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Research Article

Stimulation of p53 Transactivation Ability by Nicastrin in Mouse Fibroblasts

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Nicastrin (NCSTN), a component of the γ-secretase complex, is involved in the p53-dependent apoptosis of neurons, although its mechanism remains unclear. We analyzed the effects of NCSTN transfection on the transactivity of p53 in ras-NIH3T3 mouse fibroblasts with a luciferase assay. Luciferase activity was elevated after transfection, suggesting the stimulation of p53 transactivation ability. In addition, the protein levels of endogenous mouse p53 and transfected human p53 increased. The effects of NCSTN appeared to be independent of γ-secretase activity because it was not inhibited by the γ-secretase inhibitor DAPT. The functional domains of NCSTN were further examined with NCSTN deletion mutants. Activation of the p53-responsive promoter was completely diminished in a NCSTN mutant lacking the amino acid residues between 306 and 360. Since this domain is a γ-secretase-substrate-recognition site, the activation of p53 by NCSTN may be mediated by γ-secretase-substrate-like molecules.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that causes a progressive dementia. The pathological feature of AD is the accumulation of senile plaques composed of extracellular amyloid-β (Aβ) peptides in the cerebral cortex. The senile plaques are believed to result in the apoptosis and synaptic dysfunction of neurons resulting in AD [1]. Aβ is generated from amyloid precursor protein (APP) by two distinct steps of proteolytic processing. APP is first cleaved by a β-site APP cleaving enzyme, β-secretase, and subsequently by γ-secretase within its transmembrane domain [2]. γ-secretase activity resides in a high molecular weight multimeric protein complex composed of at least four core components, presenilin, nicastrin (NCSTN), anterior pharynx defective-1 (Aph-1) and presenilin enhancer-2 (Pen-2) [3]. In the active γ-secretase complex, presenilin exists as a heterodimer of the N- and C-terminal fragments generated by endoproteolysis [4]. Aph-1 is implicated in the assembly of γ-secretase complex as a scaffold protein [5], and the N-terminal domain of Pen-2 regulates the endoproteolysis in the γ-secretase complex [6]. NCSTN is a type-I membrane protein and its amino acid residues 261–502 in the ectodomain show sequence similarity to a peptidase family that includes aminopeptidases, carboxypeptidases, and transferrin receptor proteins. Amino acid residues 306–360 which span the most conserved sequence amongst nicastrin orthologs are indispensable for its function as a γ-secretase-substrate receptor [7]. Although APP, Notch, and several other transmembrane proteins undergo regulated intramembranous cleavage by γ-secretase, the substrate specificity remains unclear [6, 8–10].

The tumor suppressor protein p53, through its elevation and posttranslational modification in response to various types of cellular stresses, induces either cell cycle arrest by transactivating p21 or apoptosis by transactivating Bax, Noxa and PUMA [11–16]. It was reported that p53 is overexpressed in the brains of patients with AD, and that intracellular Aβ42 stimulated p53 expression [17, 18]. Therefore, p53 may be involved in neuronal cell apoptosis

in the brain of patients with AD. In the present study, we show that NCSTN stimulated p53 transactivation ability independently of γ-secretase catalytic activity.

2. Materials and Methods

2.1. Plasmids. Eukaryotic expression vector pME18S-FL3 containing human NCSTN, Aph-1, Pen-2 or presenilin 1 cDNA was purchased from Toyobo (Osaka, Japan). pcDNA3-FLAG-APP$_{695}$ [19] was provided by Dr. Toshiharu Suzuki (Hokkaido University, Japan). pCMV-p53 [20] and reporter plasmids, pG13-Luc [21], pG15-Luc [21] and pBV-PUMA Frag2-Luc [16], were provided by Dr. Bert Vogelstein (Howard Hughes Medical Institute). pCS2$^+$-Notch-ΔE [22] was provided by Dr. Tasuku Honjo (Kyoto University). pTP1-Luc [23] was provided by Dr. Lothar J. Strobl (GSF National Research Center). pGL3-Bax-Luc and pGL3-p21-Luc [24] were provided by Dr. Mian Wu (University of Science and Technology of China). pGV-B2 Noxa-Luc [15] was provided by Dr. Nobuyuki Tanaka (Nippon Medical School). pKM2L-phP53-Luc was provided by Riken BRC (Tsukuba, Ibaraki, Japan). Control reporter plasmids such as pRL-CMV and SV40-Rluc were purchased from Promega (Madison, WI). pcDNA3.1-Rho GDIα was purchased from Guthrie cDNA Resource Center (Sayre, PA). cDNA for the constitutively active catalytic subunit of cAMP-dependent protein kinase (PKA-CS) [25] was provided by Dr. G. Stanley McKnight (University of Washington).

2.2. Reagents. Dibutyl cAMP (DBcAMP), IBMX and forskolin were purchased from Sigma (St. Louis, MO). γ-Secretase inhibitors, DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester) [26] and L-685,458 [27] were purchased from Biomol International (Plymouth Meeting, PA) and Peptide Institute (Osaka, Japan), respectively.

2.3. Cell Culture and Luciferase Assay. Mouse ras-NIH3T3 fibroblasts [28] were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5% bovine serum (Bio West, Nuaille, France) and 100 μg/mL kanamycin as described [29]. Human gastric carcinoma cell line MKN45 was cultured in DMEM supplemented with 10% fetal bovine serum.

The cells seeded on 24-well plates were then transfected with test genes together with firefly luciferase and *Renilla* luciferase reporter plasmids by using LipofectAMINE-Plus (Invitrogen, San Diego, CA). Two days after the transfection, firefly and *Renilla* luciferase activities were determined with a Dual Luciferase Assay System (Promega) and a luminescencer (Atto, Tokyo, Japan). Firefly luciferase activities were normalized with the *Renilla* luciferase control activities.

