The effect of Molecular Crowding on interaction of alpha-Synuclein with Biological Membranes

Chi A Yi

The Graduate School of Health and Environment
Yonsei University
Department of Biomedical Science

The effect of Molecular Crowding on interaction of alpha-Synuclein with Biological Membranes

A Masters Thesis

Submitted to the Department of Biomedical Science

and the Graduate School of Health and Environment

Yonsei University

in partial fulfillment of the requirements for the degree of Master

Chi A Yi

FEBRUARY, 2008

This certifies that the masters thesis
of Chi A Yi is approved.

_____

Thesis Supervisor: Yong Serk Park

_____

Tae Ue Kim: Thesis Committee Member

_____

Hyeyoung Lee: Thesis Committee Member

The Graduate School
Yonsei University
FEBRUARY, 2008

Dedicated to my mom, my husband, my son, Minjae, and my family who help me succeed and continue to inspire me.

CONTENTS

LIST OF FIGURES

ABBREVIATION

α-Syn : alpha-Synuclein

cDNA : complimentary Deoxyribonucleic acid

CHO cell : Chinese hamster ovary cell

DSG : Disuccinimidyl glutarate

EDTA : Ethylenediaminetetraacetic acid

HEPES : 4-(2-Hydroxyethyl)-1-piperazineethanesulfronic acid

HB : Homogenization buffer

LB : Lewy body

NS : Non-specific

M.O.I : Multiplicity of infection

PBS : Phosphate buffered saline

PD : Parkinson's disease

PDI : Protein disulfide isomerase

PEG : Polyethylene glycol

RA : Retinoic acid

RT : Room temperature

SDS-PAGE : Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

SNCA : α-Synuclein gene

TBST : Tris-buffered saline tween 20

WT : Wild-type

The effect of Molecular Crowding on interaction of alpha-Synuclein with Biological Membranes

Abstract

α-Synuclein is known to be implicated in the pathogenesis of Parkinson's disease. Recently, it has shown that α-synuclein can transiently bind to biological vesicles through α-helix formation in its N-terminal repeat region. The intracellular environment is crowded with macromolecules. In particular, approximately 40% of the neuronal cytoplasm volume may be occupied by macromolecules. Some cellular events including protein folding and protein - protein interaction can be affected in this crowded environment. Crowding by cytoplasmic macromolecules has been experimentally modeled with inert polymers. In this study, the effect of molecular crowding on the interaction of α-synuclein with biological vesicles was examined by adding inert polymers. In short, the addition of various kind of polymers such as polyethylene glycol, dextran, and ficoll enhanced binding of α-synuclein to biological vesicles in a concentration-dependent manner. And the interaction of α-synuclein with vesicles was proportionally augmented by increased expression of α-synuclein. However, molecular crowding had a neglectable effect on the vesicle binding of α-synuclein mutants (A30P; Ala30Pro which is known as point mutation in the α-synuclein gene is a cause of familial Parkinson disease, TG6; ThrGly6 that is a mutant replacing six threnonine residues (Thr22, Thr33, Thr44, Thr59, Thr81, and Thr92) with glycine in its N-terminal region) which have been

reported to show reduced membrane binding capacity. Also, aggregation of α-synuclein seems to be accelerated by α-synuclein bound to vesicles. These results suggest that transient interaction of α-synuclein with vesicles occurs more commonly in cells than expected in the previous reports and imply the interaction of α-synuclein with vesicles may be one of the physiological processes in which α-synuclein is involved.

Keyword : Parkinson's disease, alpha-Synuclein, vesicle, Molecular crowding, inert polymer

I. INTRODUCTION

Parkinson's disease (PD) is a chronic progressive neurodegenerative movement disorder and clinical manifestations of this disease includes resting tremor, bradykinesia, postural instability, and rigidity. PD is characterized by selective degeneration of dopaminergic neurons in substantia nigra pars compacta and the presence of proteinateous inclusion bodies called Lewy bodies (LB) in remaining cells[1-2]. Although the cause of the disease is still unknown, both of genetic and environmental factors seem to play a critical role in pathogenesis of PD. Among genetic factors, α-synuclein has been known to be implicated in the development of PD[3]. Three missense mutations in α-synuclein gene (A53T, A30P, and E46K) and triplications of the α-synuclein gene (SNCA) are known to be associated with PD[4-7]. There is a growing body of evidence that abnormal accumulation and aggregation of α-synuclein may play critical roles in pathogenesis of PD.

