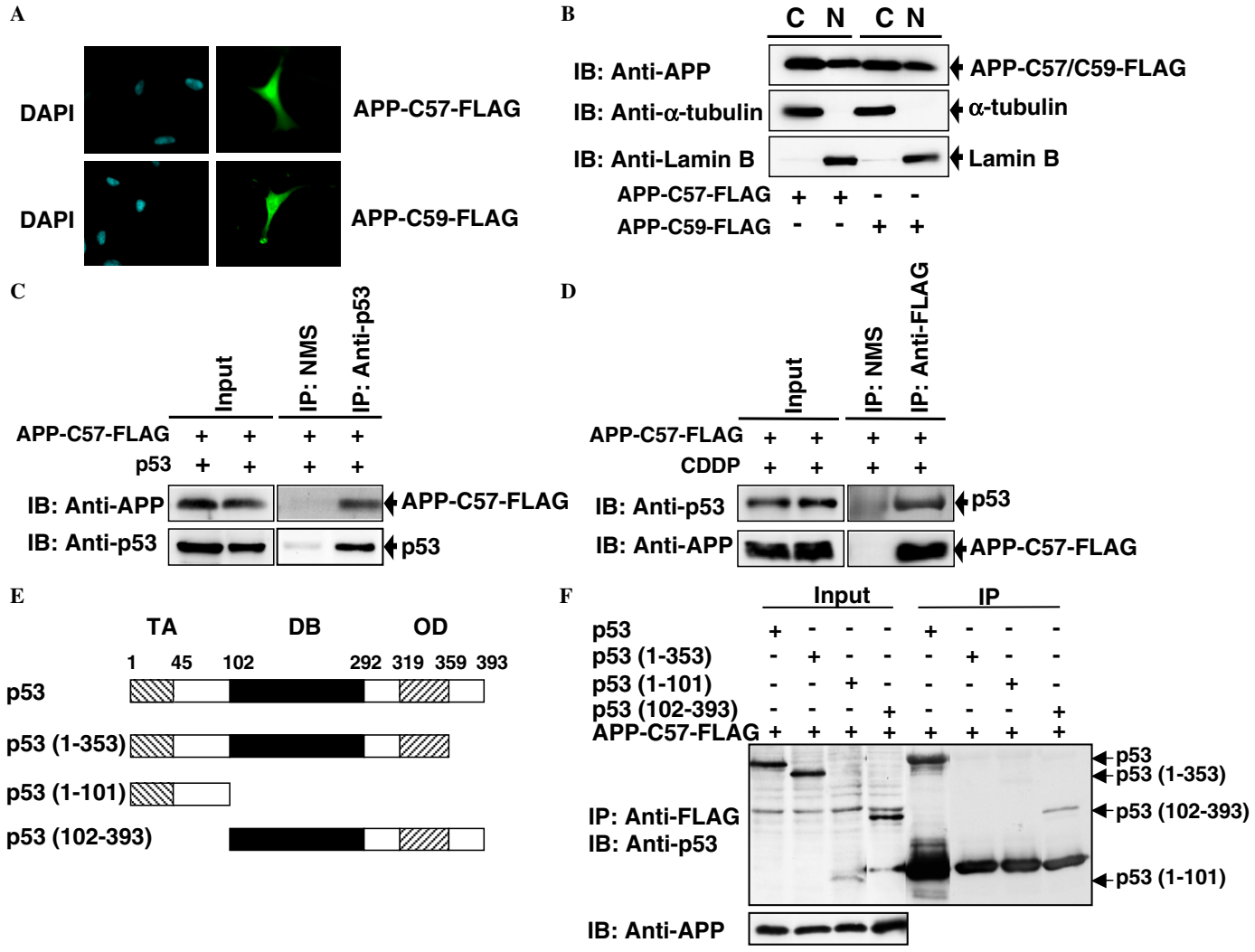

Fig. 2. Complex formation between nuclear AICD and p53 in cells. (A,B) Subcellular localization of AICD. H4 cells were transfected with the expression plasmid for APP-C57-FLAG or APP-C59-FLAG. Forty-eight hours after transfection, cells were fixed and incubated with anti-FLAG antibody. Cell nuclei were stained with DAPI (A). For subcellular fractionation, H4 cells were transfected as in (A). Transfected cells were fractionated into cytoplasmic (C) and nuclear (N) fractions. Each fraction was subjected to immunoblotting with anti-APP antibody. α-Tubulin and lamin B were used as cytoplasmic and nuclear markers, respectively. (C,D) AICD interacts with p53. H4 cells were co-transfected with expression plasmids encoding APP-C57-FLAG plus p53. Forty-eight hours after transfection, cell lysates were immunoprecipitated with normal mouse serum (NMS) or with anti-p53 antibody followed by immunoblotting with anti-APP or with anti-p53 antibody (C). For the interaction of AICD with the endogenous p53, H4 cells were transfected with the expression plasmid for APP-C57-FLAG. Twenty-four hours after transfection, cells were exposed to CDDP (20 μM) for 24 h. Cell lysates were subjected to immunoprecipitation with NMS or anti-FLAG antibody. The immunoprecipitates were analyzed by immunoblotting with anti-p53 or anti-APP antibody (D). (E,F) The COOH-terminal region of p53 is required for the interaction with AICD. H1299 cells were co-transfected with the expression plasmid for APP-C57-FLAG together with the indicated expression plasmids for p53 deletion mutants (E). Cell lysates were subjected to the immunoprecipitation with anti-APP and immunoblotted with anti-p53 antibody (F).

cells were transfected with the APP-C57-FLAG expression plasmid followed by exposure to CDDP for 24 h. As seen in Fig. 2D, the endogenous p53 was co-immunoprecipitated with APP-C57-FLAG. APP-C59-FLAG was also co-immunoprecipitated with p53 (data not shown). To identify the region(s) of p53 required for the interaction with AICD, p53-deficient H1299 cells were co-transfected with the APP-C57-FLAG expression plasmid together with the indicated expression plasmids for p53 deletion mutants (Fig. 2E). Immunoprecipitation demonstrated that APP-C57-FLAG binds to the COOH-terminal region of p53 (Fig. 2F). Thus, it is likely that nuclear AICD can interact with p53 and might modulate its function.

AICD promotes apoptosis in a p53-dependent manner