2.4. Western Blot Analysis. ras-NIH3T3 cells were transfected with expression plasmids and cultured for two days. Cell extracts were prepared by treatment with 0.5% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, 50 μM antipain, 50 μM pepstatin A, and 50 μM ALLN for 10 minutes at 4°C [30]. The cell lysate was centrifuged at 13 000 g for 10 minutes and the supernatant (cytoplasmic fraction) was used for detection of NCSTN, presenilin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The pellet (nuclear fraction) was used for detection of p53 as described previously [31]. Proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. The filters were blocked using 0.5% nonfat dry milk and were subsequently probed with primary antibodies such as anti-NCSTN (N-19; Santa Cruz Biotechnology, Santa Cruz, CA), anti-presenilin (American Research Products, Belmont, MA), anti-mouse/human p53 (DO-1; Santa Cruz Biotechnology), anti-human p53 (Bp53-12; Santa Cruz Biotechnology), anti-phospho-p53 (Ser-6) (Cell Signaling, Danvers, MA), anti-phospho-p53 (Ser-9) (Cell Signaling), anti-phospho-p53 (Ser-15) (Cell Signaling), anti-phospho-p53 (Thr-18) (Santa Cruz Biotechnology), anti-phospho-p53 (Ser-20) (Santa Cruz Biotechnology), anti-phospho-p53 (Ser-37) (Cell Signaling), anti-phospho-p53 (Ser-315) (Medical & Biological Laboratories, Nagoya, Japan), anti-phospho-p53 (Ser-392) (Cell Signaling), and anti-GAPDH (FL-335; Santa Cruz Biotechnology). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies such as donkey anti-goat IgG, donkey anti-mouse IgG and goat anti-rabbit IgG (Santa Cruz Biotechnology) followed by treatment with a Immobilon Western chemiluminescent substrate (Millipore Corporation, Billerica, MA). The bands were quantified by CS Analyzer version 3.0 for Windows (Atto).

2.5. Reverse Transcription-PCR (RT-PCR). Total cellular RNA was isolated from cultured cells using the Aqua-Pure RNA Isolation kit (Bio-Rad, Hercules, CA). Reverse transcription was performed with an oligo $(dT)_{20}$ primer using the ThermoScript RT-PCR System (Invitrogen). PCR amplification using the following primers: p53-841F, 5′-GCAACTATGGCTTCCACCTG-3′; p53-1040R, 5′-CTC-CGTCATGTGCTGTGACT-3′; β-actin-684F, 5′-ACCACA-GCTGAGAGGGAAATC-3′; and β-actin-976R, 5′-AGC-ACTGTGTTGGCATAGAGG-3′. PCR amplification using the p53 primers was performed using KOD-Plus-DNA polymerase (Toyobo) as follows: an initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation step at 94°C for 30 seconds, annealing at 61°C for 30 seconds, and extension at 68°C for 30 seconds with a final extension at 68°C for 5 minutes. PCR amplification using the β-actin primers was as follows: an initial denaturation at 94°C for 3 minutes, followed by 20 cycles of denaturation step at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 30 seconds with a final extension at 68°C for 5 minutes.

2.6. Construction of the NCSTN Deletion Mutants. NCSTN mutants (NCSTN Δ195–305, NCSTN Δ306–360, NCSTN Δ361–418, NCSTN Δ419–458 and NCSTN Δ625–662) were constructed from NCSTN cDNA using KOD-Plus-Mutagenesis kit (Toyobo) and following primers: hNC-STN-Δ195–305-F, 5′-CTGGCTGCTGCTGAAGCTTTG-3′;

hNCSTN-Δ195–305-R, 5′-CTGCTTGATGACTTTGGTTTC-3′; hNCSTN-Δ306–360-F, 5′-TCATTTGTGGAGCTGGGACAGGT-3′; hNCSTN-Δ306–360-R, 5′-CTGGGTGACAAAGGAAGCCACTG-3′; hNCSTN-Δ361–418-F, 5′-TCCCAGCCTCTCCCACCATCTTC-3′; hNCSTN-Δ361–418-R, 5′-GTCAACATTCTCTAACTGCACGG-3′; hNCSTN-Δ419–458-F, 5′-ACTGCTGAGAACATTAATGTGAG-3′; hNCSTN-Δ419–458-R, 5′-CTGATTTGGCCTCCTGAGGATGA-3′; hNCSTN-Δ625–662-F, 5′-ATCGCCAGCAAAGAGCTTGAGTT-3′; and hNCSTN-Δ625–662-R, 5′-AGTAGAACGCACACACCGGGGGA-3′. Deletions were confirmed by DNA sequencing.

3. Results

3.1. Stimulation of p53 Transactivation Ability by NCSTN. Because p53 is overexpressed in the brains of patients with AD [17], we examined the effects of APP and γ-secretase components on the transactivation ability of p53 in ras-NIH3T3 mouse fibroblasts with a luciferase assay using p53-responsive reporter plasmid pG13-Luc. Luciferase activity was stimulated considerably by NCSTN and slightly by Aph-1 and Pen-2 (Figure 1(a)). Presenilin and APP did not increase the luciferase activity significantly. Co-transfection with two of these γ-secretase components showed additive but not synergistic stimulation of the luciferase activity (data not shown). NCSTN activated the p53-responsive promoter in a dose dependent manner not only in ras-NIH3T3 cells but also in MKN45 human gastric carcinoma cells (Figures 1(b) and 1(c)). As compared to pG13-Luc, control pG15-Luc that contained mutations in the p53-responsive consensus sequence [21] showed much lower (less than 1%) luciferase activity (data not shown), indicating that most, if not all, of the observed luciferase activity derived from pG13-Luc is p53 dependent. These results suggested that the transactivation ability of p53 is stimulated by forced expression of NCSTN.