α-Synuclein is a natively unfolded presynaptic protein. It is highly expressed in neuron and localizes to nerve terminal[8]. α-Synuclein consists of an N-terminal repeat region, a hydrophobic middle region, and an acidic C-terminal region. It has been proposed that α-synuclein is involved in synaptic vesicle recycling, storage and compartmentalization of neurotransmitters, leading to affecting synaptic transmission[9-12]. Although the precise mechanism of how α-synuclein functions in synaptic transmission is not clear, its functions in lipid metabolism and vesicle trafficking were previously addressed[13-14]. A genome-wide screening in yeast showed that nearly one-third of genes that enhance the toxicity of α-synuclein were functionally related to lipid metabolism and vesicle trafficking[15]. Expression profiling studies in transgenic flies showed that expression of lipid and membrane transport genes were associated with

α-synuclein expression[16]. In addition, overexpression of α-synuclein in a neuronal cell line and homozygous deletions of α-synuclein in mice led to changes in cellular fatty acid uptake, metabolism and membrane fluidity[17-19].

α-Synuclein interacts transiently with biological vesicles[20]. This interaction is mediated by the N-terminal of protein. Whereas the free form of α- synuclein is natively unfolded, the N-terminal region adopts an α-helical conformation upon binding to vesicles[21]. The binding of α-synuclein to biological vesicles is rapidly reversible and occurs only in the presence of non-protein and non-lipid cytosolic components. It has been also shown in numerous studies that the interaction of α-synuclein with phospholipid membranes, fatty acids, or detergent micelles alters the kinetics of its aggregation[22-28].

A common characteristic of the cytoplasm is the presence of macromolecules in a high concentration. Especially in neuronal cytoplasm, approximately 40% of its volume may be occupied by macromolecules, predominantly RNAs and proteins[29-33]. Therefore, several cellular events including interaction of molecules may be affected by the crowded macromolecules. A typical diluted solution may not provide an appropriate environment to study cellular functions of cytoplasmic proteins *in vitro*[34]. Therefore, to mimic the intracelluar environment, crowding by cytoplasmic macromolecules has been experimently modeled with inert polymers such as polyethylene glycol, dextran, and ficoll[35-42]. The level of nonspecific crowding in a mammalian CHO cell cytoplasm has been approximated by the solutions containing 20% polyethylene glycol (PEG) 8,000 and 20% dextran 11,000[43-45].

In this study, the effect of molecular crowding on interaction of α-synuclein with biological vesicles was examined. Molecular crowding environment with macromolecules influenced the binding of α-synuclein to vesicles and the expression level of α-synuclein affected the interaction of α-synuclein with vesicles. Meanwhile, the molecular crowding had little effect on the vesicle

interaction of mutants. Also, the aggregation of soluble α-synuclein was augmented by α-synuclein bound to vesicles.

These results suggest that a significant amount of α-synuclein transiently binds to intracellular vesicles and their transient interactions with vesicles may be important in physiological or pathophysiological process.

II. MATERIALS AND METHODS

1. Reagents

All-*trans* retinoic acid, polyethylene glycol, dextran, ficoll, and protease inhibitor cocktail were purchased from Sigma (St,Lous, MO). OPTI-PREP iodixanol reagent was purchased from Accurate Chemicals & Scientific Corp. (Westbury, NY). Monoclonal antibodies for α-synuclein (Syn-1) and protein disulfide isomerase were purchased from BD Biosicences (SanDiego, CA). Polyclonal antibody specific for Myc tag is a product of Abcam Inc. (Cambridge, MA). Disuccinimidyl glutarate (DSG) was purchased from Pierce Biotechnology Inc. (Rockford, IL) (Fig. 1). Lipofectamine 2000 is a product of Invitrogen Corp. (Carlsbad, CA).

2. Cell culture and α-synuclein expression

The neuroblastoma cell line SH-SY5Y (SK-N-SH-SY5Y) cells which was subcloned from human neuroblastoma SK-N-SH were split to about 10% confluency and the next day, were induced to differentiate with 50 μM all-*trans* retinoic acid. The cells were incubated in fresh medium containing retinoic acid which was changed every other day[46]. On day 5 of differentiation, the cells were infected with appropriate adenoviral vectors containing cDNA for human α-synuclein (adeno/α-syn), C-terminal tagged human α-synuclein WT (adeno/α-syn-MycHis), A53T (adeno/A53T-MycHis), or A30P (adeno/A30P-MycHis) at a multiplicity of infection (m.o.i.) of 10[47]. The next day, the infection medium was replaced with fresh medium with RA. After an

additional 24 h, the cells were homogenized and subjected to the crosslinking reaction. For transfection, COS-7 cells were split to about 45% confluency and incubated for 24 h. The cells grown in 100 mm culture dishes were transfected with 5 μg of plasmids (MycHis-tagged α-synuclein WT and TG6 mutant) complexed with 15 μl of lipofectamine 2000. At two days after transfection, the cells were homogenized and subjected to the crosslinking reaction.