To examine whether the AICD-mediated apoptosis could be dependent on p53, H1299 cells were co-transfected with the constant amount of GFP expression plasmid together with the expression plasmid for p53, APP-C57-FLAG or APP-C59-FLAG. Forty-eight hours after transfection, transfected cells were identified by the presence of green fluorescence and the number of GFP-positive cells with apoptotic nuclei was scored. Consistent with the previous observations [21], enforced expression of p53 led to a significant induction of apoptosis (Fig. 3A). In contrast, APP-C57-FLAG and APP-C59-FLAG did not promote apoptosis. The ectopically expressed APP-C57-FLAG and APP-C59-FLAG induced apoptosis in U2OS cells (Fig. 3B). Since U2OS cells carry wild-type p53, our present results showed a good correlation between an ability of AICD to induce apoptosis and the p53 status.

AICD enhances the transcriptional and pro-apoptotic activities of p53

To address whether AICD could enhance the transcriptional activity of p53, U2OS cells were co-transfected with the constant amount of p53 expression plasmid and the luciferase reporter construct containing p53-responsive promoter derived from $p21^{WAF1}$ or MDM2 gene together with or without the increasing amounts of the expression plasmid for APP-C57-FLAG or APP-C59-FLAG. As shown in Fig. 4A, APP-C57-FLAG enhanced the p53-mediated transcriptional activity toward $p21^{WAF1}$ and MDM2 promoters in a dose-dependent manner. Similar results were also obtained in cells expressing APP-C59-FLAG (Fig. 4B).

Next, we examined a possible effect of AICD on the pro-apoptotic activity of p53. H1299 cells were transfected with the expression plasmid for p53, APP-C57-FLAG or APP-C59-FLAG. Following selection in G418, there was a drastic reduction of colony formation after transfection with p53 expression plasmid as compared with the empty plasmid, whereas APP-C57-FLAG or APP-C59-FLAG alone had undetectable effects (Fig. 4C). Intriguingly, co-expression of p53 with APP-C57-FLAG or APP-C59-FLAG reduced the colony formation as compared with p53 alone (Fig. 4D and E). Taken together, our present findings strongly suggest that AICD transduces apoptotic signals from cell surface to cell nucleus and might act as a co-activator of p53.

Discussion

AICD modulates gene transactivation in collaboration with Fe65 and Tip60 [9–11] and induces apoptosis in certain cells, which is dependent on its nuclear access [6]. Thus, it is likely that AICD has a potential role in transducing an apoptotic signal from cell surface to the nucleus, however, the detailed molecular mechanisms behind the AICD-mediated apoptotic response remain to be determined. In this study, we demonstrated that AICD interacts with p53 and enhances its transcriptional and pro-apoptotic functions. Our present findings provide a novel insight into understanding how γ-secretase cleavage of APP could lead to the neurodegeneration.