Then, we analyzed the protein expression levels in transfected mouse ras-NIH3T3 cells by Western blot analysis. As a control, presenilin-transfected cells as well as the empty-vector-transfected cells were also examined. The protein levels of NCSTN were much higher in NCSTN expression plasmid-transfected cells as compared with control plasmid- or presenilin expression plasmid-transfected cells (Figure 1(d)). The expression of presenilin was observed exclusively in presenilin-transfected cells. The levels of endogenous mouse p53 increased with transfection of NCSTN expression plasmid (Figures 1(d) and 1(e)).

When human p53 cDNA was cotransfected, the protein expression levels of human p53 were also elevated by NCSTN (Figures 1(f) and 1(g)). Thus, the elevated expression levels of p53 protein may account for the activation of p53 reporter by NCSTN. Analysis using anti-p53 phospho-specific antibodies revealed that NCSTN increased phosphorylation levels at multiple sites such as Ser-6, Ser-9, Ser-315 and Ser-392. On the other hand, presenilin stimulated the phosphorylation only at Ser-392 but decreased the levels at multiple sites.

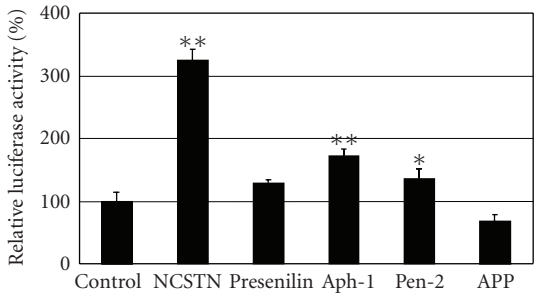
We also analyzed the mRNA levels of p53 in the NCSTN-expressing cells by RT-PCR. The levels of p53 mRNA in NCSTN-expressing cells were similar or slightly lower as compared to those of control plasmid-transfected cells (Figures 1(h) and 1(i)). The effects of NCSTN on the transcription of the p53 gene were also examined with a luciferase assay using a reporter plasmid containing the promoter region of the p53 gene. Transfection with NCSTN cDNA did not increase but rather decreased luciferase activity (Figure 1(j)). These results suggest that the elevated p53 protein levels in NCSTN-expressing cells were not attributed to the increased transcription of the p53 gene but were caused by posttranscriptional regulation.

The target specificity of p53 was examined with reporter plasmids containing p53-responsive sequences in the promoter region of p21, Bax, Noxa and PUMA. Bax and Noxa promoters were significantly activated by transfection with NCSTN cDNA whereas the increases of gene expression via p21 and PUMA promoters were only marginal and under the detectable level, respectively (Figure 1(k)).

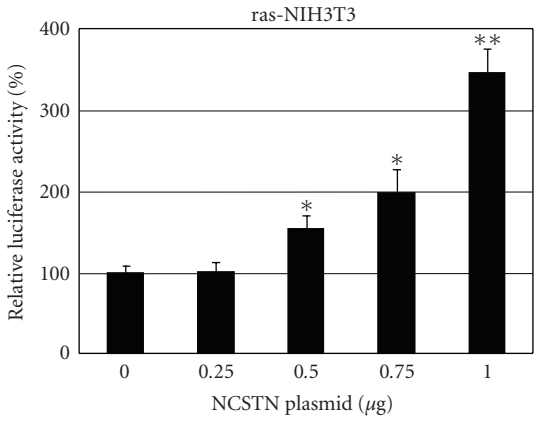
3.2. γ-Secretase-Independent Activation of p53 by NCSTN. Notch is sequentially cleaved by TNFα-converting enzyme (TACE) and γ-secretase to produce Notch intracellular region (Notch-IC) which functions as a transcription factor [9]. The transactivation ability of Notch-IC can be estimated by luciferase assay using the pTP1-Luc reporter plasmid which contains the Notch-binding domains [23]. The luciferase activity was markedly elevated by transfection with the positive control Notch-ΔE (Figure 2(a)) which lacks the TACE-eliminated domain and can, therefore, be activated only by γ-secretase [22]. The expression of Notch-ΔE increased the reporter luciferase activity 100-fold, whereas without Notch-ΔE, the reporter activity was only marginal irrespective of the enforced expression of NCSTN (Figure 2(a)), suggesting that the activation of endogenous Notch by NCSTN was only limited. In the presence of Notch-ΔE, NCSTN caused no further increase of the reporter activity. Thus, the enforced expression of NCSTN may not increase the endogenous γ-secretase activity. This also implies that the activation of p53 by NCSTN is not mediated by Notch.

We then examined whether the γ-secretase activity is necessary for the activation of p53 by NCSTN by using DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester), which is a γ-secretase-specific inhibitor [26]. DAPT inhibited Notch-ΔE-mediated expression of Notch reporter pTP1-Luc dose dependently (Figure 2(b)). This indicated that the γ-secretase activity was sufficiently inhibited by DAPT at concentrations higher than 25 μM. Then, we examined whether the activation of p53 by NCSTN was suppressed by 25 μM DAPT. No apparent suppression by DAPT was observed for the activation of p53-reporter pG13-Luc (Figure 2(c)). Therefore, the activation of p53 by NCSTN may be independent of the catalytic activity of γ-secretase.

