Amino Acid Sequence Motifs and Mechanistic Features of the Membrane Translocation of α-Synuclein

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Amino Acid Sequence Motifs and Mechanistic Features of the Membrane Translocation of α-Synuclein

Directed by Professor Jongsun Kim

The Doctoral Dissertation submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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This certifies that the Doctoral Dissertation of Ahn, Keun Jae is approved.

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The Graduate School

Yonsei University

December 2005

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부족한 사위를 아들처럼 챙겨주시고 늘 사랑과 기도로 채워주시며 긍정적 사고로 한번 더 생각할 수 있게 해주시는 장인어른과 장모님께 감사와 사랑을 전하고 싶습니다.

지금에 제가 있게 해주신, 걱정만 끼치는 아들이지만 항상 믿음으로 대해주신 어머니, 지금쯤 저 위 어디선가 보고 계실 아버지, 이제 당신들께 조금 더 나아진 모습 보여드릴 수 있게 된걸 다행이라고 생각하며 감사에 마음을 전합니다.

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ABSTRACT

Amino Acid Sequence Motifs and Mechanistic Features of the Membrane Translocation of α-Synuclein

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(Directed by Professor Jongsun Kim)

Many lines of evidence suggest that α-synuclein can be secreted from cells and can penetrate into them, although the detailed mechanism is not known. In this study, we investigated the amino acid sequence motifs required for the membrane translocation of α-synuclein, and the mechanistic features of the phenomenon. We first showed that not only α-synuclein but also β- and γ-synucleins penetrated into live cells, indicating that the conserved N-terminal region might be responsible for the membrane translocation. Using a series of deletion mutants, we demonstrated that the 11-amino acid imperfect repeats found in synuclein family members play a critical role in the membrane translocation of these proteins. We further demonstrated that fusion peptides

containing the 11-amino acid imperfect repeats of α-synuclein can transverse the plasma membrane, and that the membrane translocation efficiency is optimal when the peptide contains two repeat motifs. α-Synuclein appeared to be imported rapidly and efficiently into cells, with detectable protein in the cytoplasm within 5 min after exogenous treatment. Interestingly, the import of α-synuclein at 4°C was comparable to the import observed at 37°C. Furthermore, membrane translocation of α-synuclein was not significantly affected by treatment with inhibitors of endocytosis.

These results suggest that the internalization of α-synuclein is temperature-insensitive and occurs very rapidly via a mechanism distinct from normal endocytosis.

Key Words: α-synuclein, membrane translocation, 11-amino acid imperfect repeats, endocytosis

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I. INTRODUCTION

α-Synuclein is an acidic neuronal protein which is highly expressed in brain tissues and is primarily localized at the presynaptic terminals of neurons.[1-3] α-Synuclein is also expressed in hematopoietic cells,[4,5] and in other tissues, such as the heart, skeletal muscle, pancreas, and placenta, but it is less abundant than in the brain.[1,2] In addition to α-synuclein, β- and γ-synucleins constitute the synuclein family in humans.[2,6,7] α-Synuclein has been identified as a major component of intracellular fibrillar protein deposits (Lewy bodies) in several neurodegenerative diseases, including Parkinson's disease (PD), diffuse Lewy body disease and multiple systemic atrophy.[8-10,59] Particularly, accumulating evidence suggests that aggregation of α-synuclein may contribute to disease pathogenesis.[11-13] Although significant progress has been made in

understanding the pathological role of α-synuclein in neurodegenerative diseases,[14-17] the biological function of α-synuclein remains to be clarified. Recent studies suggest that α-synuclein may function in the regulation of synaptic plasticity, neural differentiation, and the regulation of dopamine synthesis.[18-22]

α-Synuclein is known to associate with membranous compartments in cultured cells and in brain tissue.[18,23-25] In vitro studies also showed that α-synuclein can interact with lipid layers, such as artificial liposomes containing phospholipids with acidic head groups, lipid droplets, and lipid rafts.[26-29] This binding is supposedly mediated by the 11-amino acid imperfect repeats at the N-terminal region of the protein.[25,30-32] The binding interaction between α-synuclein and lipid layers is dynamically regulated[28,33] and accompanied by conformational changes to the α-helical structure of α-synuclein.[26,30,31,34] Recently, Lee et al. reported that part of α-synuclein is either associated with the outer surface of vesicles or even localized in the lumen of vesicles, although the majority of the protein is localized in the cytoplasm.[35] Consistent with these observations, α-synuclein has been implicated in lipid metabolism and vesicle trafficking.[36,37]

Many studies have shown that α-synuclein can be secreted from cells, although the protein has no conventional signal sequence for secretion. For example, Borghi et al. showed that full-length α-synuclein might be released

from neurons into the extracellular space as part of its normal cellular processing.[38] Furthermore, α-synuclein can be detected in serum from both normal and PD subjects.[39,40] Recently, transfection studies also demonstrated that a portion of α-synuclein can be constitutively secreted from cells through an unconventional exocytic pathway.[35,41] α-Synuclein is also known to penetrate into cells by an unknown mechanism. In previous papers, we demonstrated that α-synuclein could penetrate inside neuronal cells by Rab5A-dependent endocytosis and induce cell death,[42] and that α-synuclein could penetrate into platelets and subsequently inhibit α-granule release upon stimulation.[43] In addition, Forloni et al. showed that the non-Aβ component of Alzheimer's disease amyloid (NAC) peptide derived from α-synuclein can penetrate inside cells and accumulate in the perinulcear region.[44]

As described above, many lines of evidence suggest that α-synuclein can be secreted from cells and can penetrate into them, although details of the mechanism are not known. In this study, we investigated the amino acid sequence motifs required for the membrane translocation of α-synuclein using a series of deletion mutants and recombinant peptides. We also addressed the mechanistic features of the cellular import of α-synuclein.

II. MATERIALS AND METHODS

1. Materials

DEAE-Sepharose Fast Flow, CM-Sepharose Fast Flow and Sephacryl S-200 High Resolution were purchased from Amersham biosciences (Uppsala, Sweden), and the Ni-NTA resin from Invitrogen (Carlsbad, CA). α-, β- and γ-synucleins were obtained from ATGen (Sungnam, Korea). β-D-thiogalactopyranoside (IPTG) was purchased from Sigma (St. Louis, MO). A fluorescein 5-isothiocyanate (FITC) labeling kit was purchased from Pierce (Rockford, IL). Brefeldin A (BFA) was purchased from Epicenter Technologies (Madison, WI), and Cytochalasin D was purchased from Sigma. Leupeptin, pepstatin, phenyl-methyl sulfonyl fluoride (PMSF) and imidazole were purchased from Boehringer Manheim (Manheim, Germany).

2. Construction of expression vectors for α-synuclein deletion mutants

A series of α-synuclein deletion mutant constructs were generated by PCR amplification of the α-synuclein gene with the specific primer sets described below. The protein coding regions of the N-terminal amphipathic portion (residues 1-60; Syn1-60 in Fig. 1A) and the N-terminal amphipathic portion plus the NAC peptide (residues 1-95; Syn1-95) were amplified by PCR

with the 5'-oligonucleotide primer CGAGCTC**CATATG**GATGTATTCATGA AAGG and the 3'-oligonucleotide primers CGAGCTC**AAGCTT**CTATTTGG TCTTCTCAGCCACTGTTGC and AGAGCTC**GAATTC**CTAGACAAAGCC AGTGGCTGCTGCAAT containing the restriction enzyme sites underlined above, respectively. The protein coding regions of the C-terminal acidic tail (residues 96-140; Syn96-140) and the NAC plus acidic tail (residues 61-140; Syn61-140) were amplified by PCR with the 5'-oligonucleotide primers CGATCGC**CATATG**AAAAAGGACCAGTTGGGCAAGAATGAA and CGA TCGC**CATATG**GAGCAAGTGACAAATGTTGGAGGAGCA, and the 3'-oligonucleotide primer GAGCTC**AAGCTT**TTAGGCTTCAGGTTCGTAGT CTTGATA containing the restriction enzyme sites underlined above, respectively. The amplified DNAs were gel purified, digested with appropriate enzymes, and then ligated into the pRSET A bacterial expression vector (Invitrogen) that had been digested with the appropriate restriction enzymes. pSynΔNAC, which lacks the NAC portion, was generated by consecutive cloning of the N-terminal amphiphatic portion (residues 1-60) and the C-terminal acidic tail (residues 96-140) into the pRSET A vector (Invitrogen). All constructs were confirmed by DNA sequencing.

