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α-Synuclein functions as a negative regulator for expression of tyrosine hydroxylase

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Abstract

Previous studies have shown that over-expression of α-Synuclein (α-Syn), a protein whose abnormality is implicated in the pathogenesis of Parkinson's disease (PD), reduces tyrosine hydroxylase (TH) expression and dopamine synthesis. To explore the possible mechanism for the regulation of TH expression by α-Syn, luciferase reporter gene carrying a -493/+27bp fragment of human TH gene (pGL3-TH520) and pcDNA carrying hα-Syn gene (pcDNA-hα-Syn) were co-transfected into T293 cells. The results showed that α-Syn was only detected in pcDNA-hα-Syn-transfected cells but not in pcDNA vector control cells. In α-Syn-transfected cells, the luciferase activity was dramatically reduced compared with the vector control cells. These results suggest that α-Syn may function as a negative regulator for TH expression by affecting the activity of TH promoter.

Key words: α-synuclein; tyrosine hydroxylase; dopaminergic neurons; promoter; luciferase.

Introduction

α-Synuclein (α-Syn) is a 140-amino acid protein richly expressed in the brain(George, 2002; Ma *et al*., 2003). Several evidences suggest that α-Syn is implicated in the pathogenesis of Parkinson's disease (PD). For example, mutations in the α-Syn gene (A30P, A53T, and E46K) are associated with autosomal dominant early-onset forms of PD (Krüger *et al*., 1998; Polymeropoulos *et al*., 1997; Zarranz *et al*., 2004). Multiple copies of wild-type α-Syn are identified in some families with this disease (Singleton *et al*., 2003; Chartier-Harlin *et al*., 2004; Ibáñez *et al*., 2004). Fibrillated α-Syn is shown to be the major component of Lewy bodies and Lewy-related neurites in both sporadic and familial PD (Arima *et al*., 1998; Baba *et al*., 1998; Spillantini *et al*., 1997). α-Syn involved in PD pathogenesis was supported by studies in α-Syn transgenic animals and gene-transfected cells (Perez□ *et al*., 2002; Abeliovich *et al*., 2000; Lotharius and Brundin, 2002; Perez *et al*., 2004; Rochet *et al*., 2004). These studies showed that over-expression of α-Syn caused preferential damage of dopaminergic (DA) neurons in the brain. In what manner abnormally expressed α-Syn may selectively damage the DA neurons remains yet unknown.

Since dopamine neurotransmitters and tyrosine hydroxylase (TH) is the rate-limiting enzyme for DA synthesis (Nagatsu *et al*., 1964; Zigmond *et al*., 1989), we hypothesize that some relationship may exist between α-Syn and TH, which may play an important role in the selective damage of DA neurons caused by abnormal α-Syn expression. The previous studies showed that the expression of TH was negatively regulated by α-Syn(Yu *et al*., 2004; Perez, 2002; Tehranian *et al*., 2006). However, the exact molecular mechanism underlying this regulation has been unclear. It is known that gene regulation often occurs in the promoter regions, whether α-Syn regulate TH is also through regulation of promoter activity. To demonstrate this, we constructed a luciferase reporter gene containing -493/+27 region of TH gene, and transfected the reporter gene with α-Syn gene into T293 cells simultaneously. Our results demonstrated that the TH promoter activity was significantly reduced in the α-Syn-transfected T293 cells.

Materials and Methods

Plasmid construction

Genomic DNA was isolated from a healthy human adult. Briefly, 10 ml of blood was anticoagulated with 1.0 mmol/L EDTA-Na_2. The erythrocytes

were lysed in a buffer containing 10 mmol/L Tris-HCl (pH 7.5), 0.32 mol/L sucrose, five mmol/L $MgCl_2$ and 1% (V/V) Triton X-100. The leukocytes were isolated by centrifugation (16,000 *g*, 30 s) and lysed in a buffer containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 25 mmol/L $MgCl_2$, 0.1 mg/mL gelatin, 0.45% (V/V) Nonidet P40 and 0.45% (V/V) Tween 20. The lysate was incubated with 20 mg of SDS and 1mg of protease K at 55°C overnight. The genomic DNA was isolated and purified with ethanol precipitation.