3.3. Analysis of Functional Domains in NCSTN. Next, we analyzed the functional domains by preparing NCSTN deletion mutants which lacked the residues 195–305, 306–360, 361–418, 419–458 or 626–662 in the NCSTN ectodomain



(a)



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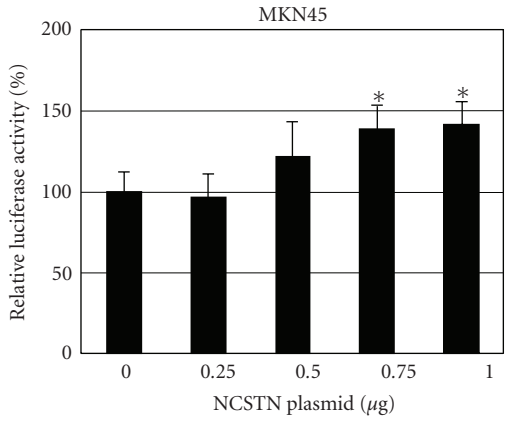

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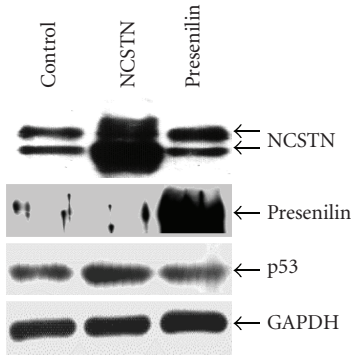

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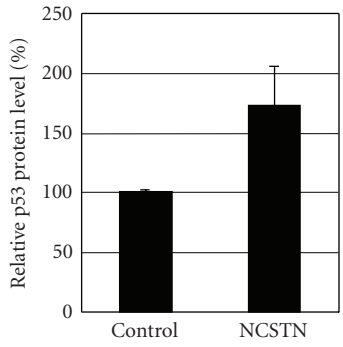

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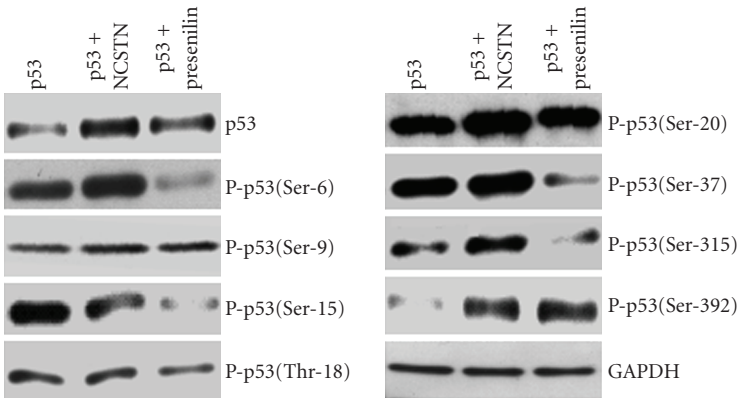
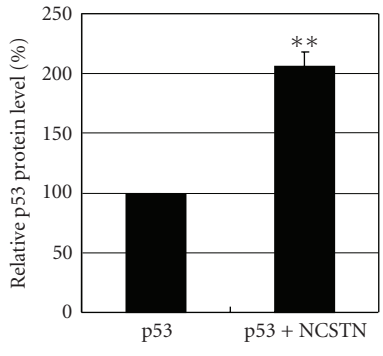


(f)



(g)

Figure 1: Continued.