3. Construction of expression vector for KTKEGV truncated mutants of α-synuclein

Four N-terminally truncated mutant constructs of α-synuclein,

(Syn16-140, Syn28-140, Syn38-140 and Syn51-140 in Fig. 4A) which serially lacked the 11-mer repeats were generated by the PCR amplification method. The protein coding regions were amplified by PCR with the 5'-oligonucleotide primers CGAGCTC**CATATG**GTGGCTGCTGCTGAGAAAACCAAA for pSyn16-140, CGAGCTC**CATATG**GAAGCAGCAGGAAAGACAAAAGAG for Syn28-140, CGAGCTC**CATATG**CTCTATGTAGGCTCCAAAACCAAG for pSyn38-140, CGAGCTC**CATATG**GGTGTGGCAACAGTGGCTGAG AAG for Syn51-140, and the 3'-oligonucleotide primer GAGCTC**AAGCTT** TTAGGCTTCAGGTTCGTAGTCTTGATA containing the restriction enzyme sites underlined above. The amplified DNAs were gel purified, digested with appropriate enzymes, and then ligated into the pRSET A vector that had been digested with the appropriate restriction enzymes. All constructs were gel purified and confirmed by DNA sequencing.

Three expression vectors which encode one, two, and three 11-amino acid repeat sequence motifs of α-synuclein, respectively, were similarly constructed by the PCR amplification method. The protein coding regions were amplified by PCR, and the amplified DNAs were ligated into the *NdeI* and *HindIII* sites of the pRSET A expression vector.

4. Bacterial expression and purification of mutant proteins

The α-synuclein deletion mutants were overexpressed in *Escherichia coli* (BL21), and the recombinant proteins were purified to apparent

homogeneity. This was done by taking advantage of the thermosolubility of the protein and using conventional column chromatography, as described previously.[43] Briefly, the transformed bacteria were grown in LB medium with 0.1 mg/ml ampicillin at 37°C to an A_{600} of 0.8, and then cultured for an additional 4 h after being induced with 0.5 mM IPTG. The cells were harvested by centrifugation at 10,000 rpm for 10 min, resuspended in 20 mM Tris-HCl pH 7.4, and then disrupted by ultrasonication. The supernatant was subjected to heat treatment at 100°C for 20 min. After removing the precipitates, the supernatant was purified with DEAE anion-exchange chromatography, and subsequently with CM cation-exchange chromatography in 20 mM Tris-Cl, pH 7.4. The bound proteins were eluted with a linear gradient between 0.1 M and 0.5 M NaCl at a flow rate of 1.5 mL/min. All proteins were further purified on an FPLC gel-filtration column pre-equilibrated with 20 mM Tris-HCl, pH 7.4. The proteins were concentrated and buffer-changed with a Centricon apparatus (Amicon, Beverly, MA). The proteins were quantitated with the BCA assay and stored at −30°C. The identity of each deletion mutant proteins was confirmed by mass spectroscopy (Table 1).

Table 1: Molecular weight, charged residue, isoelectric point (pI) and hydropathy values of α-synuclein and its deletion mutants.

Protein	Molecular weight (MW)		Charged Residues		pI Value*	Hydropathy Value*
	Predicted*	Mass Spectroscopy	Positive	Negative		
α-synuclein	14460.1	14462.23	15	24	4.67	-0.403
Syn1-60	6149.1	6150.44	11	7	9.52	-0.188
Syn1-95	9391.8	9392.72	12	9	9.26	0.148
Syn61-140	8460.1	8462.66	4	17	3.85	-0.533
Syn96-140	5217.5	5216.1	3	15	3.76	-1.491
SynΔNAC	11935.3	11935.42	14	23	4.63	-0.729

* The prediced molecular weight, the pI values and the hydropathy values were calculated by using the ProtParam program (www.expasy.ch).

5. Cell culture

Chinese hamster ovary cells (CHO-K1) and human leukemia K562 cells were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin, and maintained at 37°C in an atmosphere containing 5% CO_2. Human neuroblastoma SH-SY5Y cells and rat adrenal pheochromocytoma PC12 cells were routinely maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin in a humid atmosphere of 5% CO_2 at 37°C.

6. Western blot analysis

Western blot analysis was performed using anti α-synuclein antibody, as described previously.[43] Briefly, cells were lysed using the following lysis buffer: 50 mM Tris (pH 8.0), 110 mM NaCl, 5 mM EDTA, 1% Triton X-100, PMSF (100 μg/ml), 10% glycerol, 1.5 mM $MgCl_2$ and protease inhibitor cocktail (Roche). Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). Whole cell lysates containing 50 μg of protein were boiled in equal volumes of loading buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% of 2-β-mercaptoethanol). Proteins were separated electrophoretically on 12–15% denaturing polyacrylamide gels and subsequently transferred to Hybond ECL nitrocellulose membranes (Amersham, Uppsala, Sweden) using the BioRad Mini-Gel system(CA, USA). For immunoblotting, membranes were blocked with 5% non-fat dried milk in

Tris-buffered saline (TBS) for 1 h. Primary antibody against α-synuclein (Syn 211 for Fig.3B and Syn N-19 for Fig. 3C ; Santa Cruz Biotechnology, CA) was applied at a 1:1000 dilution for 1 h. After washing twice with TBS containing 0.05% Tween 20 (TTBS), secondary antibody (peroxidase-conjugated goat anti-mouse IgG for Fig. 3B, Zymed, CA; peroxidase-conjugated donkey anti-goat IgG for Fig. 3C, Santa Cruz Biotechnology, CA) was applied at a 1:2000 dilution for 1 h. Blots were washed twice in TTBS for 10 min, incubated in commercial enhanced chemiluminescence reagents (ECL, Pierce, Rockford, IL), and exposed to photographic film.

7. Preparation of FITC-labeled proteins

Proteins were labeled with N-Hydroxysuccinimide (NHS) - Fluorescein-isothiocyanate (FITC) according to the manufacturer's instruction (Pierce). Briefly, proteins (1 mg/ml in PBS buffer) were buffer-changed into borate buffer (pH 8.5) by using desalting columns. The fluorescent dye was dissolved in DMSO, and a 24-fold molar excess of the fluorescent dye was mixed rapidly with each protein solutions. The reaction mixtures were incubated for 1 h at room temperature, then overnight at 4°C in the dark. After coupling, protein solutions were loaded onto a Sephadex G-25 column to remove the unbound dyes. Fractions were carefully monitored for protein content by measuring the absorbance of each fraction at 280 nm. The labeled protein fractions were pooled and concentrated with a Centricon apparatus

(Amicon, Beverly, MA). Protein concentration was determined with a BCA assay kit (Pierce, Rockford, IL) using bovine-serum albumin as a standard. To standardize the labeled proteins, the efficiency of the FITC labeling was estimated from the absorbance at 495 nm and from the protein concentration. Labeled proteins were also separated on an SDS polyacrylamide gel and the fluorescence of each sample was additionally standardized on an image analysis system (Molecular Analyst II, version 1.5, Bio-Rad).

8. Protein internalization and visualization

Protein internalization was measured by flow cytometry and confocal microscopy on living cells. For flow cytometry, cells were seeded at 8 x 10^4 cells·cm^{-2} in 12-well plates and grown for 24 h in complete medium. Before incubation with the FITC-labeled proteins, the cells were washed and preincubated in DMEM or RPMI for 1 h at 37°C. Cells were then incubated with 5 μM FITC-labeled protein at either 37°C or 4°C for various periods of time, and then washed and placed in ice-cold PBS (pH 7.4). The cell pellet was washed twice before a 5 min incubation with trypsin (1 mg/ml) at 37°C. For determination of intracellular FITC fluorescence alone, cells were then washed once more with NaCl/Pi and incubated for 5 min with 150 μl of a freshly prepared 0.1% Tween 20 solution (pH 7.4). Cells were further washed and placed in ice-cold PBS. FITC-labeled proteins were excited at 488 nm and fluorescence was measured at 525 nm using a FACScalibur (Becton Dickinson)

yielding the mean fluorescence intensity per cell, which is a measure of the amount of cell-associated peptide. Data are reported at least triplicate samples.

For confocal microscopic analysis, cells were seeded at 1 x 10^4 cells·cm^{-2} in Laboratory-Tek® German borosilicate coverglass with eight chambers (Nalge Nunc International) and grown for 48 h in complete medium. Subsequently, cells were washed and preincubated in 200 μl DMEM or RPMI (30 min at 37°C). Cells were subsequently incubated with the FITC-labeled proteins for 30 min at either 4°C or 37°C, washed, and placed in 300 μl ice-cold PBS (pH 7.4). For determination of the intracellular FITC fluorescence alone, cells were placed at 4°C and incubated for various times with 50 μl of a freshly prepared 0.1% Tween 20 solution (pH 7.4). Cells were further washed and placed in ice-cold PBS. Photographs were taken with a Zeiss model LSM510 invert on the base of a Zeiss Axiovert 100 microscope (Carl Zeiss B.V., Sliedrecht, Netherlands).

Fluorescence intensity was quantitated using Metamorph software (version 4.6r5, Universal Imaging Corporation, Downingtown, PA). Mean fluorescence intensity was measured on each image, and total mean fluorescence was calculated for each sample. To exclude background fluorescence, mean fluorescence intensity of the negative images was subtracted from those of positive images.