Cytosolic AICD has a short half-life and its stability is enhanced through the interaction with Fe65 [7,8]. Fe65 is an adaptor protein containing a central WW domain and two COOH-terminal phosphotyrosine-binding domains (PTB1 and PTB2) [22], and has a transactivation potential

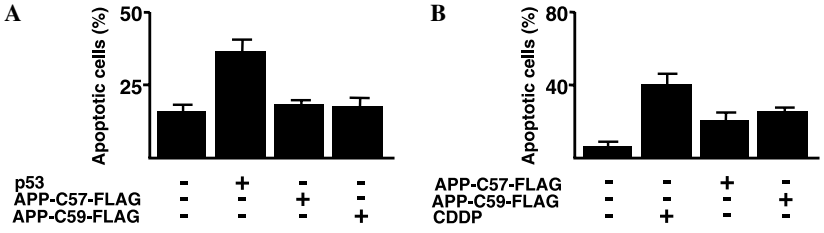

Fig. 3. AICD induces apoptosis in a p53-dependent manner. (A) AICD has undetectable effect on H1299 cells. H1299 cells were co-transfected with the constant amount of GFP expression plasmid (200 ng) together with the empty plasmid (800 ng), expression plasmid for p53 (200 ng), APP-C57-FLAG (800 ng) or APP-C59-FLAG (800 ng). Forty-eight hours after transfection, transfected cells were identified by the presence of green fluorescence. Cell nuclei were stained with DAPI to reveal nuclear condensation and fragmentation. The number of GFP-positive cells with apoptotic nuclei was scored. (B) AICD induces apoptosis in U2OS cells. U2OS cells were co-transfected with the constant amount of GFP expression plasmid (200 ng) along with the empty plasmid (800 ng), 800 ng of the expression plasmid encoding APP-C57-FLAG or APP-C59-FLAG. Twenty-four hours after transfection, cells were treated with 20 μM of CDDP for 24 h or left untreated and then the number of apoptotic cells was measured as in (A).