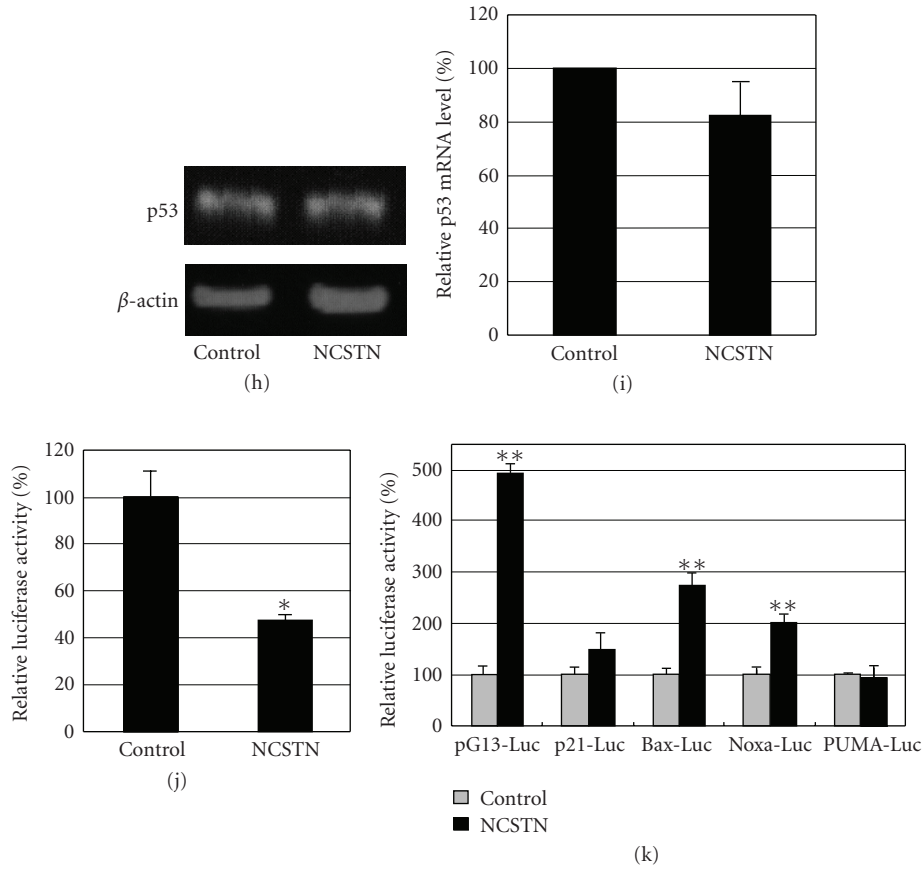


Figure 1: p53 reporter activation by NCSTN. (a) Effects of AD-related genes on p53 transactivity. Mouse ras-NIH3T3 cells (1×10^5 cells) were cotransfected with p53-responsive reporter plasmid pG13-Luc (10 ng), transfection standard SV40-Rluc (5 ng), and test effector plasmid (1 μg) for NCSTN, presenilin, Aph-1, Pen-2 or APP cDNAs, or control empty vector pME18S-FL3. Cells were harvested 48 hours after transfection and luciferase activities in the cell extracts were measured. The error bars represent SD ($n = 3$, t-test; $^*P < .05$; $^{**}P < .01$). ((b) and (c)) Dose-dependent activation of the p53 reporter by NCSTN. Ras-NIH3T3 (b) and MNK45 (c) cells were transfected with increasing amounts of NCSTN cDNA plasmid together with pG13-Luc and control SV40-Rluc. Appropriate amounts of control empty plasmid pME18S-FL3 were included to make equivalent amounts of total DNA (1 μg) for each transfection. ((d)–(g)) Expression of NCSTN and p53 in NCSTN-transfected cells. ras-NIH3T3 cells were transfected with pME18S-FL3 (control), NCSTN or presenilin expression plasmid alone (d), or with human wild-type p53 expression plasmid alone or in combination with NCSTN expression plasmid (f), and incubated for 48 hours. Cell lysates were prepared and subjected to Western blot analysis using anti-NCSTN, anti-mouse/human p53 and anti-GAPDH antibodies (d) or anti-human p53 (Bp53-12), anti-phospho-p53 (Ser-6), anti-phospho-p53 (Ser-9), anti-phospho-p53 (Ser-15), anti-phospho-p53 (Thr-18), anti-phospho-p53 (Ser-20), anti-phospho-p53 (Ser-37), anti-phospho-p53 (Ser-315), anti-phospho-p53 (Ser-392) and anti-GAPDH antibodies (f). Densitometric quantification of endogenous mouse p53 (e) and ectopic human p53 (g) protein levels was performed from the results of Figures 1(d) and 1(f), respectively. The data were normalized by the values of GAPDH. The error bars represent SD ($n = 3$, t-test; $^{**}P < .01$). ((h) and (i)) NCSTN did not affect p53 mRNA levels. The expression levels of p53 and β-actin mRNAs in control vector- or NCSTN-transfected ras-NIH3T3 cells were examined by RT-PCR (h). Primers p53-841F and p53-1040R were used. For control, β-actin-684F and β-actin-976R primers were used. Relative expression levels of p53 versus β-actin were quantitated and shown in (i). The error bar represents SD ($n = 3$). (j) NCSTN attenuated promoter activity of the p53 gene. ras-NIH3T3 cells were cotransfected with luciferase reporter plasmid under the control of the p53 gene promoter and control vector or NCSTN expression plasmid. Relative luciferase activities are shown. The error bars represent SD ($n = 3$, t-test; $^*P < .05$). (k) Specificity for activation of p53-responsive promoters by NCSTN. ras-NIH3T3 cells were cotransfected with reporter plasmids such as pG13-Luc, pGL3-p21-Luc, pGL3-Bax-Luc, pGV-B2 Noxa-Luc and pBV-PUMA Frag2-Luc together with control empty vector or NCSTN expression plasmid. Cells were harvested 48 hours after transfection and luciferase activities in the cell extracts were measured. The error bars represent SD ($n = 3$, t-test; $^{**}P < .01$).

(Figures 3(a) and 3(b)). It is known that the amino acid residues 306–360, 419–458 and 626–662 of NCSTN are highly conserved amongst NCSTN orthologs [32], and that amino acid residues 306–360 are the receptor domain for γ-secretase-substrates [7]. NCSTN Δ195–305, NCSTN Δ361–418 and NCSTN Δ625–662, as well as wild-type NCSTN, significantly activated p53-reporter pG13-Luc. On the other hand, NCSTN Δ306–360 did not increase but rather significantly reduced the luciferase activity, and NCSTN Δ419–458 showed no apparent effect (Figure 3(c)), despite the comparable protein expression levels of these NCSTN mutants (Figure 3(b)). Thus, it is conceivable that

