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The nuclear accumulation of alpha-synuclein is mediated by importin alpha and promotes neurotoxicity by accelerating the cell cycle

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ABSTRACT

α-Synuclein (α-syn), a 14 kDa pre-synaptic protein, is widely involved in the Parkinson's disease (PD) pathogenesis. Recent studies have shown that the nuclear accumulation of α-syn might have a toxic effect. The main purpose of the present study was to explore which amino acid residues in α-syn are associated with its nuclear accumulation, the molecule(s) mediated the nuclear import of α-syn, and the role of α-syn accumulated in the nucleus. It has been noted that the nuclear import of α-syn may be mediated by importin α and that both the amino acid residues 1–60 and 103–140 of α-syn were indispensable for its nuclear import. After imported into the nucleus, the accumulated α-syn played a toxic role in both the PC12 cells and the C57 mice. Furthermore, α-syn-nuclear localization signal-injected mice showed behavioral symptoms associated with PD. Further studies performed *in vitro* showed that the toxicity of α-syn in the nucleus might be due to an interference of the cell cycle. Thus, it can be concluded that α-syn can accumulate in nucleus, which is mediated by importin α, and promote neurotoxicity by accelerating the cell cycle.

1. Introduction

α-Synuclein (α-syn), a 14 kDa pre-synaptic protein, is widely involved in the Parkinson's disease (PD) pathogenesis due to its presence in Lewy bodies (LB) in PD (Dev et al., 2003). As its name implies, α-syn is initially localized in the pre-synaptic nerve terminals and the nucleus (Maroteaux et al., 1988). A large number of subsequent studies have focused on the role of α-syn that is localized to the pre-synaptic nerve terminals. Little attention has been paid to the role of α-syn in the nucleus, although the nuclear localization of the protein has been found in all kinds of experimental systems, including cultured cells (Ma et al., 2011; McLean et al., 2000; Specht et al., 2005; Yuan et al., 2008), α-syn transgenic Drosophila (Takahashi et al., 2003) and mice (Goers et al., 2003; Masliah et al., 2000).

Recent studies have shown that α-syn can be transported into the nucleus in the presence of H_2O_2. When treated with 200 μM H_2O_2, the increased intra-nuclear accumulation of the C-terminal fragment of α-syn in dopaminergic neurons has been observed in Xu's study (Xu et al., 2006). Further studies have indicated that the nuclear translocation of α-syn can increase the susceptibility of MES23.5 dopaminergic cells to oxidative stress (Zhou et al., 2013). The interaction between oxidative stress and nuclear translocation of α-syn may form a positive feed-back cycle, leading to the death of dopaminergic cells. However, the mechanism of α-syn nuclear translocation is still unknown. Thus, this issues was the key point we focused on in this study and it was explored from two aspects, the amino acid residues associated with the nuclear accumulation (α-syn itself) and the molecule(s) mediating the nuclear accumulation (associated cellular molecules).

In view of the extensive localization of α-syn in the neuronal nucleus throughout the brain in normal conditions, it might play an important physiological function in the nucleus of brain neurons (Yu et al., 2007). However, the role of α-syn under physiological and pathological conditions remains poorly understood. Recent studies have reported that α-syn might play an import role in pathogenesis of PD when localized in nucleus. Hegde et al. have demonstrated the interaction of DNA with α-syn (Hegde et al., 2010), and Boyer's

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results further showed that α-syn within the nucleus accumbens induced changes in cocaine-associated behaviors in rats (Boyer and Dreyer, 2007). The other studies also reported that α-syn can associate with histone *in vitro* (Goers et al., 2003), and the following study demonstrated that it can promote neurotoxicity by inhibiting histone acetylation in the nucleus (Kontopoulos et al., 2006). Thus, the nuclear accumulation of α-syn might be a key factor in the pathogenesis of synucleinopathies. It was in line with the report that several proteins associated with neurodegenerative diseases exerted their pathogenic effects when localized in the nucleus (Klement et al., 1998; Peters et al., 1999; Saudou et al., 1998; Takeyama et al., 2002). But so far now, the function of α-syn in the nucleus was still uncertain. Thus, the role of α-syn in the nucleus was also studied in this paper.