TH gene promoter region was amplified by PCR method using the following primers: upstream primer 5´-CAGATGGCACTCCTAGGAACCAC-3´, downstream primer 5´-gaagatctTCA GTGTGGAGGTCCGGGCT-3´, which were designed according to the human TH promoter sequence (M23597, GenBank) (Chu and Kordower, 2007). A 5´-*gaagatct*-3´ sequence containing restriction endonuclease *Bgl*II site, was subjoined to 5´-flanking of the downstream primer. TH gene promoter region was amplified by PCR method using the following primers: upstream primer 5´-CAGATGGCACTCCTAGGAACCAC-3´, downstream primer 5´-gaagatctTCA GTGTGGAG-GTCCGGGCT-3´, which were designed according to the human TH promoter sequence (GenBank no:M23597) (Chu and Kordower, 2007). A 5´-*gaagatct*-3´ sequence containing restriction endonuclease *Bgl*II site, was subjoined to 5´-flanking of the downstream primer. The PCR products were purified and inserted to the pMD 18-T vector (TaKaRa, Japan) to generate the recombinant plasmid pMD, which was digested by *Sma* I and *Bgl* IIto release an approximately 500 bp DNA fragment to subclone into pGL_3-Basic luciferase vector (promega, USA) to produce the resultant vector pGL_3-TH520. All vectors were identified by sequencing.

Cell transfection

Human embryonic kidney (HEK) 293T cells were grown in DMEM (GIBCO) supplemented with 100 U/ml of penicillin, 100 U/ml streptomycin and 10% heat-inactivated FBS under 5% CO_2/95% air in a humidified incubator. Cells were kept at exponential phase of growth. The culture medium for 293T cells with hα-Syn transfectants was similar to that of 293T control cells except containing 200 μg/ml of G418 (Sigma).

Cells were transfected using Lipofectamine 2000 reagent according to the protocol. Briefly, the cells were seeded in 96-well plates at the density of 1×10^4 cells/100 μl/well, and cultured for 24 h. Each of the plasmids (pGL3, pcDNA, pEGFP) in the Lipofectamine 2000 (w/v, 1:4) was added, the plasmid phRL-TK (promega, USA) was co-transfected (w/w, phRL/pGL3 or pcDNA, 1:25) to the cells was used as internal control. Five hours later, the cells were changed with fresh medium, and cultured for additional 24 h continually. The luciferase activities were then measured in a GLOMAXTM Microplate Luminometer using Dual-Luciferase Reporter Assay System kit (promega, USA).

For immunofluorescence labeling, 293T cells were maintained in $35cm^2$ culture dishes at a density of 2×10^5 cells/ml. 2.5 μg of each DNA construct was transfected into the cells by Lipofectamine 2000 reagent as describes above. 24 h after transfection, the cells were fixed in 4% paraformaldehyde containing 0.1% glutaraldehyde and 0.3% Triton X-100. The cells were then incubated overnight at 4 °C with anti-human α-Syn monoclonal antibody (3D5, 1:3,000) (Yu *et al*., 2007), followed by 1 h incubation with the FITC-conjugated goat anti-mouse IgG (1:200, Santa cruz) at room temperature. After several washes with PBS, the cells were coverslipped and observed with a confocal laser microscope (Bio-Rad, MRC, 1024, USA).

Western blot

For Western blot analysis, 293T cells were maintained in 25 cm^2 flasks at a density of 7×10^4 cells/ml. 20 μg of each DNA construct was transfected into the cells by Lipofectamine 2000 reagent as describes above. After transient transfection for 24 h, the cells were collected and lysed with a lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1 mM PMSF, 1 mM EDTA and 0.01% (w/v) SDS. The cell lysates were then centrifuged at 12,000 × g for 20 min and the protein concentration in the supernatant was determined using BCA method (pierce). Proteins (60 μg /lane) were separated by 12.5% SDS-PAGE and transferred to PVDF membrane (Life Science). The membrane was blocked for 30 min with 5% skim milk in TBS containing 0.1% Tween-20 (TBST), and then incubated with anti-human α-Syn monoclonal antibody (3D5, 1:5,000), or anti-β-tubulin monoclonal antibody (1:20,000, sigma) overnight at 4°C, followed by horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000, Santa Cruz) for 1 h, and developed with ECL reagents (Santa Cruz).