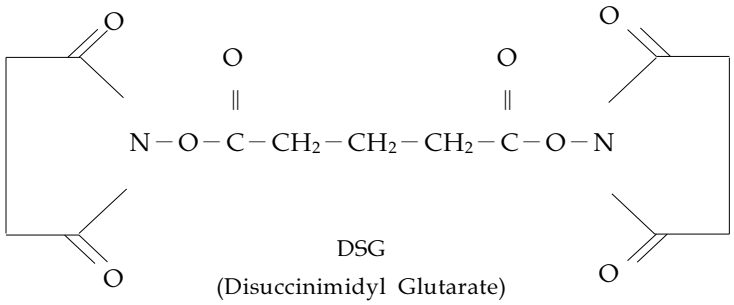
3. Cell homogenization and flotation centrifugation

Cells grown in a 100-mm culture dish were washed twice with ice-cold phosphate-buffered saline (PBS) and scraped in 500 ml homogenization buffer (HB; 10 mM HEPES, pH 7.2, 1 mM EDTA, 250 mM Sucrose) with protease inhibitor cocktail. The cells were disrupted using the Dounce homogenizer. The cell extract was centrifuged at 10,000 × g for 10 min. Then, the supernatant was collected and defined as S2 fraction (Fig 2). The S2 fraction was mixed with 60% iodixanol to obtain a final concentration of 40% and was layered under 1.8 ml of 30% iodixanol/HB. Then, 0.1 ml of 5% iodixanol/HB was gently layered on top and the samples were centrifuged at 200,000 × g for 2 h. The top fraction (300 μl) containing vesicles and 200 μl of the bottom fraction with cytosolic proteins were obtained and used for further study (Fig. 3).

4. Treatment of polymer and Crosslinking

For cell-free crosslinking, cell homogenates (S2 fractions) were incubated with inert polymers such as polyethylene glycol, dextran, and ficoll at room temperature for 30 min. Then, the mixture were crosslinked with 1 mM DSG

at room temperature for 30 min and the reaction was stopped by adding 1 M glycine (pH 8.0) to a final concentration of 100 mM.