Our results indicate that the nuclear import of α-syn might be mediated by importin α and that both the amino acid residues 1–60 and 103–140 of α-syn were indispensable for its nuclear import. The cell viability assay also showed that α-syn can promote neurotoxicity when targeted to the nucleus. Additionally, the cell cycle of α-syn-NLS overexpression cells was accelerated, as indicated by an increased number of cells in S phase in the cell cycle assay. These findings are consistent with the results from the Western blot analysis for cyclin and from the CFDA-SE staining. Furthermore, AAV1/2-α-syn, AAV1/2-α-syn-NLS and AAV1/2-α-syn-NES recombinant adeno-associated viruses were acquired and stereotaxically injected into the substantia nigra of the C57 mice. The PD-like animal behavior and histopathology were observed in the AAV1/2-α-syn-NLS injected C57 mice. All of these findings support the conclusion that, the nuclear accumulation of alpha-synuclein is mediated by importin alpha and promotes neurotoxicity by accelerating the cell cycle.

2. Methods

2.1. Animal

The C57 mice (22–24 g) were purchased from Vital River Laboratories (VRL), Beijing, China. All of the animal studies were approved by the Institutional Ethics Committee and were performed in accordance with the (NIH) Guide for the Care and Use of Laboratory Animals.

2.2. Plasmid construction

All reactions were performed in an Applied Biosystems Veriti Thermal Cycler. The oligonucleotide primers (Table 1) were synthesized by Invitrogen (Shanghai, China). The template was pDsRed-α-syn (Ma et al., 2011). Bimax2 was inserted into the pDsRed-N1 Vector, and siRNA specific for importin α was cloned into pSilencer 3.1-H1 hygro Vector. The remaining DNA fragments were cloned into the pEGFP-N1 vector. The recombinant plasmids were confirmed by DNA sequencing.

2.3. Transfection

All the recombinant plasmids were transfected into cells according to the product manual provided by Invitrogen (Lipofectamine 2000 Transfection Reagent, Invitrogen). At 48 h post transfection, the following tests were performed.

2.4. Confocal analysis of the distribution of an α-Syn fragment

Pheochromocytoma (PC12) cells, MN9D cells and SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, 12800-017) containing 5% (vol/vol) FBS (Gibco, 10099-141) and 5% (vol/vol) ES (Gibco, 16050-122), at 37 °C with 5% (vol/vol) CO_2. Cells were seeded on chamber slides and cultured for 24 h. Forty-eight hours post-transfection, transfected cells were imaged on a laser scanning confocal microscope (Leica TCS SP2) to capture images.

2.5. Generation of stable transfectants

PC12 cells were transfected with the recombinant vectors pEGFP-N1-α-syn, pEGFP-N1-α-syn-NLS, pEGFP-N1-α-syn-NES or plasmid pEGFP-N1/pcDNA3.1 and screened with G418. Stably transfected cell lines were maintained in DMEM supplemented with 5% fetal bovine serum, 5% horse serum, and 200 μg/ml G418 (Sigma, A1720-5G) at 37 °C in a 5% CO_2 atmosphere.

2.6. Immunoprecipitation

For immunoprecipitation (Monti et al., 2007), cells were washed with PBS and harvested in cold buffer (50 mmol/L Tris–HCl, pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mmol/LEDTA, 1 mmol/L Na_3VO_4, 1 mmol/LNaF, protease, and phosphatase inhibitors cocktails). The lysate was sonicated, pre-cleared for 1/2 h at 4 °C with protein A/G Plus agarose (Santa Cruz) and centrifuged at 1000 *g*. The supernatants were incubated either with 2 μg (10 μL) of antibodies against α-syn or of antibodies against importin α, together with 5 μg (20 μL) of protein A/G plus agarose and rocked at 4 °C overnight. The protein G beads were pelleted and washed three/four times with immunoprecipitation buffer. The precipitates were resolved on 12% sodium dodecylsulfate–poly acrylamide gel electrophoresis and subjected to western blot analysis as described above.