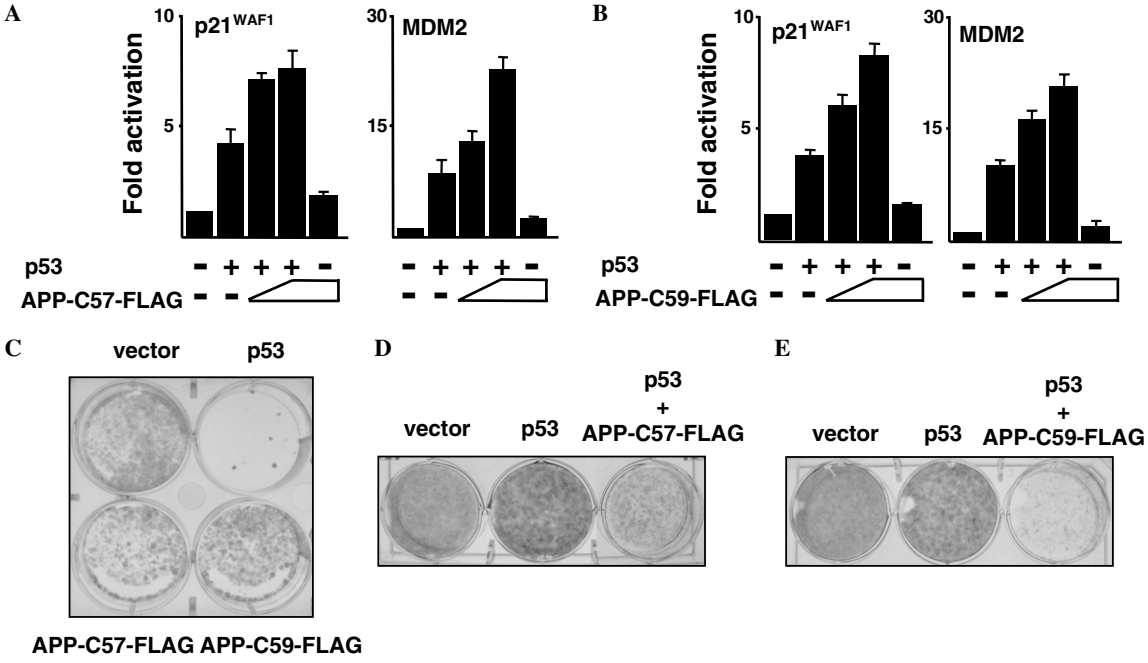

Fig. 4. AICD enhances the p53-mediated transcriptional and pro-apoptotic activities. (A,B) Luciferase reporter assay. U2OS cells were co-transfected with the constant amount of the expression plasmid for p53 (25 ng), p53-responsive luciferase reporter construct carrying the $p21^{WAF1}$ or MDM2 promoter (100 ng), *Renilla* luciferase cDNA (10 ng) together with or without the increasing amounts of the expression plasmid for APP-C57-FLAG (100 and 200 ng) (A) or APP-C59-FLAG (100 and 200 ng) (B). Forty-eight hours after transfection, cells were lysed and their luciferase activities were analyzed. Results are shown as fold-induction of the firefly luciferase activity compared with control cells. (C–E) Colony formation assay. H1299 cells were transfected with pcDNA3 (1 μg), the expression plasmid for p53 (200 ng), APP-C57-FLAG (800 ng) or APP-C59-FLAG (800 ng). Total amount of the expression plasmids was kept constant (1 μg) with pcDNA3. At 48 h post-transfection, cells were maintained in the culture medium containing G418 (400 μg/ml). After 2 weeks of selection, the plates were stained with Giemsa's solution (C). To examine the effect of APP-C57 or APP-C59 on p53, H1299 cells were transfected with the constant amount of the expression plasmid for p53 (50 ng) together with or without the expression plasmid encoding APP-C57-FLAG (400 ng) (D) or APP-C59-FLAG (400 ng) (E). Forty-eight hours after transfection, cells were kept in the medium containing G418 for 2 weeks and surviving colonies were stained as described in (C).

depending on its WW domain [23]. Tip60 with a histone acetyltransfecrase activity acts as a co-activator for AICD/Fe65 complex [9]. Tip60 alone has no transactivation function [9]. Additionally, Tip60 is part of a large nuclear protein complex, which possesses a DNA-binding activity [24]. To understand the molecular mechanisms underlying the nuclear AICD/Fe65/Tip60-mediated transcriptional regulation, it is necessary to identify nuclear protein(s) with a sequence-specific DNA-binding activity. According to our present results, AICD interacts with p53 and enhances its transcriptional activity, suggesting that p53 is one of the sequence-specific transcription factors in the nuclear AICD/Fe65/Tip60 complex. Of note, Legube et al. [17] described that Tip60 is associated with p53 and functions as a co-activator for p53. Deletion analysis revealed that AICD binds to the COOH-terminal region of p53. Since the p53 COOH-terminal region has an inhibitory effect on its DNA-binding activity, it is possible that AICD reduces its inhibitory effect.

Alternatively, Kim et al. [25] reported that AICD forms a complex with Fe65 and CP2/LSF/LBP1 family, and induces the expression of glycogen synthase kinase 3β (GSK3β). According to their results, AICD-mediated up-regulation of GSK3β led to neuronal apoptosis. Intriguingly, Watcharasit et al. [26] demonstrated that nuclear GSK3β interacts with p53 and promotes its apoptotic response. Therefore, it is possible that AICD contributes to the formation of p53/GSK3β complex, thereby enhancing the p53-mediated pro-apoptotic activity. Additionally, c-Abl binds to Fe65 and stimulates the AICD/Fe65-mediated transactivation [27]. Considering that c-Abl enhances the transcriptional activity of p53 [28], it is likely that c-Abl might be involved in the AICD-dependent activation of p53.

Recently, Esposito et al. [16] reported that the inhibition of β-secretase cleavage of FAD-linked APP mutant significantly reduces the p53-mediated transcriptional activation. They also described that the treatment of FAD-associated APP mutant-expressing cells with γ-secretase inhibitor can confer resistance to apoptotic stimuli. APP missense mutations found in FAD led to an increased production of Aβ42, which might be due to the increased cleavage of APP by γ-secretase [29]. The accumulation of Aβ42 caused the neuronal apoptosis and this process was mediated through the activation of p53/Bax cell death pathway [30], suggesting that there could exist a functional interaction between intracellular Aβ42 and proximal effector(s) of this pathway. However, the precise molecular mechanisms by which p53 is activated by intracellular Aβ42 remain to be clarified. During the preparation of our

manuscript, Alves da Costa et al. [31] described that AICD enhances the transcriptional activity of p53 through the up-regulation of p53 at mRNA level. Under our experimental conditions, AICD had negligible effects on the mRNA level of p53 as examined by RT-PCR (data not shown). It might be due to the different cell systems. Collectively, it is likely that the intracellular Aβ and/or nuclear AICD might induce neuronal apoptosis at least in part through the activation of the p53-dependent pro-apoptotic pathway.

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