Statistical analysis

Data were analyzed by one-factor analysis of variance, and independent-sample T Test by SPSS

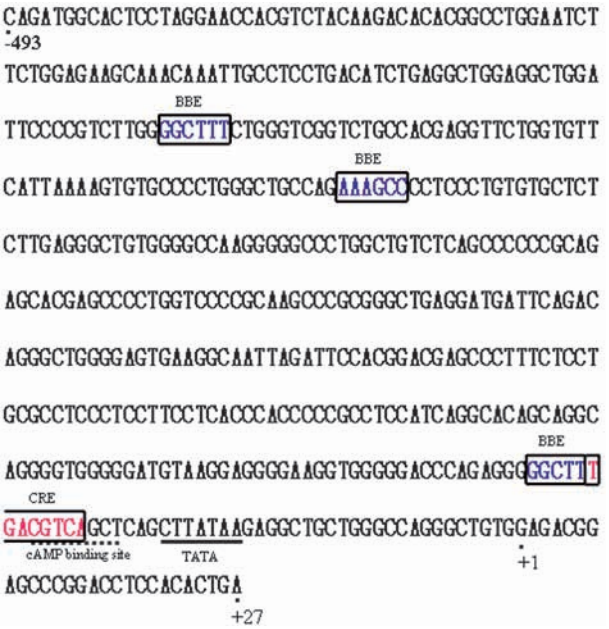

FIG. 1. — Nucleotide sequence of the amplified 5´-flanking region of the human TH gene. The transcription start site is designated +1. Exon 1 extends from nucleotides +1 to +27. Putative consensus sequences for transcription factors including bicoid-type element (BBE), cAMP response element (CRE), and canonical TATA box are boxed with their names indicated above.

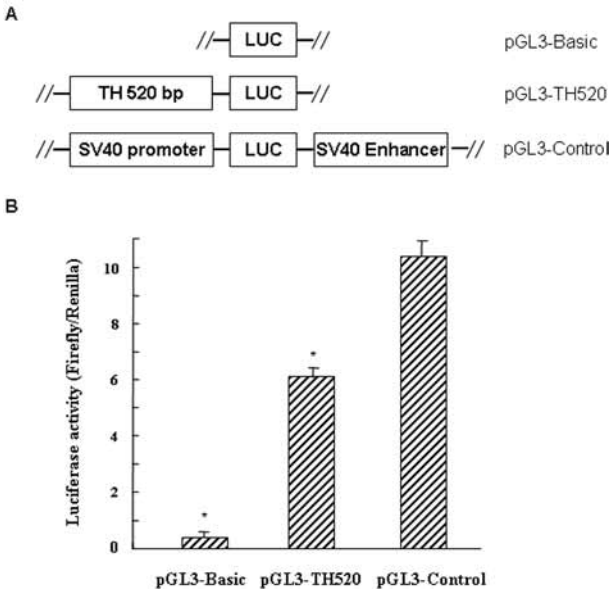

FIG. 2. — Promoter assay of the amplified -493/+27 region of the human TH gene. A: Schematic representation of the human TH promoter reporter constructs. The amplified 5´-flanking regions were subcloned into a promoterless luciferase construct (pGL3-Basic). B: Constructs were transiently co-transfected with a synthetic Renilla luciferase reporter plasmid (phRL-TK) into 293T cells. Aliquots corresponding to identical Renilla luciferase activity were used for each luciferase assay.

11.5 statistical software. Data were expressed as mean ± SEM, representing the average value over 6 independently repeated experiments.