III. RESULTS

1. Intracellular delivery of α-, β- and γ-synucleins

Recent studies showed that α-synuclein can translocate across the plasma membrane by a mechanism that has yet to be elucidated. As a first step in understanding the membrane translocation mechanism of α-synuclein, we checked whether other synuclein family members (β- and γ-synucleins) could also translocate across the cell membrane. For this purpose, purified recombinant α-, β- and γ-synucleins were FITC-labeled and exogenously added into cell culture media. Cells were then harvested and the intracellular delivery of FITC-labeled synucleins was visualized using a confocal microscope. As shown in Fig. 1A, not only α-synuclein, but also β- and γ-synucleins penetrated the cell membrane and were localized primarily in the cytoplasm, although internalized proteins might also be localized in vesicles. Interestingly, unlike other membrane-permeating proteins, synuclein family members did not appear to translocate across the nuclear membrane. Membrane translocation of α-, β- and γ-synucleins was also confirmed by flow cytometric analysis (Fig. 1B). The membrane translocation efficiencies of all the synuclein family members appeared to be slightly different (Fig. 1C), and the membrane translocation of these proteins can be detected even at a concentration of 0.1 μM (data not shown). The C-terminal acidic tails of synuclein family members are very diverse in size as well as in sequence, although the N-terminal amphipathic

regions of the synuclein family members are well conserved among species. Thus, it seems likely that the N-terminal part (residues 1-95) may play a critical role in the membrane translocation of synuclein family members.

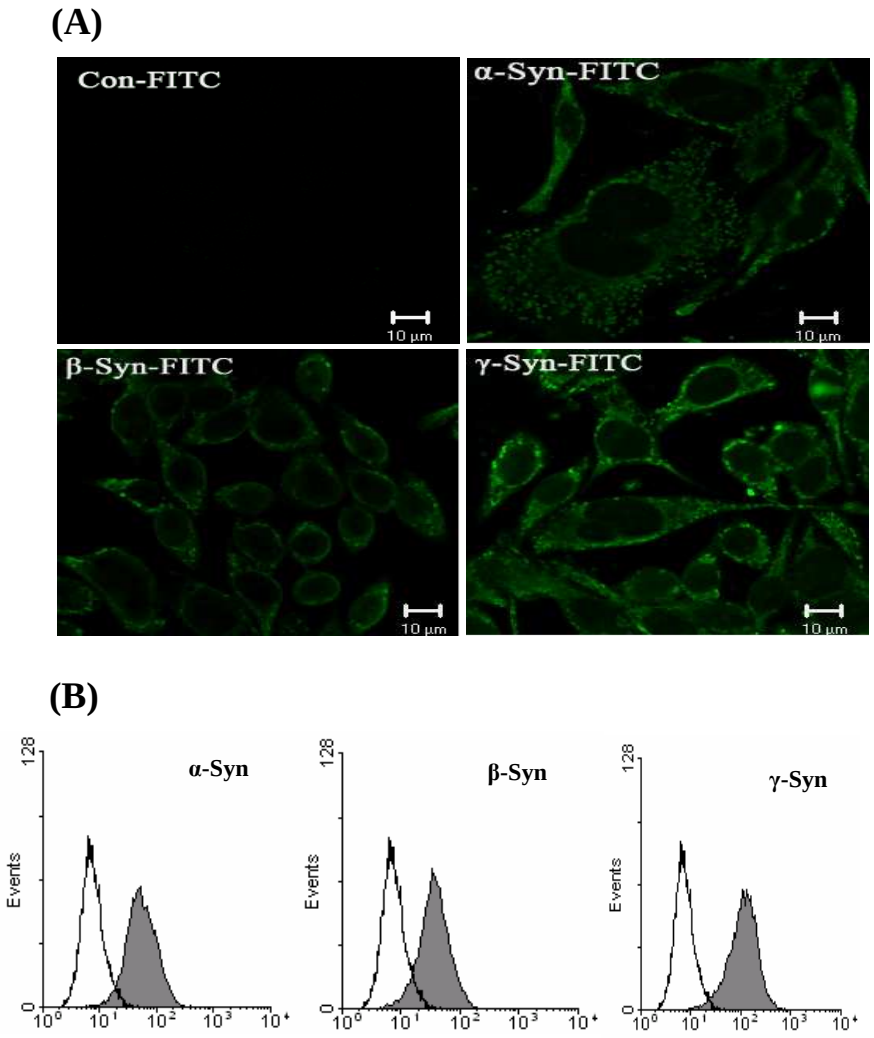


Figure 1-1. Intracellular delivery of α-, β- and γ-synucleins.

(A) Confocal microscopic analysis. CHO-K1 cells were incubated for 30 min with PBS alone (Con-FITC), 5 μM of FITC-labeled α-synuclein (α-Syn-FITC), 5 μM of FITC-labeled β-synuclein (β-Syn-FITC) and 5 μM of FITC-labeled γ-synuclein (γ-Syn-FITC), respectively. Cells were washed extensively with PBS prior to confocal microscopic analysis.

(B) Flow cytometric analysis. CHO-K1 cells were incubated with 5 μM of FITC-labeled α-synuclein (α-Syn), β-synuclein (β-Syn) and γ-synuclein (γ-Syn), respectively, for 30 min. Cells were washed extensively with a trypsin-EDTA solution and with PBS prior to flow cytometry analysis. Solid white histograms are untreated control cells, and solid grey histograms are cells treated with FITC-labeled synucleins.

(C)

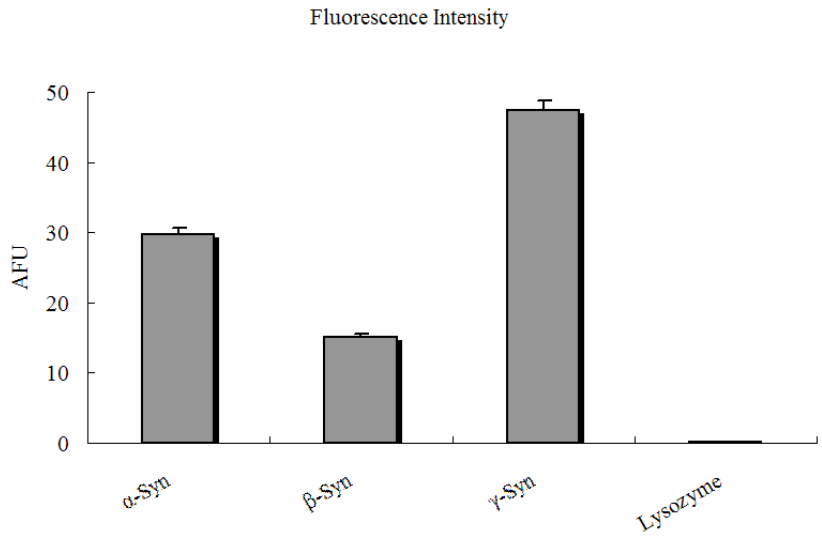


Figure 1-2. Intracellular delivery of α-, β- and γ-synucleins.

(C) Average cell fluorescence intensity. Average cell fluorescence intensity measurements of 30 cells labeled with FITC over the total section area ($P<0.05$). Values are expressed as mean ± SE.

2. The N-terminal and NAC regions contain the motifs for the intracellular delivery of α-synuclein

α-Synuclein consists of three distinct regions: the N-terminal amphipathic region (residues 1-60), the hydrophobic NAC region (residues 61-95), and the C-terminal acidic region (residues 96-140). To investigate which region is responsible for the membrane translocation of α-synuclein, we produced five deletion mutant constructs (Fig. 2A). Syn1-60 encodes the entire region of the amphipathic region, Syn1-95 encodes the amphipathic and NAC regions, Syn61-140 encodes the NAC and acidic tail regions, Syn96-140 encodes the acidic tail region, and SynΔNAC lacks the NAC region. The deletion mutant proteins were overexpressed in E. coli and purified using conventional column chromatography methods. The protein samples used in this study were highly purified as determined by SDS-PAGE (Fig. 2B), and the characteristics of these mutant proteins are summarized in Table 1.