DSG
(Disuccinimidyl Glutarate)
MW 326.26
Spacer Arm Lenth 7.72Å

Figure 1. Structure of crosslinker, DSG

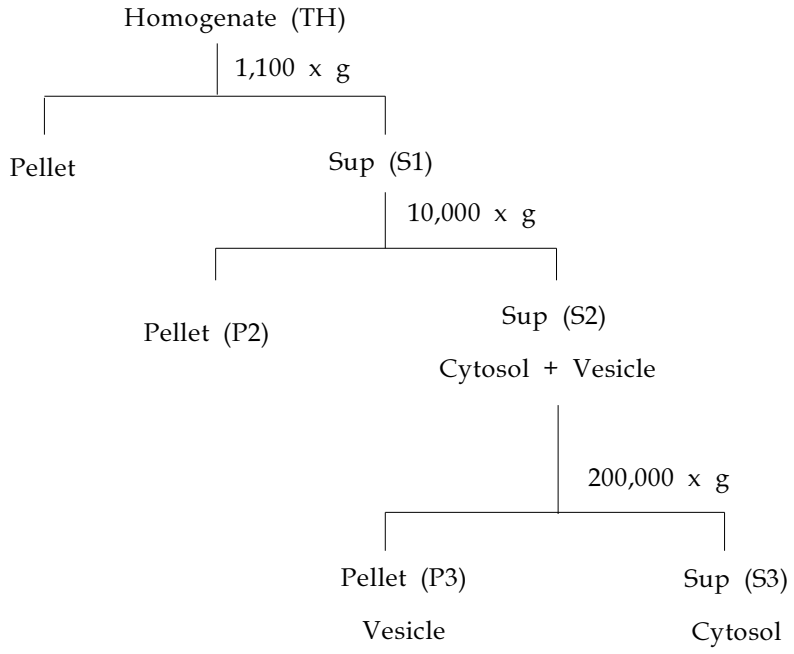


Figure 2. Procedure for fractionation

5. Western blotting

Cytosolic and vesicle fraction were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel was transferred to a nitrocellulose filters. The blots were blocked by 10 ㎖ of tris-buffered saline tween 20 solution (TBST, Tris 0.05 M, NaCl 0.15 M, Tween 20 0.05%, pH 7.4) containing 5% skim milk for 1 h at room temperature. It was then washed with TBST solution 3 times for each 10 min and then submerged into 10 ㎖ of TBST solution containing mouse anti-α-synuclein monoclonal antibody (1:2,000 dilution) for 1 h. Then, the blots were washed with TBST solution 3 times for each 10 min. They were incubated in the presence of the secondary antibody (10 ㎖ of TBST solution containing horse radish peroxidase-conjugated goat anti-mouse IgG, 1:3,000 dilution) for 40 min. After washing with TBST solution 3 times for each 10 min, the blots were submerged in the enhanced chemiluminescence solution using a PicoEPD™ (Enhanced Peroxidase Detection) Western blot detection kit (Elpis Biotech, Korea). For quantitative analysis, the Western results were quantified by computer-assisted densitometry using ImageQuaNT software (Molecular Dynamics, USA) under equal light and power settings.