2.7. Western blot analysis

The cells were washed with PBS and lysed in NP-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.4, and 1 mM EDTA with protease inhibitors). The protein concentrations were measured using a BCA kit (Pierce, 23227). The cell lysates (20 mg) were solubilized in SDS sample buffer, separated on an SDS–PAGE gel, and then transferred to a PVDF membrane (Millipore, IPVH00010). The membrane was blocked with 3% BSA (Calbiochem, 12659), incubated with primary antibody (anti-alpha-synuclein from Santa Cruz, sc-7011-R (1:500); anti-importin α from Everest Biotech Ltd., EB06233 (0.2 μg/mL); anti-Actin from Santa Cruz, sc-1616 (1:500); anti-GAPDH from MBL, M171-3 (3 μg/mL); anti-Cyclin B1 from MBL, K0128-3 (0.1 μg/mL); anti-Cyclin RB from Abcam, ab6075 (1:300); anti-Cyclin D3 from MBL, K0013-3S (1 μg/mL)), followed by horseradish peroxidase (HRP)-conjugated secondary antibody (KPL, 474-1506 (1:3000); MBL, 330 (1:5000); Santa Cruz, sc-2020 (1:5000)) and detected with the enhanced chemiluminescence (ECL) plus detection system (Molecular Device, Lmax) (Ma et al., 2011).

2.8. Quantitative RT-PCR

To detect the mRNA levels of importin α, a quantitative RT-PCR approach was used (Zhang et al., 2010). Total RNA was extracted using Trizol (Invitrogen, 15596-018), according to the manufacturer's protocol, and cDNA was prepared with 3 μg total RNA using a reverse transcription system (Takara, D2639A). Equal amounts of cDNA were subjected to PCR in the presence of the SYBR green dye using the SYBR® Premix Ex Taq™ II kit (Takara, DRR081S), and the samples were run in an ABI Fast quantitative PCR 7000HT system (Applied Biosystems). A PCR reaction without a template was used as a negative control. The β-actin mRNA level was used as an internal control. Importin α, β-actin and the negative control reactions were amplified on the same plate. The importin α- and β-actin-specific primers were designed using the Primer Express 2.0 software and were as follows: importin α sense: 5′-CTGATGCACAATGATTATAAAGTGGTTT-3′, antisense: 5′-TGAATGTCATCCCCTGTAACAATG-3′. β-actin: sense: 5′-GAA ATC GTG CGT GAC ATT AAA GAG-3′, antisense: 5′-GCG GCA GTG GCC ATC TC-3′. The PCR was performed for 40 cycles of 30 s at 95 °C and 31 s at 60 °C after an initial denaturation of 30 s at 95 °C. Each sample was normalized using the difference in critical thresholds (CT) between importin α and β-actin. The following equation was used to describe the result:

$$CT(\text{importin } \alpha) = CT(\text{importin } \alpha 1) - CT(\beta - \text{actin})$$

CT(importin α1) was the difference in the CT values between importin α1 and the negative control, and CT(β-actin) was the difference between β-actin and negative control. The mRNA levels of each sample were then compared using the expression CT(importin α). All experiments were independently performed three times, and the average was used for comparison.

2.9. MTT assays for cell survival

The MTT assay for cell viability was performed according to the procedure: ①fixed incubation time (48 h) and varied concentration of rotenone (from 0 μM to 5 μM); ②fixed concentration of rotenone (2 μM) and varied incubation time (from 24 h to 78 h)) (Ma et al., 2011). The stable cells (transfected with the recombinant vectors pEGFP-N1-α-syn, pEGFP-N1-α-syn-NLS, pEGFP-N1-α-syn-NES or plasmid pEGFP-N1) were incubated with MTT (3-(4, 5)-dimethylthiazol (-z-y1)-3, 5-diphenytetrazoliumromide) (Amresco, 0798) for 4 h at 37 °C, and the media was then carefully removed. The formazan crystals were dissolved in dimethyl sulphoxide (DMSO), and the absorbance was determined at 570 nm. All experiments were performed at least three times using 4 cultures per trial.

2.10. Cell cycle analysis

The cells were synchronized following the protocol of Cecchi et al. (2008). Briefly, cells were cultured in medium without serum for 24 h to induce cells to arrest in quiescence or G0 phase. The synchronized cells were harvested 20 h after the addition of medium supplemented with 10% FBS; the cells were trypsinized, washed with PBS and pelleted by centrifugation. Aliquots of 1×10^6 cells were fixed