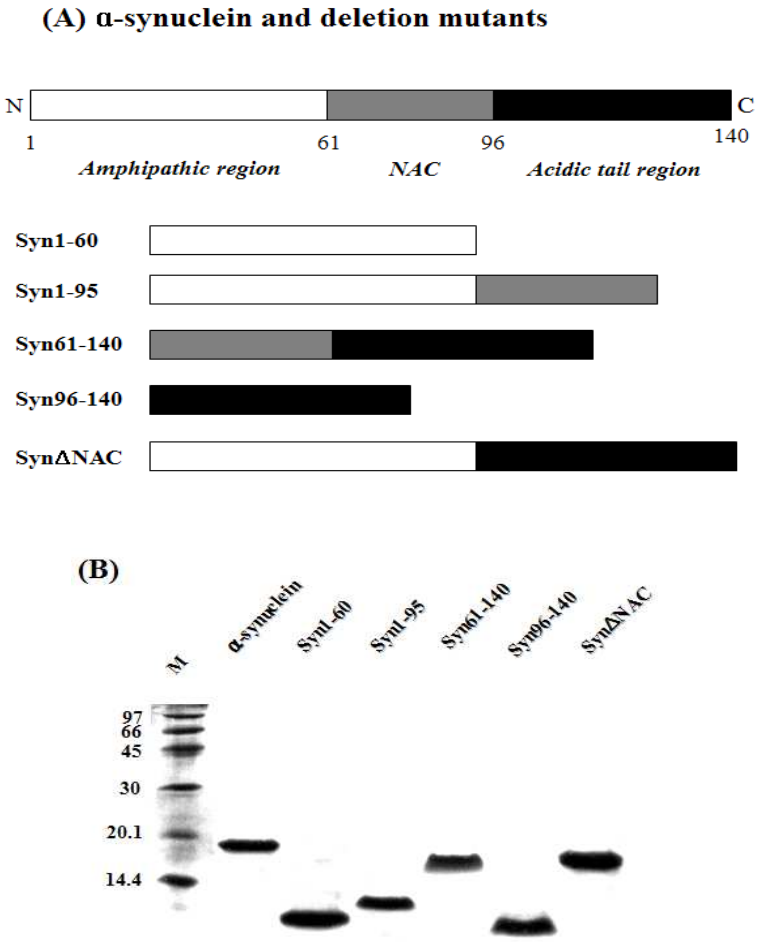


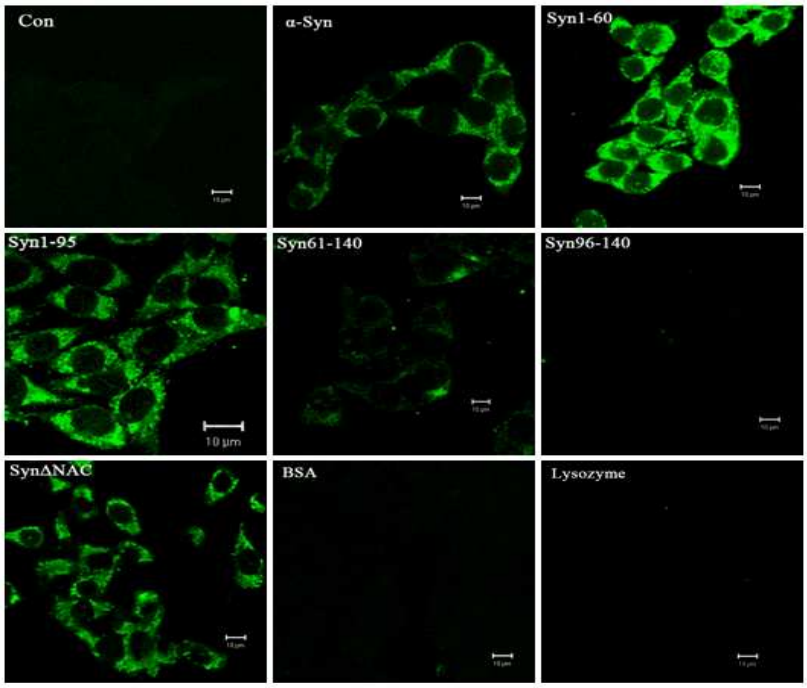
Figure 2-1. Membrane translocation of α-synuclein and its deletion mutants.

(A) A schematic diagram of α-synuclein and deletion mutant constructs. α-Synuclein consists of three distinct regions: the N-terminal amphipathic region (residues 1-60), the hydrophobic NAC region (residues 61-95), and the C-terminal acidic tail (residues 96-140). Five deletion mutant constructs encoding the amphipathic region (Syn1-60), the amphipathic region and the NAC region (Syn1-95), the NAC and acidic tail regions (Syn61-140), the acidic tail region (Syn96-140), and the NAC deleted α-synuclein named SynΔNAC were used in this study.

(B) SDS PAGE analysis of the purified α-synuclein and its deletion mutant proteins. The purified proteins were analyzed on a 15% SDS polyacrylamide gel, and the protein bands were stained with Coomassie Brilliant Blue R250.

We first investigated the intracellular delivery of the α-synuclein deletion mutants using confocal microscopy. α-Synuclein and its deletion mutants were labeled with FITC, and CHO-K1 cells were treated with the FITC-labeled proteins. Cells were harvested and then visualized using confocal microscopy. As shown in figure 2C, FITC-labeled α-synuclein, Syn1-60, Syn1-95 and SynΔNAC displayed bright fluorescence, indicating that FITC labeled proteins were delivered into the cells. Interestingly, the fluorescence of WT, Syn1-60, Syn1-95 and SynΔNAC proteins appeared to be localized mainly in the cytoplasm and not in the nucleus. The relative fluorescence intensity of Syn61-140 was lower, and that of Syn96-140, which lacks both the N-terminal amphipathic and the hydrophobic NAC regions, was almost invisible (Figs. 2C and 2D). Similar results were obtained in SHSY-5Y cells and PC12 cells (data not shown).

(C)



(D)

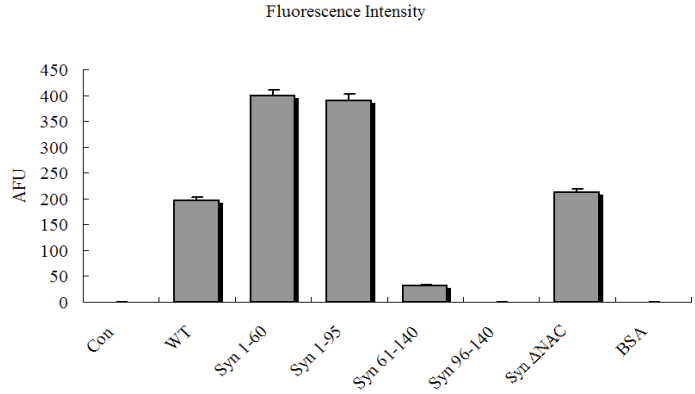


Figure 2-2. Membrane translocation of α-synuclein and its deletion mutants.

(C) The internalization of α-synuclein and deletion mutants analyzed by confocal microscopy. Cells were incubated with 5 μM of FITC-labeled proteins for 30 min at 37°C. BSA and Lysozyme were used as negative controls. Cells were not fixed to avoid fixation artifacts, but were washed extensively with PBS before confocal microscopic analysis. The scale bar represents 10 μm.

(D) Average cell fluorescence intensity. Average cell fluorescence intensity measurements of 30 cells labeled with FITC over the total section area (P<0.05). Values are expressed as mean ± SE.

Membrane translocation of α-synuclein deletion mutants was also demonstrated by flow cytometric analysis (Fig. 3A). Consistent with the confocal microscopic studies mentioned above, flow cytometric analysis of cells incubated with WT, Syn1-60 Syn1-95, Syn61-140 and SynΔNAC proteins showed a bright green fluorescence. However, control cells and the cells incubated with Syn96-140 did not show any fluorescence.

Membrane translocation of α-synuclein deletion mutants was finally verified by western blot analysis. As shown in Fig. 3B, exogenously added α-synuclein, Syn61-140 and SynΔNAC appeared to penetrate the cell membrane; however, Syn96-140 did not. In addition, the membrane translocation of the α-synuclein deletion mutants was observed in every cell type tested, such as PC12, K562, SHSY-5Y, and CHO-K1 cells (Fig. 3B). Because we used a monoclonal antibody that can only detect the C-terminal acidic tail of α-synuclein, membrane translocation of Syn1-60 and Syn1-95 was not demonstrated in this experiment. To demonstrate the membrane translocation of Syn1-60 and Syn1-95, polyclonal antibodies were used for western blot analysis. As shown in Fig. 3C, Syn1-60, Syn1-95, and SynΔNAC appeared to penetrate cell membranes. Taken together, our data demonstrated that α-synuclein, Syn1-60, Syn1-95, Syn61-140, and SynΔNAC were able to penetrate cell membrane, but Syn96-140 could not. This suggests that the N-terminal and NAC regions contain the motifs for the intracellular delivery of α-synuclein, although the NAC is less effective than the N-terminal region.