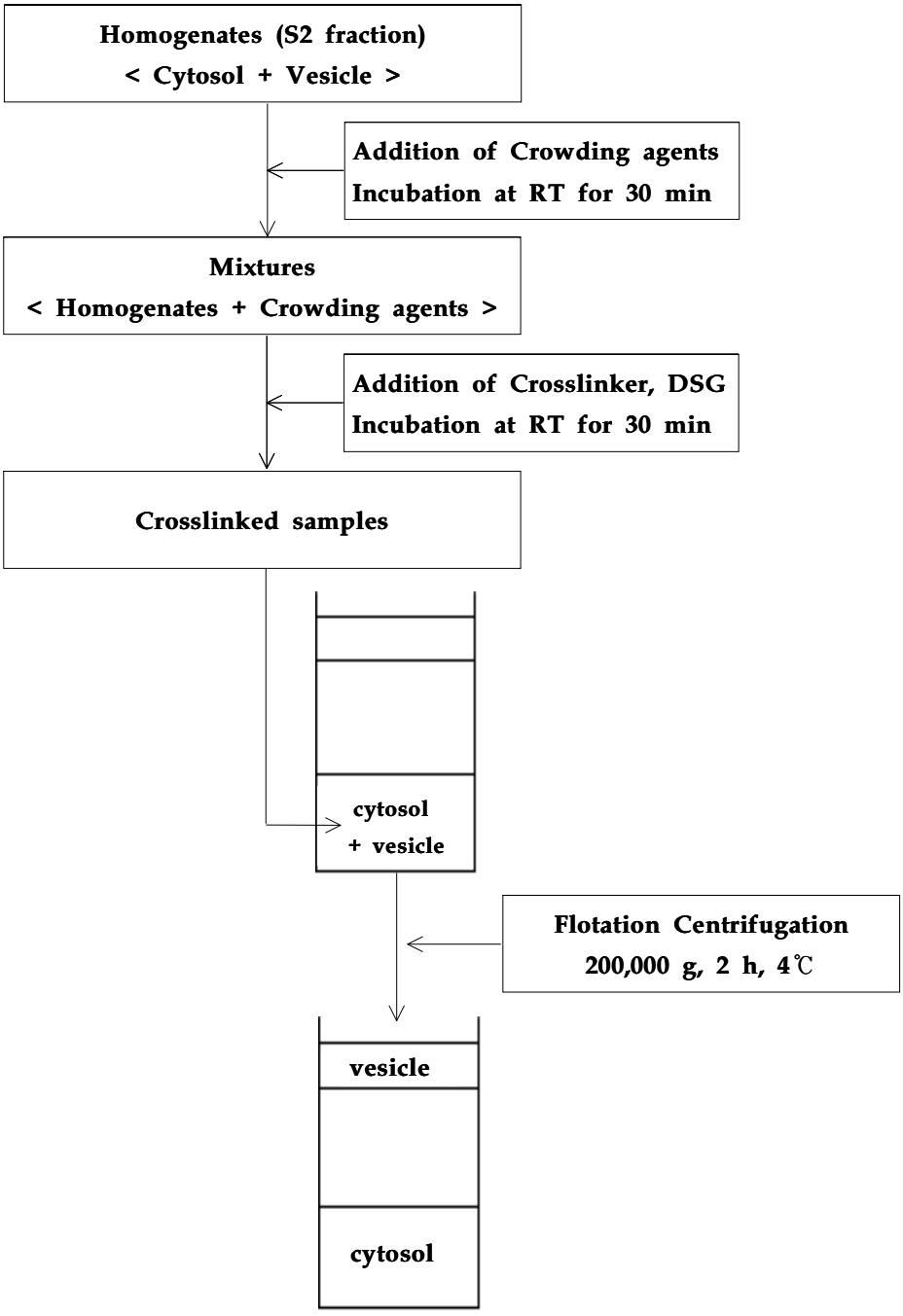


Figure 3. Schematic diagram of crosslinking and fractionation procedures

III. RESULTS

1. PEG-induced molecular crowding increases the interaction of α-synuclein with vesicles.

To investigate the effect of molecular crowding on the interaction of α-synuclein with intracellular vesicles *in vitro*, differentiated human neuroblastoma SH-SY5Y cells were infected by adenovirus expressing α-synuclein and then homogenized. Varied concentrations (0, 5, 10, 15, 20%) of PEG 8K, one of crowding agents (the inert polymers), were added to the homogenates (S2 fraction) which were then incubated at room temperature for 30 min. Then, DSG was added to stabilize transient binding of α-synuclein to vesicles in the homogenates which were fractionated by flotation centrifugation into the cytosol and vesicles. Binding of α-synuclein to vesicles were detected by Western blotting of the vesicle fraction using anti-α-synuclein antibody. As shown in Fig. 4, DSG was able to crosslink between α-synuclein molecules with vesicles (compare lane 1 with lane 2) and the crosslinkage was increased by molecular crowding with PEG 8K in a dose-dependent manner. This result shows molecular crowding in cells influences the interaction of α-synuclein with vesicles.

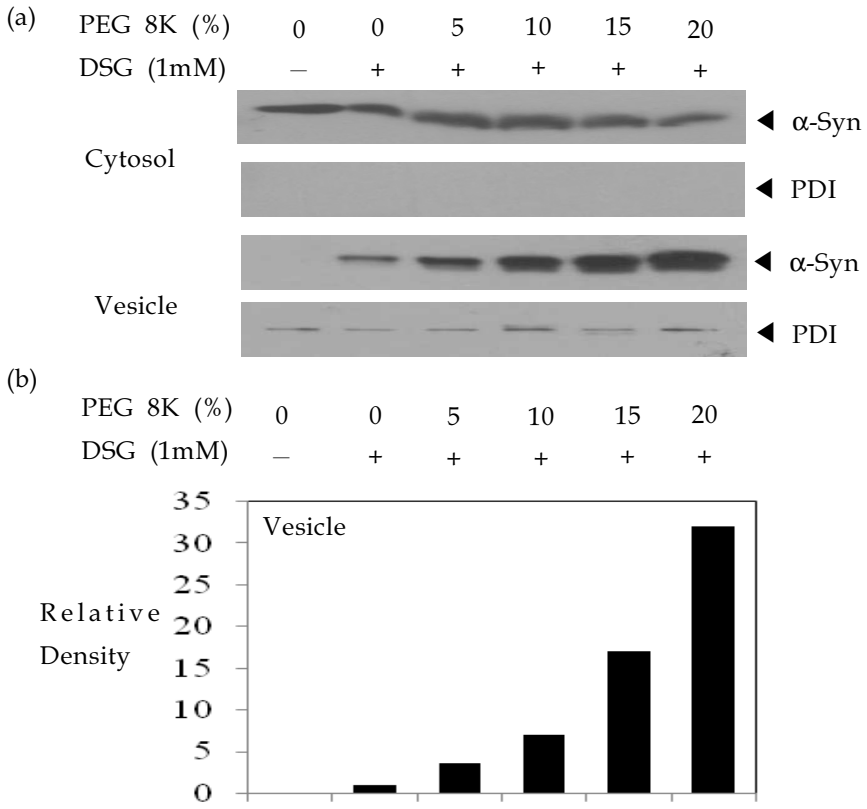


Figure 4. The interaction of α-synuclein with intracellular vesicles was increased by the PEG-induced molecular crowding. (a) The homogenates of differentiated SH-SY5Y cells expressing α-synuclein were incubated in the presence of the indicated concentrations of PEG 8K at room temperature for 30 min and crosslinked using DSG. Then, the crosslinked mixtures were fractionated into cytosol and vesicles by flotation centrifugation. α-Synuclein in the each fraction was identified by Western blotting with anti-α-synuclein antibody. Protein disulfide isomerase (PDI), a vesicular protein, was used as an internal control. (b) Densitometric quantification of the Western blotting results.

2. The interaction of α-synuclein with vesicles is also augmented by addition of other inert polymers.

To confirm that molecular crowding affects the binding of α-synuclein to vesicles, other polymers which have different chemical properties from PEG were used to mimic crowded environment. SH-SY5Y cells expressing α-synuclein were homogenized and the homogenates (S2 fraction) were incubated with varied concentrations (0, 2.5, 5, 7.5%) of dextran or ficoll. Then, the cell homogenates were crosslinked in the presence of DSG and then fractionated by flotation centrifugation. As expected, dextran- or ficoll-induced molecular crowding increased the interaction of α-synuclein with intracellular vesicles (Fig. 5). The effect of dextran or ficoll was similar to that of PEG. This result suggests the increased interaction of α-synuclein with vesicles is resulted from molecular crowding, not from the unique chemical properties of used polymers.