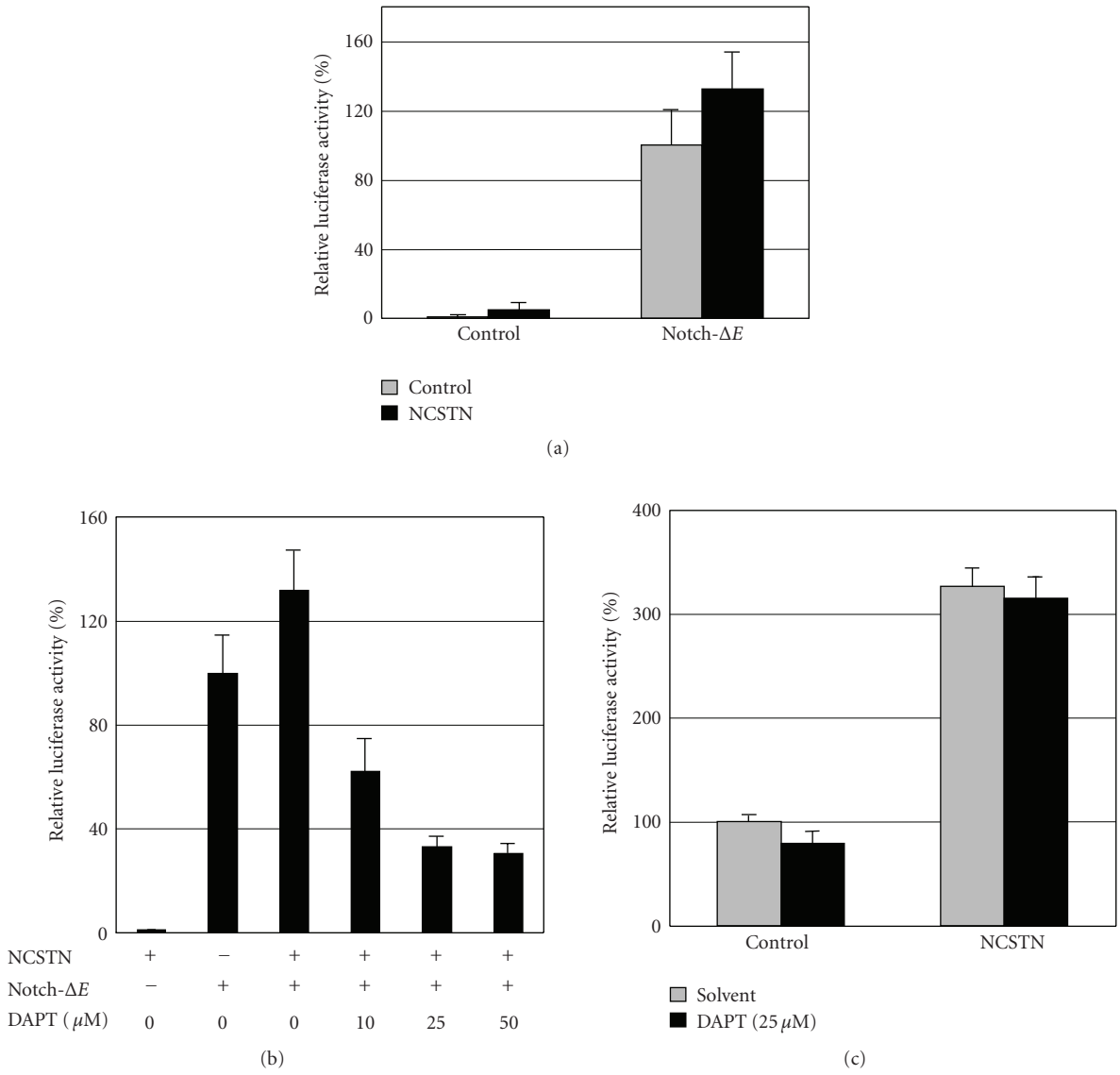


Figure 2: γ-secretase inhibitor did not suppress NCSTN-induced activation of the p53-responsive promoter. (a) γ-secretase is active in ras-NIH3T3 cells. ras-NIH3T3 cells were cotransfected with Notch-responsive reporter plasmid pTP1-Luc and expression vectors of control, NCSTN or Notch-ΔE as indicated. Cells were harvested 48 hours after transfection and luciferase activities in the cell extracts were measured. The error bars represent SD ($n = 3$). (b) Inhibitory effects of γ-secretase inhibitor DAPT on Notch transactivation ability. ras-NIH3T3 cells were transfected with a Notch-responsive reporter plasmid pTP1-Luc and expression vectors of NCSTN, Notch-ΔE or control as indicated. Twenty-four h after transfection, cells were treated with DAPT at the indicated concentrations for another 24 hours. Cells were harvested 48 hours after transfection and luciferase activities in the cell extracts were measured. The error bars indicate SD ($n = 3$). (c) Lack of inhibition of DAPT on NCSTN-induced p53 transactivation ability. ras-NIH3T3 cells were transfected with a p53-responsive reporter plasmid pG13-Luc and an expression vector for NCSTN or its empty vector, and treated or not treated with DAPT (25 μM). Cells were harvested 48 hours after transfection and luciferase activities in the cell extracts were measured. The error bars represent SD ($n = 3$).

the substrate receptor domain, amino acid residues 306–360, as well as amino acids 419–458, of NCSTN is indispensable for the stimulation of p53 transactivity ability.

3.4. Involvement of cAMP Signal in the Activation of p53 by NCSTN. We then examined the signaling pathway of p53 activation by NCSTN using various probes that affect signal transduction pathways such as protein kinase C, cAMP-dependent protein kinase (PKA), MAP kinase, Akt kinase, NF-κB, Cdk and Ras family pathways [33]. It was shown thus far that the effects of NCSTN were attenuated by DBcAMP, IBMX and forskolin which increased intracellular cAMP concentration (Figure 4(a)). The involvement of cAMP was further confirmed by cotransfection with cDNA of the constitutively active catalytic subunit of PKA (PKA-CS), which reduced both basal and NCSTN-stimulated p53 activity (Figure 4(b)). cDNA of Rho-GDI also reduced the