(A)

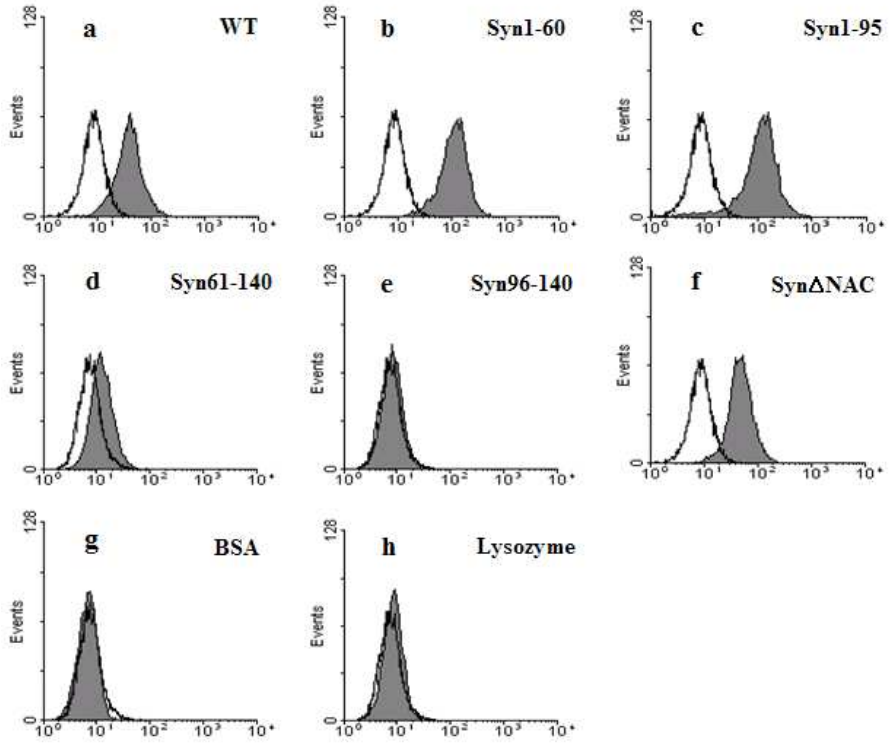


Figure 3-1. Flow cytometry and Western blot analysis for the membrane translocation of α-synuclein and its deletion mutants.

(A) Flow cytometry analysis of cells incubated with FITC-labeled proteins. CHO-K1 cells were incubated with FITC labeled WT α-synuclein (a), FITC-labeled Syn1-60 (b), FITC-labeled Syn1-95 (c), FITC-labeled Syn61-140 (d), FITC-labeled Syn96-140 (e), FITC-labeled SynΔNAC (f), FITC labeled-BSA (g) and FITC labeled-lysozyme (h) for 30 min at 37°C. Cells were washed extensively with a trypsin-EDTA solution and with PBS prior to flow cytometry analysis. Solid white histograms are untreated control cells, and solid grey histograms are cells treated with FITC-labeled proteins.

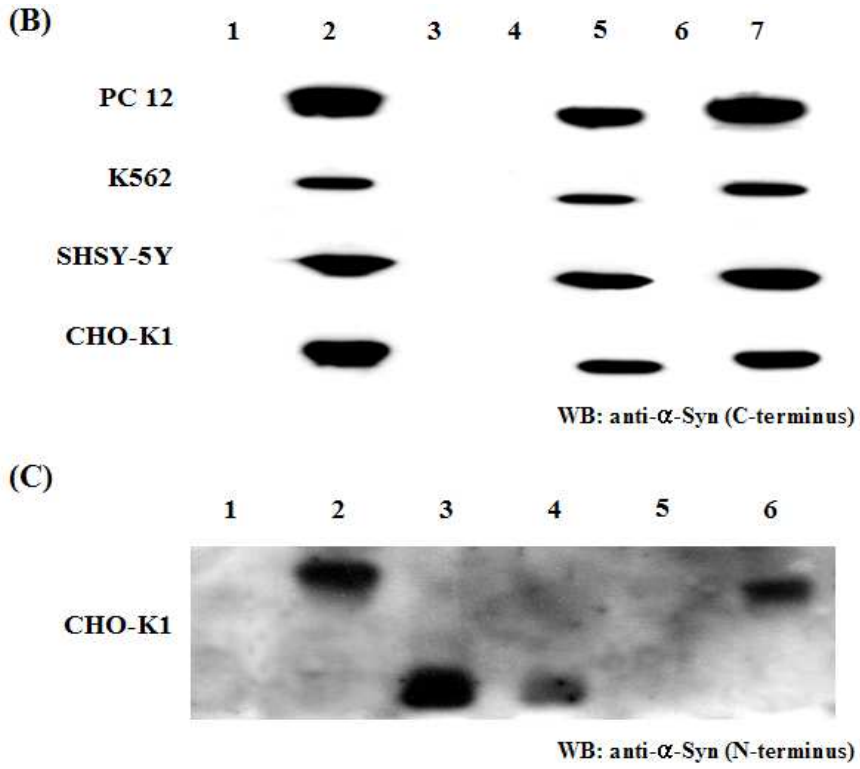


Figure 3-2. Flow cytometry and Western blot analysis for the membrane translocation of α-synuclein and its deletion mutants.

(B) Western blot analysis of the membrane translocation of exogenously added α-synuclein and its deletion mutants with a monoclonal antibody (Syn211). Cells were treated with PBS alone (lane 1), 10 μM WT α-synuclein (lane 2), 10 μM Syn1-60 (lane 3), 10 μM Syn1-95 (lane 4), 10 μM Syn61-140 (lane 5), 10 μM Syn96-140 (lane 6) and 10 μM SynΔNAC (lane 7) for 1 h at 37°C. Cells were carefully washed with a trypsin-EDTA solution and with PBS before the preparation of cell lysates.

(C) Western blot analysis of the membrane translocation of exogenously added α-synuclein and its deletion mutants with polyclonal antibodies (SynN-19). Cells were treated with PBS alone (lane 1), 10 μM WT α-synuclein (lane 2), 10 μM Syn1-60 (lane 3), 10 μM Syn1-95 (lane 4), 10 μM Syn96-140 (lane 5) and 10 μM SynΔNAC (lane 6) for 1 h at 37°C. Cells were carefully washed with a trypsin-EDTA solution and with PBS before the preparation of cell lysates.

3. Membrane translocation of a series of N-terminally truncated mutants

The N-terminal and NAC regions (amino acid residues 1-95) of α-synuclein contain seven 11-amino acid imperfect repeats with a highly conserved hexamer motif (KTKEGV). The repeat sequence motifs are also found in β- and γ-synucleins. Interestingly, these repeat regions are structurally homologous to the amphipathic, lipid-binding α-helical domains of apolipoproteinA-I (apoA-I). Apo A-I has eight 22-mer amphipathic helical repeat domains as the major protein component of human HDL. A recent report suggested that the lipid binding or membrane interaction is affected by partial deletion of the amphipathic helical domains of ApoA-I. Based on this information, we investigated whether the 11-amino acid imperfect repeats of α-synuclein were responsible for its membrane translocation. For this purpose, we produced a series of N-terminally truncated forms of α-synuclein (Fig. 4A). Δ1Syn lacks one repeat sequence motif, Δ2Syn lacks two repeat sequence motifs, Δ3Syn lacks three repeat sequence motifs, and Δ4Syn lacks four repeat sequence motifs at the N-terminus. These N-terminally truncated forms were purified from *E. coli*, and the purified proteins were FITC-labeled (Fig. 4B).

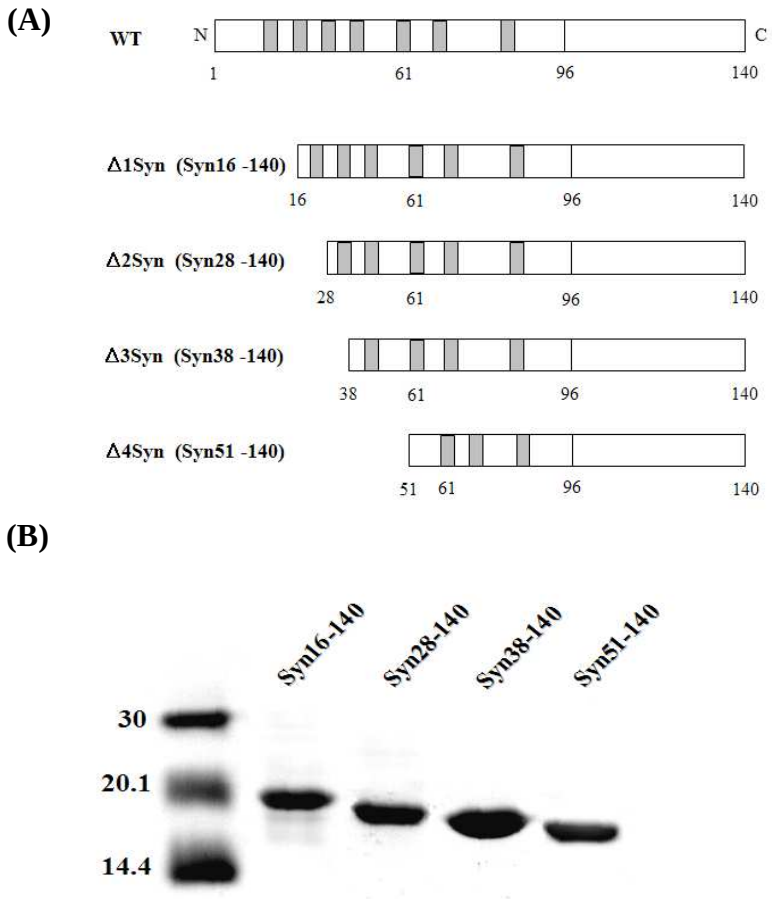


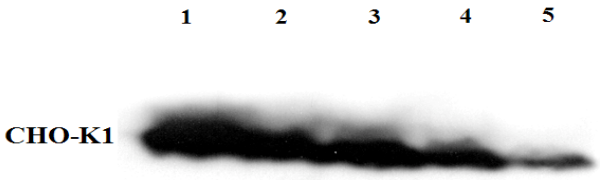
Figure 4-1. Membrane translocation of a series of N-terminally truncated α-synuclein mutants.