Results

Using human genomic DNA as a template, we amplified an approximately 500 bp DNA fragment by PCR, and inserted the DNA fragment into the promoter site of pGL3-Basic reporter plasmid vector. The sequence of the inserted DNA was identified and showed in Fig. 1.

To determine the receptivity of 293T cells for extrinsic DNA, plasmid pEGFP-C1 (Clontech) was used to evaluate the transfection efficiency of 293T cells. Around 70% of the cells were shown to be positive for GFP (data not shown). With this transfection efficiency, the construct containing -493/+27 bp region of the human TH promoter (plasmid pGL3-TH520) was transiently transfected into 293T cells. At the same time, the pGL3-Basic (promoterless luciferase reporter vector) and the pGL3-control (luciferase reporter vector containing the SV40 promoter and SV40 enhancer) were used as negative or positive control, respectively. pGL3-TH520 could express luciferase in 293T cells, approximately 68-fold higher than the pGL3-Basic (Fig. 2).

We determined the expression of α-Syn by anti-α-Syn monoclonal antibody 3D5, which was well characterized before (Yu *et al*., 2007). Immunofluorescence labeling illustrated that the signal for α-Syn in the hα-Syn-transfected cells was higher than those in the vector control cells (Fig. 3A). Western blot analysis showed that the cells having the α-Syn-construct expressed distinct protein levels of α-Syn. However, no signals of α-Syn were detected in the samples derived from the non-transfected cells and the vector control cells (Fig. 3B).

We assayed the functional alteration of human TH gene -493/+27 region in different 293T cells. As shown in Fig. 3C, the dual luciferase activity of the pGL3-TH520 in the 293T/pcDNA (6.73 ± 0.26) was significantly higher than that in the 293T/pcDNA-hα-syn (4.54 ± 0.26), $p < 0.01$.

Discussion

In the present study, we isolated a human TH gene fragment, which had 520 bp, containing the first -493 bp of the promoter immediately upstream of the

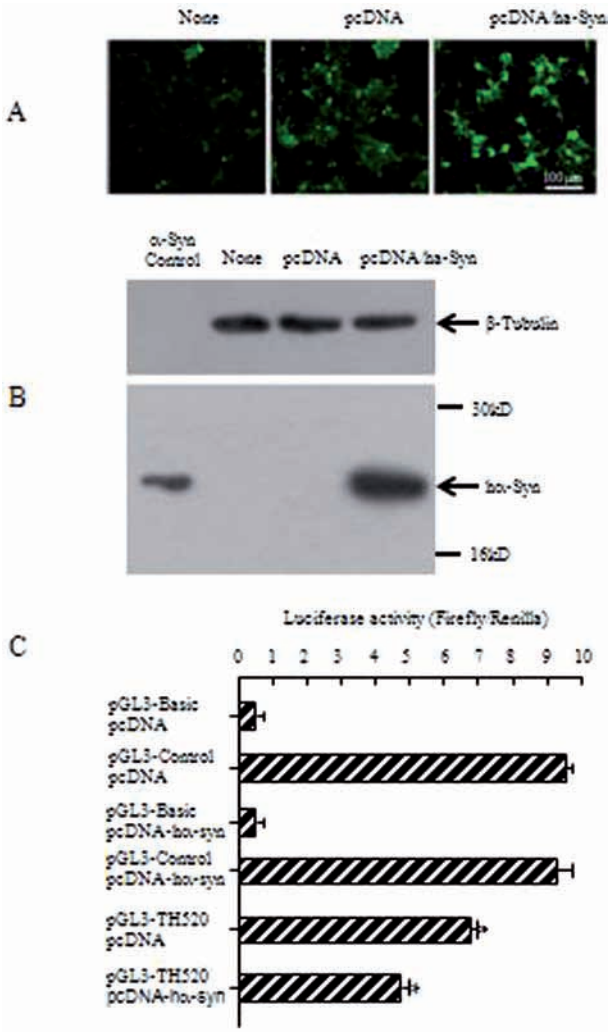

FIG. 3. — Expression of α-Syn and luciferase in 293T cells. A: after transfection with the indicated plasmids, the cells are immunofluorescently labeled with anti-α-Syn antibody 3D5 (green). Scal bar = 100mm. B: the cells are lysed and Western blots are performed with anti-β-tubulin antibody and anti-α-Syn antibody 3D5, respectively. C: the cells are assayed with the dual-luciferase reporter assay system. *$p < 0.01$.