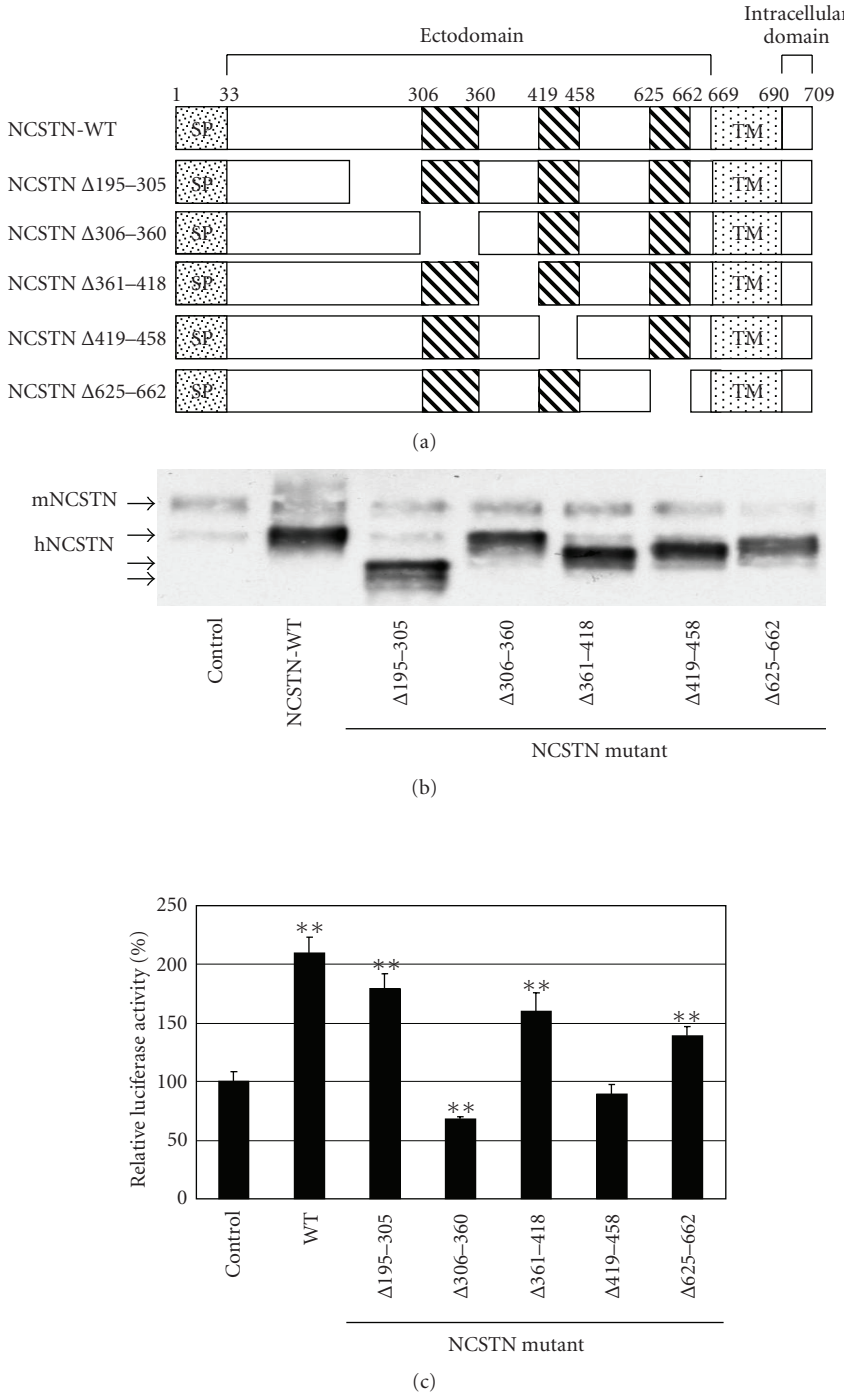


Figure 3: Analysis of NCSTN domain functions. (a) Structures of wild-type (WT) and mutant NCSTN. Deletion mutants of NCSTN were constructed as described in the Materials and methods. Deleted regions in NCSTN Δ195–305, NCSTN Δ306–360, NCSTN Δ361–418, NCSTN Δ419–458 and NCSTN Δ625–662 are shown. SP and TM represent the signal peptide and the transmembrane domain, respectively. Striped regions between amino acid positions 306 and 360, 419 and 458, and 625 and 662 are highly conserved sequences amongst NCSTN orthologs. (b) Comparable expression levels of NCSTN in mutant-NCSTN transfectants. ras-NIH3T3 cells were transfected with cDNA of wild- type or mutant NCSTN. Cells were harvested 48 hours after transfection, and the NCSTN protein expression levels were examined by Western blotting. mNCSTN and hNCSTN represent endogenous mouse NCSTN and ectopically expressed human NCSTN, respectively. (c) Effects of NCSTN mutants on the p53-responsive promoter. ras-NIH3T3 cells were cotransfected with p53-responsive reporter plasmid pG13-Luc and the expression plasmid for wild-type or mutant NCSTN. Cells were harvested 48 hours after transfection and the luciferase activities in the cell extracts were measured. The error bars represent SD ($n = 3$, t-test; $^*P < .01$).