(A) Schematic diagrams of full-length and N-terminally truncated forms of α-Synuclein. Shaded regions (light gray) represent the 11-mer repeats. Δ1Syn lacks the first repeat found in α-synuclein (residues 1-15) ; Δ2Syn lacks the first two repeats (residues 1-27) ; Δ3Syn lacks the first three repeats (residues 1-37) ; and Δ4Syn lacks the first four repeats (residues 1-50).

(B) SDS PAGE of the purified N-terminally truncated mutant proteins. The purified proteins were analyzed on a 13.5% SDS polyacrylamide gel, and the protein bands were stained with Coomassie Brilliant Blue R250.

The membrane translocation abilities of these mutant proteins were assessed by Western blot analysis. As shown in Fig. 4C, all the truncated forms appeared to be delivered into CHO-K1 cells, but the translocation efficiencies were different. As the more repeated sequence motifs were deleted, membrane translocation efficiency decreased (Fig. 4C). Confocal microscopic studies also demonstrated that the membrane translocation efficiency of the N-terminally truncated forms of α-synuclein was proportional to the number of repeat sequence motifs present (Fig. 4D). These results indicate that the 11-amino acid imperfect repeats of synuclein family members play a critical role in the membrane translocation of these proteins.

(C)



(D)

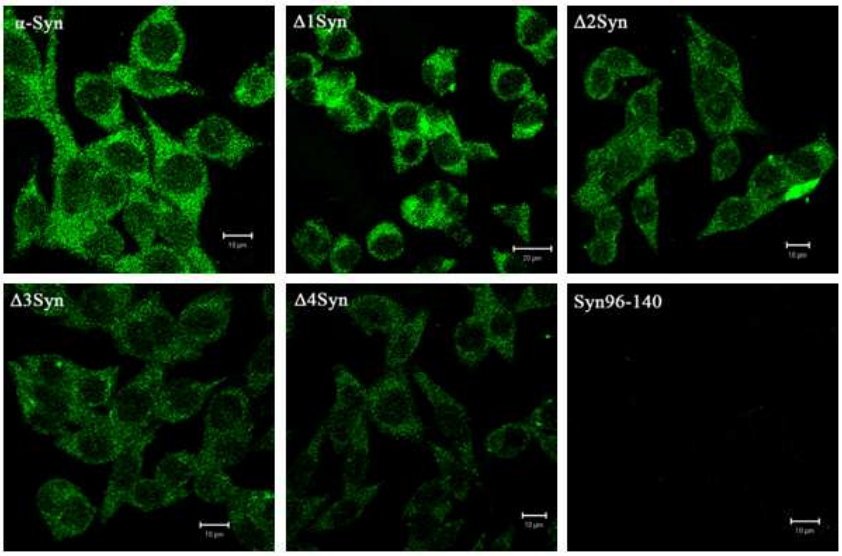


Figure 4-2. Membrane translocation of a series of N-terminally truncated α-synuclein mutants.

(C) Western blot analysis of the membrane translocation of exogenously added α-synuclein and N-terminally truncated mutants. CHO-K1 cells were treated with 10 μM of each proteins for 1 h at 37°C. Cells were carefully washed with a trypsin-EDTA solution and with PBS before the preparation of cell lysates. Lanes: lane 1, WT ; lane 2, Δ1Syn ; lane 3, Δ2Syn ; lane 4, Δ3Syn ; and lane 5, Δ4Syn .

(D) Confocal microscopic analysis of the membrane translocation of exogenously added α-synuclein and N-terminally truncated mutants. Cells were incubated with 5 μM of FITC-labeled proteins for 30 min at 37°C. Syn 96-140 was used as a negative control. Cells were not fixed to avoid fixation artifacts, but were washed extensively with PBS before confocal microscopic analysis.

4. Sequence motifs for the membrane translocation of α-synuclein

To directly demonstrate that the 11-amino acid repeat sequences can transverse the plasma membrane, we produced a series of fusion peptides (Fig. 5A) that contained the repeat sequence motif using recombinant DNA technology. 1XR contains a single repeat motif (amino acids 10-20 of α-synuclein), 2XR contains two repeat motifs (amino acid residues 10-31), and 3XR contains three repeat motifs (amino acid residues 10-42).

(A)

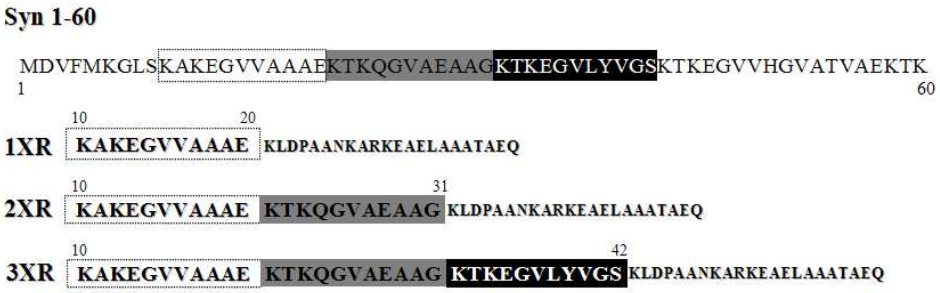
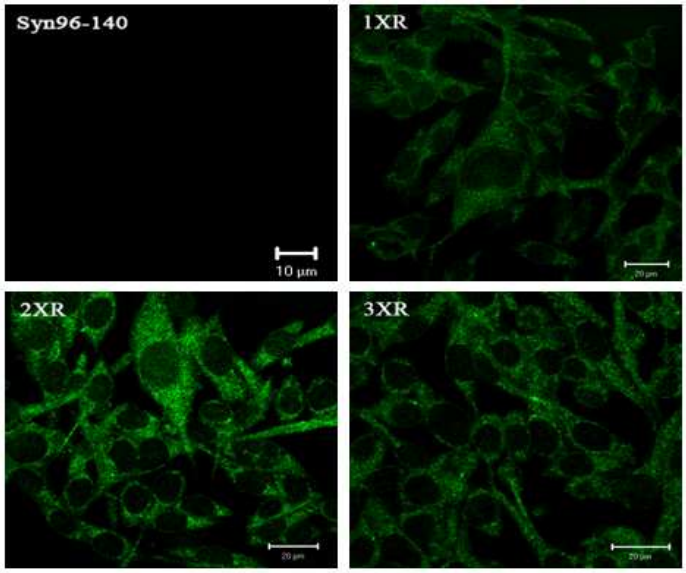


Figure 5-1. Transduction efficiency of fusion peptides containing the 11-mer repeat(s).

(A) Amino acid sequences of fusion peptides containing the 11-mer repeat(s). 1XR contains one 11-mer repeat, 2XR contains two repeats, and 3XR contains three repeats derived from the N-terminal region of α-synuclein. The C-terminal parts of fusion peptides were derived from the pRSET A expression vector.

These fusion peptides were expressed in *E. coli*, and the peptides were FITC-labeled. Using these peptides, membrane translocation abilities were compared using confocal microscopy. As shown in Fig. 5B, all the peptides appeared to be delivered into CHO-K1 cells, but the translocation efficiencies were different. Interestingly, the fluorescence intensity of 2XR treated cells was higher than those of 1XR and 3XR treated cells, suggesting that the membrane translocation efficiency of 2XR is the greatest. Similar results were obtained using flow cytometry studies (Fig. 5C). These results indicate that the 11-amino acid imperfect repeats of α-synuclein can transverse the plasma membrane, and the membrane translocation efficiency is optimal when the peptide contains two repeat motifs.

(B)



(C)

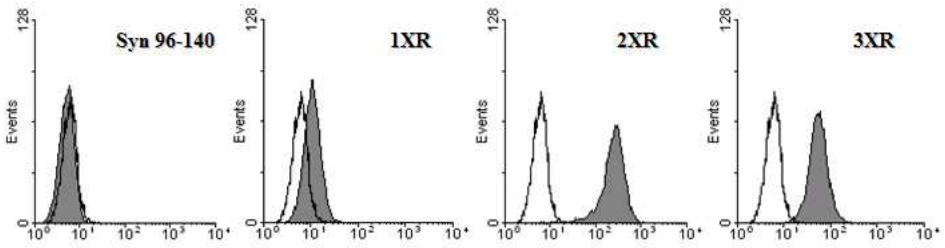


Figure 5-2. Transduction efficiency of fusion peptides containing the 11-mer repeat(s).

(B) The internalization of fusion peptides containing the 11-mer repeat(s) analyzed by confocal fluorescence microscopy. Cells were incubated with 5 μM of FITC-labeled peptides for 30 min at 37°C. Syn96-140 was used as a negative control. Cells were not fixed to avoid fixation artifacts, but were washed extensively with PBS before confocal microscopic analysis.