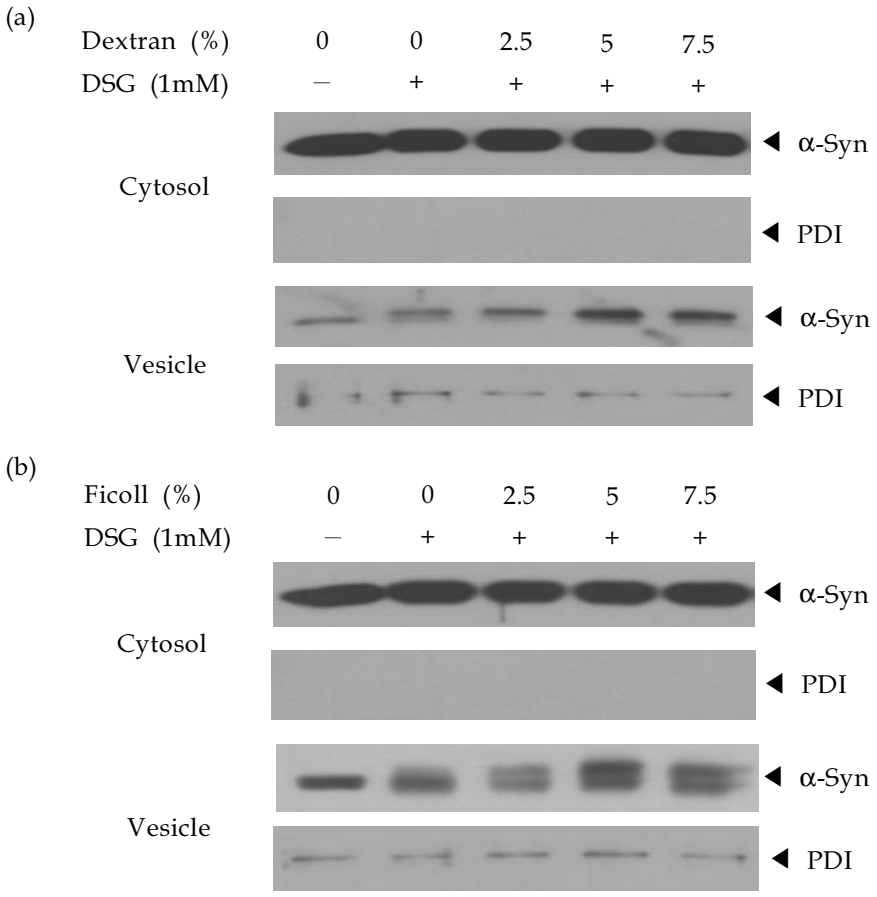


Figure 5. The binding of α-synuclein to vesicles was augmented by molecular crowding induced by dextran and ficoll. The homogenates of differentiated SH-SY5Y cells expressing α-synuclein were incubated in the presence of the indicated concentrations of dextran (a) or ficoll (b) at room temperature for 30 min. The cell homogenates were crosslinked using DSG and then fractionated into cytosol and vesicles. α-Synuclein in the each fraction was identified by Western blotting with anti-α-synuclein antibody. Protein disulfide isomerase (PDI), a vesicular protein, was used as an internal control.

3. The interaction of α-synuclein with vesicles is dependent upon the amount of intracellular α-synuclein

Molecular crowding causes the increase of local concentration of intracellular proteins. Thus, addition of crowding agents such as PEG, dextran, and ficoll results in increase of local concentration of α-synuclein. Therefore, to examine whether the amount of α-synuclein protein influences its interaction with intracellular vesicles, SH-SY5Y cells were infected with varied amounts (0, 1, 3, 10, 30, 100 m.o.i) of adenovirus expressing α-synuclein. Then, homogenates of the cells were crosslinked with DSG at room temperature for 30 min and fractionated into cytosolic and vesicle fractions. As shown in Fig. 6, the binding of α-synuclein to vesicles was proportionally augmented depending on the level of α-synuclein expression. This result indicates that molecular crowding increases the local concentration of α-synuclein and the increased local concentration of α-synuclein leads to enhanced its interaction with intracellular vesicles.