transcription start, and several basic elements of the promoter, such as canonical TATA box, cAMP response element (CRE), and consensus sequences for transcription factors bicoid-type element (BBE) (Kim *et al*., 2003). We inserted the fragment into plasmid pGL3-Basic to construct a luciferase reporter vector pGL3-TH520. pGL3-TH520 vector could express luciferase 68-folds higher than the promoterless luciferase reporter vector in the transfected 293T cells, indicating that the human TH gene -495/+27 fragment possesses the promoter function.

By co-transfecting the TH promoter and α-Syn genes into 293T cells, we showed that the activity of TH promoter was reduced in the cells transfected with α-Syn gene but not in the control cells. Western blot showed that α-Syn protein was detected only in the cells transfected with α-Syn gene. These results suggest that α-Syn may function as a negative regulator of the TH promoter.

We previously showed that in the α-Syn–transfected MES23.5 cells, increasing of α-Syn expression was accompanied by dramatic reduction of TH mRNA. The present results indicate that the inhibition of TH gene expression by α-Syn may be due to its action on the promoter activity of TH gene. It remains unclear if α-Syn can directly bind to the promoter region of TH gene to inhibit its activity, or through an indirect mechanism to affect the activity of TH promoter.

α-Syn is normally localized in the nuclei of brain neurons (Yu *et al*., 2007). The nuclear localization suggests a physiological function of this protein in the regulation of gene expression. The present results could extend our knowledge about the possible function of α-Syn in the gene regulation in the nucleus. In dopaminergic neurons, the regulation of TH promoter activity by α-Syn may have specific meanings. As described above, inhibition of TH promoter activity will lead to the suppression of TH expression in dopaminergic neurons, which may be favorable in physiological conditions for these neurons to protect themselves from the damage of oxidative stress induced by self-oxidation of dopamine. In this context, a physiological role of α-Syn is likely to work as a regulator in dopaminergic neurons to maintain a homeostasis of DA metabolism, which allows both normal functioning and self-protection of these neurons. However, in pathological conditions, such as in the case of PD, the concentration of α-Syn within the cells may be increased, leading to elevated levels of nuclear α-Syn and decreased TH gene transcription. To support this, a recent study on humans and monkeys has shown that during aging the levels of cytoplasmic α-Syn increase in dopaminergic neurons, which correlates with a decline in TH expression (Chu and Kordower, 2007). However, no apparent reduction in TH expression was found in dopaminergic neurons in the ventral tegmental area where the levels of cytoplasmic α-Syn did not increase significantly. Although the cytoplasmic α-Syn level increases in the dopaminergic neurons during normal aging, it may not reach to a sufficient magnitude to drive DA levels pass a threshold that would develop the cardinal signs and symptoms of PD. In

PD, however, for reasons still unknown, the age-related accumulation of α-Syn becomes further intensified and misfolded to form inclusions. These events may cause the cell to lose its dopaminergic phenotype, and DA levels may pass a critical threshold, causing symptoms to emerge.

Conclusion

The -493/+27 bp region of the human TH gene has a promoter function. α-Syn may function as a negative regulator for TH expression by affecting the activity of this TH promoter as a trans-acting factor.