(C) Flow cytometry analysis of cells incubated with FITC-labeled fusion peptides. CHO-K1 cells were incubated with FITC-labeled 1XR, 2XR, 3XR, or Syn96-140 for 30 min at 37°C. Cells were washed extensively with a trypsin-EDTA solution and with PBS prior to flow cytometry analysis. Solid white histograms are untreated control cells, and solid grey histograms are cells treated with FITC-labeled peptides. Syn 96-140 was used as a negative control.

5. Kinetic and mechanistic features of the membrane translocation of α-synuclein

We next addressed the kinetics of and mechanism behind α-synuclein uptake by cells. CHO-K1 cells were incubated with FITC-labeled α-synuclein at 37°C or 4°C for various time periods. The time course of α-synuclein uptake showed that the protein was imported rapidly and efficiently, with detectable protein in the cytoplasm within 5 min of exogenous treatment (Fig. 6A). Furthermore, the import of α-synuclein at 4°C was comparable to that detected at 37°C by confocal microscopy (Fig. 6A). Flow cytometric studies also resulted in the same conclusion (Fig. 6B). These results suggest that the typical endocytosis pathway might not be responsible for the membrane translocation of α-synuclein.

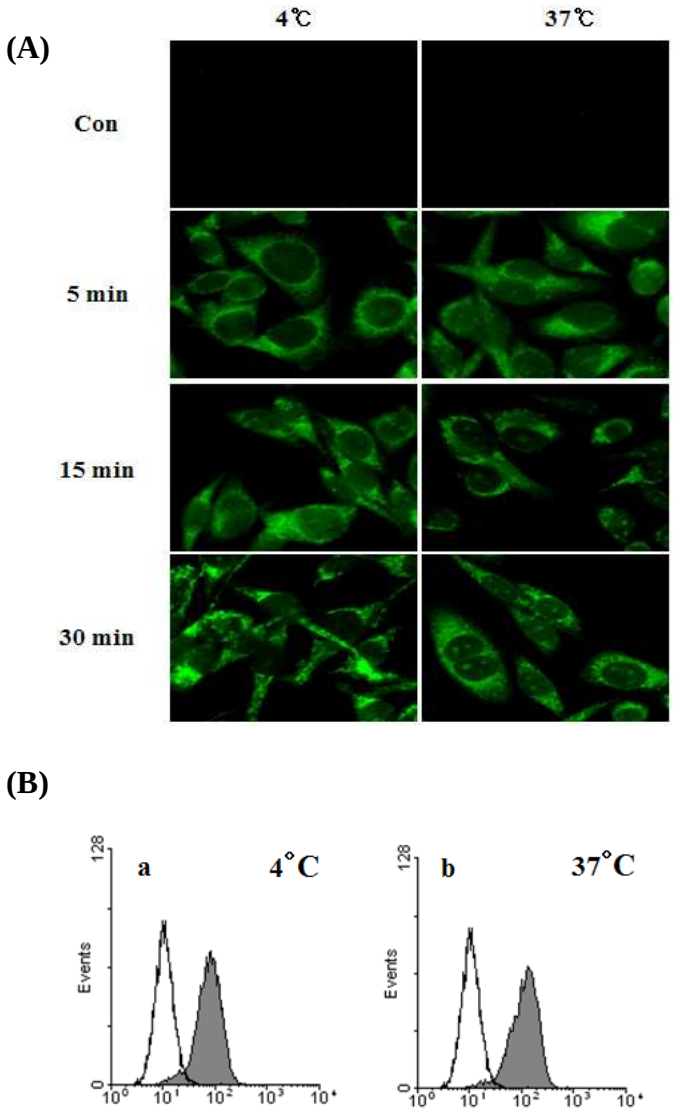


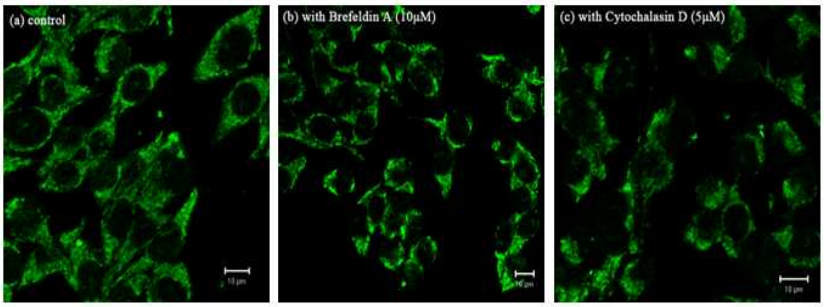
Figure 6. Effects of incubation time and temperature on the membrane permeabilization of α-synuclein.

(A) Confocal microscopic analysis. CHO-K1 cells were incubated for 5 min, 15 min, 30 min or 60 min with 5 μM of FITC-labeled α-synuclein at 4°C and 37°C, respectively.

(B) Flow cytometric analysis. CHO-K1 cells were incubated with 5 μM of FITC-labeled α -synuclein for 5 min at 4°C (a), and at 37°C (b). Cells were washed extensively with a trypsin-EDTA solution and with PBS prior to flow cytometry analysis. Solid white histograms are untreated control cells, and solid grey histograms are cells treated with FITC-labeled α-synuclein.

We further examined the effects of endocytosis inhibitors on the membrane translocation of α-synuclein. To investigate the role of the Golgi in α-synuclein transport, CHO-K1 cells were pretreated with Brefeldin A, an inhibitor of trans-Golgi transport,[50] and incubated with FITC-labeled α-synuclein for 30 min. Confocal microscopic observation indicated that the import of α-synuclein was minimally altered by Brefeldin A treatment (Fig. 7A). Internalization of α-synuclein was also minimally altered by pretreatment with Cytochalisin D (Fig, 7A), a microfilament-disrupting drug.[51] In addition, flow cytometric analysis of the cells resulted in the same conclusion (Fig, 7B). Taken together, these results suggest that the internalization of α-synuclein is temperature-insensitive and occurs very rapidly via a route distinct from normal endocytosis.

(A)



(B)

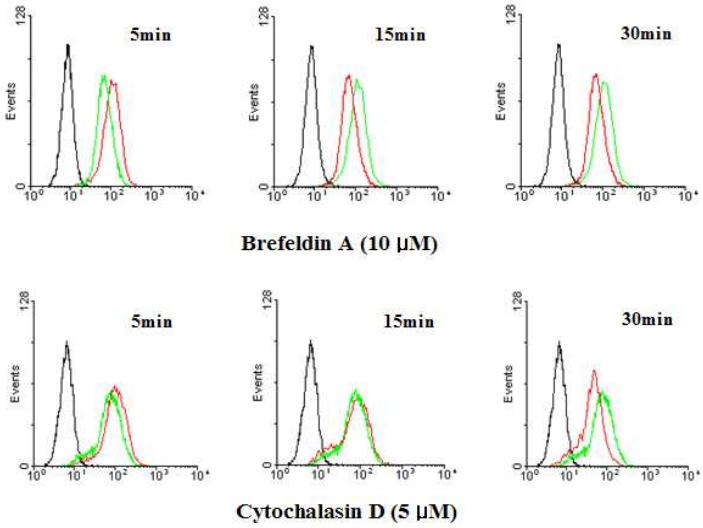


Figure 7. Effects of endocytosis inhibitors on the internalization of α-synuclein.

(A) CHO-K1 cells were pretreated with 10 μM of Brefeldin A (b) or 5 μM of Cytochalasin D (c) for 30 min, and then FITC-labeled α-synuclein was added to the medium. After 30 min incubation, cells were washed and observed on a confocal fluorescence microscopy. Control cells were treated with PBS only (a).

(B) CHO-K1 cells were pretreated with (red curves) or without (green curves) inhibitors for indicated time periods, and then FITC-labeled α-synuclein was exogenously added to the medium. After 30 min incubation, cells were extensively washed with a trypsin-EDTA solution and with PBS, and analyzed by flow cytometry. Solid white histograms are untreated control cells, and red and green histograms are cells treated with FITC-labeled α-synuclein.

IV. DISCUSSION

Many proteins with no signal sequence can be secreted through an unconventional exocytosis pathway independently of the ER/Golgi pathway.[52,53] α -Synuclein is also known to be secreted into cerebral spinal fluid (CSF) and plasma.[38-40] Furthermore, exogenous α–synuclein can be imported into cells.[42,43] In this study, we demonstrated that the N-terminal amphipathic (a.a. residues 1-60) and the NAC peptide (a.a. residues 61-95) regions are responsible for the membrane translocation of α-synuclein. Particularly, the 11-amino acid imperfect repeat sequences in these regions appear to mediate the import of α-synuclein into cells. These sequence motifs are distinct from those of other protein transduction domain (PTD) containing proteins, including Tat and VP22 (discussed below in detail). However, mechanistic features of the membrane translocation of α-synuclein appear to be very similar to other PTD containing proteins. These results extend our understanding of the secretary proteins lacking signal sequences, particularly of the PTD containing protein family.