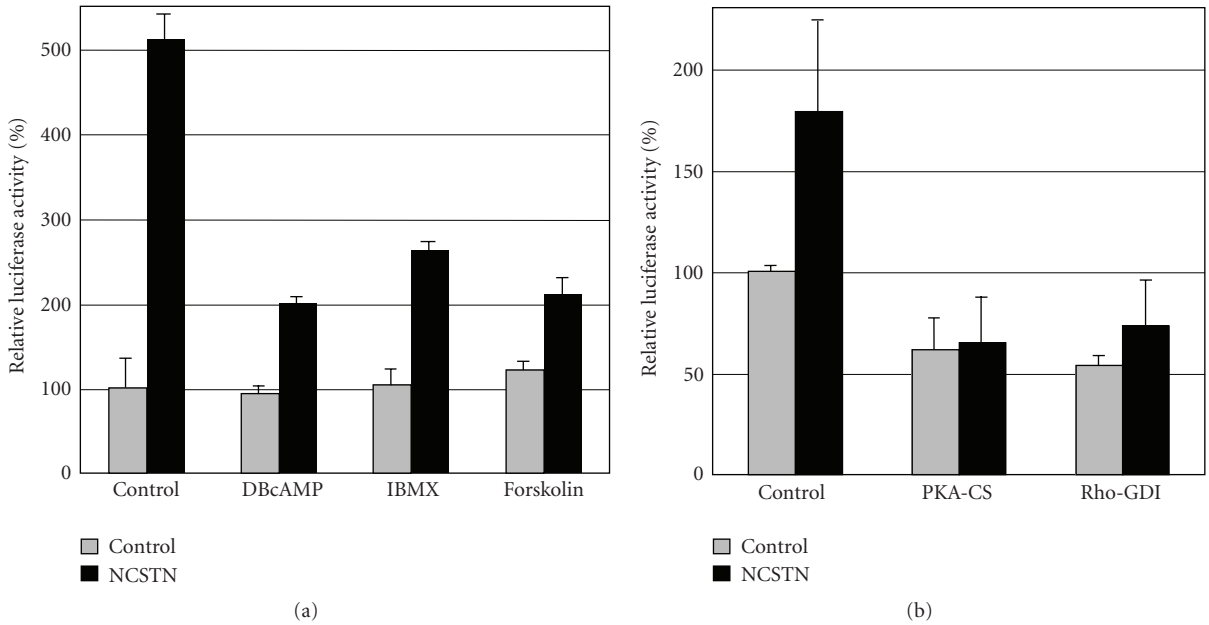


Figure 4: Involvement of cAMP signal in activation of the p53-responsive promoter by NCSTN. (a) Suppression of NCSTN-induced activation of the p53-responsive promoter by cAMP-increasing compounds. ras-NIH3T3 cells were transfected with NCSTN or control expression plasmid (1 μg). Twenty-four h after transfection, cells were treated with DBcAMP (100 μM), IBMX (100 μM), forskolin (10 μM) or the solvent dimethylsulfoxide (0.1%) (control) and incubated further for 24 hours. Cells were harvested and the luciferase activities in the cell extracts were measured. (b) Suppression of NCSTN-induced activation of the p53-responsive promoter by PKA. NCSTN expression plasmid (0.5 μg) was cotransfected with PKA-CS or Rho-GDI expression plasmid (0.5 μg). Relative luciferase activities are shown. The error bars represent SD ($n = 3$).

effects of NCSTN partly. Thus, the activation of p53 by NCSTN might be mediated by cAMP and Rho signaling pathways.

4. Discussion

Senile plaque is a typical pathological feature of AD, and thus may be implicated in the pathogenesis of AD, although the precise mechanism remains unclear. It has been shown that p53 is highly expressed in the brain of patients with AD [17, 18]. Presenilin, Aph-1, Pen-2, and NCSTN form γ-secretase complex, which cleaves amyloid precursor protein to produce amyloid β-peptide (Aβ) [32]. Generation of an excess amount of Aβ activates p53 and induces neurodegeneration [18]. p53-associated cell death in Alzheimer disease was dependent on presenilin [34]. However, on the other hand, overexpression of Aph-1 and Pen-2 reduced p53 expression and activity and suppressed apoptosis [35], and consistently, knockdown of Pen-2 resulted in p53-dependent apoptosis [36]. Dunys et al. suggested the importance of the integrity of γ-secretase complex [35]. Unusual increase of one of the components could cause distinct effects independently of γ-secretase complex.

In the present study, we examined the effects of γ-secretase components on p53 with a luciferase assay and found that p53-responsive reporter plasmid pG13-Luc was significantly activated by NCSTN (Figures 1(a)–1(c)). This result suggested that p53 transactivation ability was enhanced by NCSTN. This putative activation of p53 by NCSTN was accompanied by the increase not only in p53 protein expression levels but also in phosphorylation levels at multiple sites in p53 (Figures 1(d)–1(g)) without any increase in p53 mRNA levels (Figures 1(h) and 1(i)). NCSTN also activated the reporter under the control of p53-responsive promoter sequences of proapoptotic Bax and Noxa but not antiapoptotic p21 (Figure 1(k)). This is consistent with the neuronal cell death observed in AD brains. The increased p53 by NCSTN may be physiologically relevant because the anchorage-independent growth in soft agar medium was suppressed in NCSTN-transfected ras-NIH3T3 cells as compared to control vector-transfected cells (Supplementry Figure 1, Supplementry Material available on doi: 10.3814/2010/606391).

The reporter plasmid pTP1-Luc is specifically activated by Notch-IC [9] which is produced by cleavage of Notch-ΔE by γ-secretase [22]. A substantial activation of this reporter by transfection with Notch-ΔE (Figure 2(a)) suggested that γ-secretase activity was present in ras-NIH3T3 cells. Transfection with with NCSTN caused no further increase in the luciferase activity (Figure 2(a)), suggesting that NCSTN is not a limiting factor for γ-secretase activity in this cell. The activation of pG13-Luc by NCSTN was not inhibited by γ-secretase inhibitor DAPT despite the inhibition of Notch-ΔE-induced activation of pTP1-Luc by DAPT (Figures 2(b) and 2(c)). Similar results were obtained with another γ-secretase inhibitor L-685,458 (data not shown). Accordingly, the activation of p53 by NCSTN may be independent of the catalytic activity of γ-secretase.

Recently, Pardossi-Piquard et al. have reported that NCSTN lowers p53 expression as well as p53 transcriptional activity independently of γ-secretase activity [37]. They also reported the reduction of p53 promoter activity by NCSTN, which is consistent with our results (Figure 1(j)). We frequently observed such reduced p53 promoter activity after activation of p53 (data not shown), and therefore, decrease in the p53 promoter activity might be a result of feedback regulation after elevation of the expression and/or transactivation ability of p53. Decrease in p53 expression might be a secondary effect caused by reduced transcription of the p53 gene. It is possible that activation of p53 is the primary effect of NCSTN.

Comparison of NCSTN deletion mutants suggested that the activation of p53 by NCSTN is dependent on the substrate receptor domain, amino acid residues 306–360, and amino acids 419–458 of NCSTN (Figure 3). Thus far, many substrates for γ-secretase have been identified [38, 39]. Without proteolytic cleavage, simple binding and trapping of some substrates on the cell surface by NCSTN might provoke a signaling pathway which may lead to the increase in the protein expression levels and transactivation ability of p53.

Among the signaling pathways examined thus far, activation of PKA reproducibly reduced the stimulation of p53 transactivity by NCSTN (Figure 4). PKA activity is decreased in AD brains [40], and PKA-activating reagents were recently reported to be therapeutic drugs for AD [41]. The rationale for this therapy may be partly explained by the suppression of NCSTN-mediated activation of p53 by PKA-activating gene (PKA-CS) and drugs (DBcAMP, IBMX and forskolin).

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