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REFERENCES

1. Abeliovich A, Schmitz Y, Farinas I, Choi-Lundberg D, Ho WH. *et al*. Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. Neuron. 2000;25:239-252.
2. Arima K, Ueda K, Sunohara N, Hirai S, Izumiyama Y. *et al*. Immunoelectron-microscopic demonstration of NACP/alpha-synuclein-epitopes on the filamentous component of Lewy bodies in Parkinson's disease and in dementia with Lewy bodies. Brain Res. 1998;808:93-100.
3. Baba M, Nakajo S, Tu PH, Tomita T, Nakaya K. *et al*. Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. Am J Pathol. 1998;152: 879-884.
4. Chartier-Harlin MC, Kachergus J, Roumier C, Mouroux V, Douay X. *et al*. Alpha-Synuclein locus duplication as a cause of familial Parkinson's disease. Lancet. 2004;364: 1167-1169.
5. Chu Y, Kordower JH. Age-associated increases of α-synuclein in monkeys and humans are associated with nigralstriatal dopamine depletion: Is this the target for Parkinson's disease? Neurobiol Dis. 2007;25:134-149.
6. George JM. The synucleins. Genome Biol. 2002;3:3002.
7. Ibáñez P, Bonnet AM, Débarges B, Lohmann E, Tison F. *et al*. Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. Lancet. 2004;364:1169-1171.
8. Kim T, Park M, Choi E, Lee H, Lee S. *et al*. Cloning and cell type-specific regulation of the human tyrosine hydroxylase gene promoter. Biochem Biophys Res Commun. 2003;312:1123-31.
9. Krüger R, Kuhn W, Müller T, Woitalla D, Graeber M. *et al*. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet. 1998;18:106-8.
10. Lotharius J, Brundin P. Pathogenesis of Parkinson's disease:dopamine, vesicles and alpha-synuclein. Nat Rev Neurosci. 2002;3:932-942.
11. Ma QL, Chan P, Yoshii M, Ueda K. Alpha-synuclein aggregation and neurodegenerative diseases. J Alzheimers Dis. 2003;5:139-48.
12. Nagatsu T, Levitt M, Uderfriend S. Tyrosine hydroxylase, the initial step in norepinephrine biosynthesis. J Biol Chem. 1964;239:2910-2917.
13. Perez RG, Waymire JC, Lin E, Liu JJ, Guo F. *et al*. A role for alpha-synuclein in the regulation of dopamine biosynthesis. J. Neurosci. 2002;22: 3090-3099.
14. Perez RG, Hastings TG. Could a loss of alpha-synuclein function put dopaminergic neurons at risk? J Neurochem. 2004;89:1318-1324.
15. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A. *et al*. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science. 1997;276:2045-2047.
16. Rochet JC, Outeiro TF, Conway KA, Ding TT, Volles MJ. *et al*. Interactions among alpha-synuclein, dopamine, and biomembranes: some clues for understanding neurodegeneration in Parkinson's disease. J Mol Neurosci. 2004;23:23-34.
17. Singleton AB, Farrer M, Johnson J, Singleton A, Hague S. *et al*. alpha-Synuclein locus triplication causes Parkinson's disease. Science. 2003;302: 841.
18. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R. *et al*. Alpha-synuclein in Lewy bodies. Nature. 1997;388:839-840.
19. Tehranian R, Montoya SE, Van Laar AD, Hastings TG, Perez RG. Alpha-synuclein inhibits aromatic amino acid decarboxylase activity in dopaminergic cells. J Neurochem. 2006;99:1188-1196.
20. Xu S, Zhou M, Yu S, Cai Y, Zhang A. *et al*. Oxidative stress induces nuclear translocation of C-terminus of alpha-synuclein in dopaminergic cells. Biochem Biophys Res Commun. 2006;342:330-335.
21. Yu S, Li X, Liu G, Han J, Zhang C. *et al*. Extensive nuclear localization of alpha-synuclein in normal rat brain neurons revealed by a novel monoclonal antibody. Neuroscience. 2007;145:539-55.
22. Yu S, Zuo X, Li Y, Zhang C, Zhou M. *et al*. Inhibition of tyrosine hydroxylase expression in□ alpha-synuclein-transfected dopaminergic neuronal cells. Neurosci Lett. 2004;367:34-39.
23. Zarranz JJ, Alegre J, Gómez-Esteban JC, Lezcano E, Ros R. *et al*. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann Neurol. 2004;55:153-156.

24. Zigmond RE, Schwarzschild MA, Rittenhouse AR. Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitters via phosphorylation. Annu Rve Neurosci. 1989;12:415-461.

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