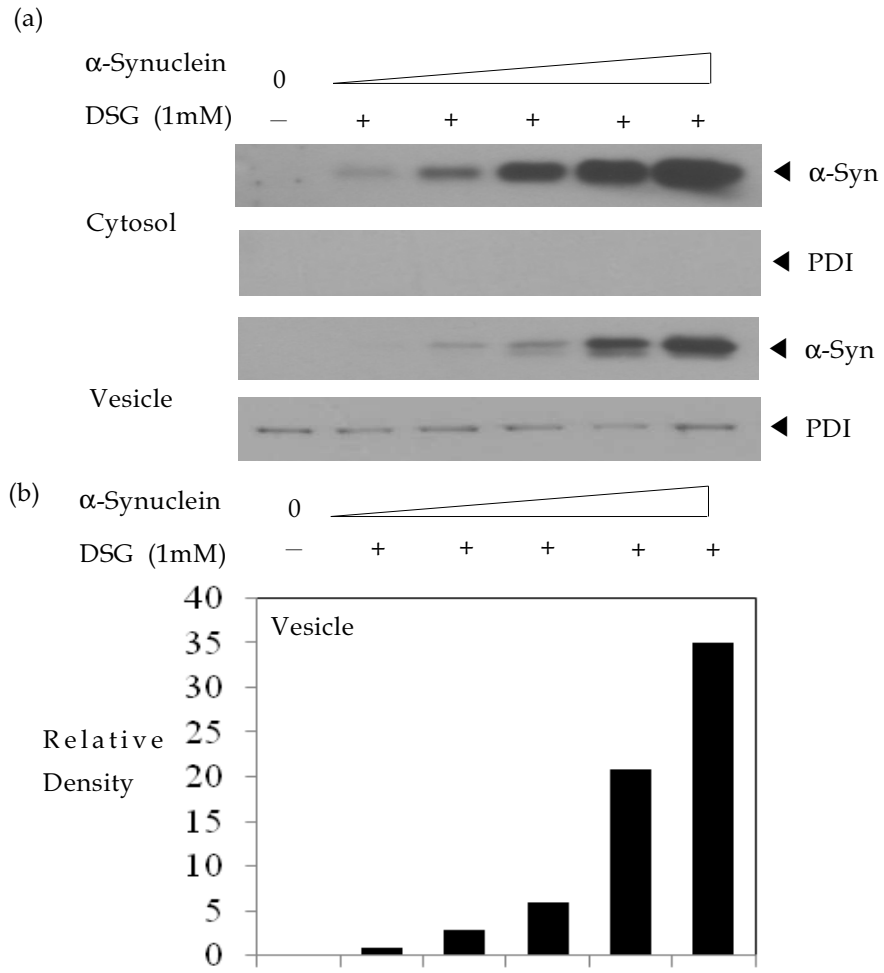


Figure 6. The interaction of α-synuclein with vesicles was enhanced by the increased expression of α-synuclein. (a) Differentiated SH-SY5Y cells were infected with varied concentrations of adenoviral vectors expressing human α-synuclein. Homogenates of the infected cells were crosslinked with DSG and fractionated into cytosol and vesicles by flotation centrifugation. α-Synuclein in the each fraction was identified by Western blotting with anti-α-synuclein antibody. (b) Densitometric quantification of the Western blotting results.

4. Molecular crowding has less effect on the binding of A30P α-synuclein mutant to vesicles.

It has been reported that an A30P mutant among the PD-linked α-synuclein mutants has a reduced capability to interact with intracellular vesicles. Therefore, it is reasonable to compare PD-linked mutants with the wild-type one in terms of the molecular crowding effect on their interaction with vesicles. SH-SY5Y cells were infected with adenoviral vectors expressing wild-type, A30P mutant, or A53T mutant of α-synuclein. The infected cells were homogenized and then incubated in the absence or presence of PEG 8K. The homogenates of the infected cells were crosslinked with DSG and then fractionated by flotation centrifugation. Binding of A53T α-synuclein mutant to intracellular vesicles was increased by addition of the crowding agent, PEG 8K (Fig. 7). The pattern of increased A53T mutant interaction with vesicles was similar to that of wild-type α-synuclein interaction. On the other hand, the molecular crowding had less effect on the interaction of A30P α-synuclein mutant with intracellular vesicles.