Although α-synuclein does not possess a hydrophobic N-terminal signal sequence for secretion, earlier studies demonstrated that α-synuclein is secreted in both PD patients and in normal subjects.[38-40] Secreted α-synuclein can be detected at nanomolar concentrations in the CSF and blood. Interestingly, the blood levels of α-synuclein have been shown to be increased in familial PD

patients with an α-synuclein gene triplication.[40] α-synuclein secretion has also been demonstrated in vitro by transfection studies.[35,41] Particularly, Lee et al. demonstrated that a portion of cellular α-synuclein is present in vesicles and is secreted from cells through an unconventional exocytic pathway in a constitutive manner.[35] Secretion of α-synuclein was temperature sensitive, but was not affected by BFA treatment, suggesting that an unconventional exocytosis mechanism might be involved.

In this study, we showed that α-synuclein can be translocated into adrenal pheochromocytoma cells (PC12), neuronal cells (SH-SY5Y), hematopoietic cells, and Chinese hamster ovary cells (CHO-K1). Previous studies also showed that α-synuclein can penetrate into undifferentiated neuronal cells and platelets.[42,43] These results suggest that the membrane translocation of α-synuclein is not specific to certain cell types. If α-synuclein uses a specific receptor for its import into cells, penetration of α-synuclein should be limited to cell types expressing the receptor(s). Therefore, it seems highly likely that α-synuclein may bind to common molecules on the cell surface. Since the N-terminal region of α-synuclein is known to interact with lipid layers in vitro as well as in vivo,[30-32] we propose that the interaction between α-synuclein and the plasma membrane is an essential step for the membrane translocation of α-synuclein. Transfection studies demonstrated that the secretion of α-synuclein is also not specific to certain cell types.[35,41] Overexpressed α-synuclein can even be secreted from yeast[54] and from *E. coli*.

(authors' unpublished results). Interestingly, Lee et al. reported that a portion of α-synuclein is stored in the lumen of vesicles in the cytoplasm, and that the α-synuclein in vesicles might be secreted through an unconventional exocytosis pathway. These results suggest that the interaction between α-synuclein and vesicle membranes is critical for the translocation of α-synuclein into vesicles, and presumably for the subsequent secretion process. Thus, our data clearly indicate that the 11-amino acid imperfect repeat motifs are responsible for the membrane translocation of α-synuclein, i.e., both secretion and penetration.

The 11-amino acid repeat motifs contain a well-conserved core sequence of KTKEGV, and these repeats are also present in the N-terminal region of β- and γ-synuclein. The 11-mer repeats of α-synuclein are supposed to form amphiphatic α-helices when the protein is bound to lipid molecules.[31] Although no significant sequence homology is found, the repeat region is structurally homologous to the lipid binding domain of exchangeable apolipoproteins, in which the repeat sequence motifs also form amphipathic α-helices.[48] In this study, all the α-synuclein deletion mutants and recombinant peptides that contained one or more of the repeat sequence motif(s) appeared to translocate the cell membrane (Fig. 2, 4, 5). On the other hand, Syn96-140 and control proteins, which have no such motif, did not permeate into cells (Fig. 2). Taken together, the data suggest that the repeat sequence motifs bind to the lipid bilayer, and the binding interaction might be critical for the membrane translocation of synuclein proteins.

We demonstrated that the cellular uptake of α-synuclein could be detected within 5 min, and that this uptake was not inhibited when cells were incubated at 4˚C. It is well established that receptor-mediated endocytosis is blocked by incubation at 4˚C.[55] The cellular uptake of α-synuclein also appeared to be insensitive to treatment with the general endocytosis inhibitors, Brefeldin A and Cytochalasin D. Brefeldin A is known to disrupt the Golgi apparatus and inhibit transport through the Golgi[50] whereas Cytochalasin D is a microfilament-disrupting drug.[51] Therefore, these results suggest that internalization of α-synuclein is temperature-insensitive and occurs via a route distinct from normal endocytosis, as is the case for other PTDs.

Basic peptides derived from translocatory proteins, such as the Tat protein, the Antennapedia protein and VP22, and even many arginine-rich peptides have been reported to have a membrane permeability and a carrier function for intracellular cargo delivery.[56-58] These peptides are called protein transduction domains (PTDs). Like other translocatory proteins and PTDs derived from them, α-synuclein appears to pass through the cell membrane in an energy-independent, non-endocytic manner, at temperatures as low as 4˚C. Unlike other translocatory proteins, however, α-synuclein does not appear to penetrate into the nucleus. Translocated α-synuclein is localized primarily in the cytoplasm. Furthermore, the amino acid sequence of the α-synuclein's PTD (STD) is distinct from those of other PTDs. No significant amino acid sequence homology exists between STD and other PTDs, but a common feature is that

they are all basic peptides. STD is composed of 11-mer repeats that contain no arginine residues. Instead, each 11-mer repeat includes one or two lysine residues. The 11-mer repeats are rather structurally homologous to those found in apolipoproteins, but it is not known whether the apolipoproteins are actually able to tanslocate across cell membranes.

In summary, not only α-synuclein, but also β- and γ-synucleins can penetrate into live cells, and the 11-amino acid imperfect repeats of synuclein family members appear to play a critical role in the membrane translocation of these proteins. Fusion peptides containing the 11-amino acid imperfect repeats of α-synuclein (STD) can transverse the plasma membrane, and the membrane translocation efficiency is optimal when the peptide contains two repeat motifs. Internalization of α-synuclein is temperature-insensitive and occurs very rapidly via a route distinct from normal endocytosis.

These features suggest that the synuclein proteins will create a useful model for analyzing unconventional import and export pathways in mammalian cells. Furthermore, STD could be a potential carrier for the efficient delivery of peptides that do not permeate living cells.

V. CONCLUSION

In this study, we investigated amino acid sequence motifs and mechanistic features for the membrane translocation of α-synuclein which has not been well known until now.

1. The N-terminal region may play a critical role for the membrane translocation of synuclein family members.

2. The N-terminal and NAC regions are critical for the intracellular delivery of α-synuclein.

3. The 11-amino acid imperfect repeats of synuclein family members play a critical role in the membrane translocation of these proteins.

4. The 11-amino acid imperfect repeats of α-synuclein can transverse the plasma membrane, and the membrane translocation efficiency is optimum when the peptide contains two repeat motifs.

5. The typical endocytosis pathway might not be responsible for the membrane translocation of α-synuclein.

6. Internalization of α-synuclein is energy-independent and occurs very rapidly via a route other than normal endocytosis.

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ABSTRACT (IN KOREAN)

α-Synuclein 의 세포막 전위에서의 아미노산 서열 모티프와 기전적 특징

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안 근 재

α-Synuclein에 세포막 전위(주; 세포 외부로의 분비나 세포 내부로의 투과)와 관련해서 많은 증거들이 제시되고 있는 반면, 그러한 일련에 과정에 대한 세분화된 기전에 대해서는 아직까지 알려져 있지 않다. 이에 본 연구자는 α-synuclein의 세포막 전위에 중요하게 작용하는 아미노산 모티프를 찾고 이를 바탕으로 세포막 전위에 세분화된 기전을 찾고자 하였다. 우리는 α-synuclein 뿐만 아니라 β-와 γ-synuclein 역시 세포 내로 투과되는 현상을 관찰할 수 있었다. 이러한 현상은 synuclein 그룹간 높은 유사성을 갖는 N-말단 부위에 의한 것으로 생각된다. α-Synuclein 구조를 기초로 제작한 결실 변이형을 이용한 실험 결과, α-synuclein의 세포막 전위는 N-말단 쪽에

존재하는 불완전한 11 개의 아미노산 반복 모티프에 의한 것임을 확인할 수 있었다. 또한 이러한 11 개의 불완전 아미노산 모티프 만으로 구성된 펩타이드들 역시 세포막 전위와 관련해서 중요한 역할을 하는 것을 알 수 있었다. 더욱이 세포막 전위에 있어 가장 효율적인 펩타이드는 두 개의 불완전 모티프를 가지는 경우로 나타났다. 또한 α-synuclein의 세포막 전위는 단백질 처리 5 분 후 세포질에서 관찰 할 수 있을 정도로 비교적 빠른 시간 내에 세포 내로 투과됨을 확인 할 수 있었다. 흥미로운 것은 37°C와 비교해서 4°C에서도 별다른 영향 없이 세포 내로 투과됨을 확인하였다. 게다가 endocytosis inhibitor를 처리한 후 살펴본 α-synuclein의 세포막 전위 역시 아무런 영향을 받지 않는 것을 확인 할 수 있었다.

이상에 결과들을 종합해 보면 α-synuclein의 세포막 전위는 정상적인 endocytosis 외에 다른 경로를 통해 energy-independent하며 매우 빠르게 일어나는 것으로 생각된다.

이러한 결과들을 볼 때 α-synuclein은 향후 PTD (Protein Transduction Domain)와 유사한 방식으로 여러 가지 물질들을 세포 내로 유입시키는 하나의 도구가 될 것으로 사료된다.

핵심되는 말 : α-synuclein, 세포막 전위, 세포 투과, 11-불완전 아미노산 모티프, endocytosis, energy-independent