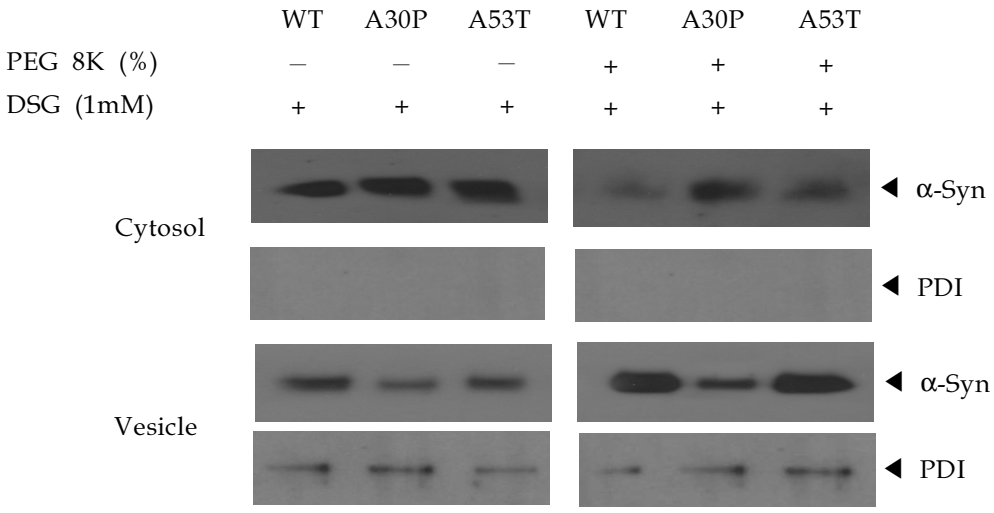


Figure 7. Effects of Molecular crowding on the interaction of PD-linked α-synuclein mutants with intracellular vesicles. SH-SY5Y cells were infected with adenoviral vectors for wild-type (WT), A30P, or A53T mutants of α-synuclein. Homogenates of the infected cells were incubated with or without 20% PEG 8K at room temperature for 30 min. The cell homogenates were crosslinked using DSG and then fractionated into cytosol and vesicles. α-Synuclein in the each fraction was identified by Western blotting with anti-α-synuclein antibody. Protein disulfide isomerase (PDI), a vesicular protein, was used as an internal control.

5. Molecular crowding little affect the interaction of TG6 α-synuclein mutant with vesicles.

It has been known that the interaction of α-synuclein with intracellular vesicles requires the formation of an α-helix in its N-terminal repeat region. Therefore, it was examined whether molecular crowding affects the binding of of α-synuclein mutants defective in the α-helical N-terminal repeat region to vesicles. TG6 mutant of α-synuclein is a mutant replacing six threnonine residues (Thr22, Thr33, Thr44, Thr59, Thr81, and Thr92) with glycine in its N-terminal region by multiple site-directed mutagenesis. Because the glycine does not favor α-helix formation, these Thr to Gly substitutions can interfere with the helix formation in α-synuclein. COS-7 cells were transfected with plasmid DNAs encoding wild-type and TG6 mutant of α-synuclein. The homogenates of the transfected cells were incubated with or without PEG 8K, a crowding agent. Then, the mixtures were subjected to crosslinking and fractionation by flotation ultracentrifugation. As shown in Fig. 8, TG6 α-synuclein mutant proteins were not able to interact with intracellular vesicles and addition of crowding agent did not increase the vesicular interaction either.

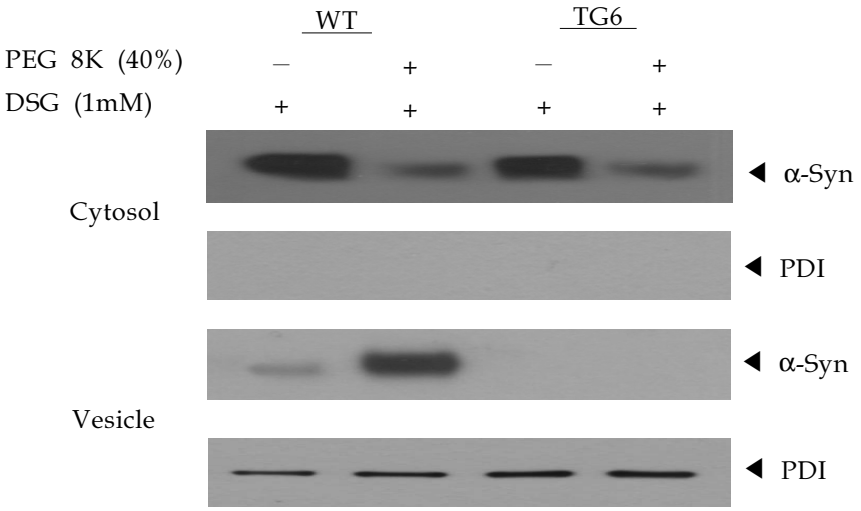


Figure 8. Effects of molecular crowding on the binding of TG6 α-synuclein mutant to intracellular vesicles. COS-7 cells were transfected with plasmid DNA encoding either wild-type (WT) or the TG6 mutant (TG6) α-synuclein, both of which were tagged with Myc epitope. Homogenates of the transfected cells were treated with or without 40% PEG 8K at room temperature for 30 min. The cell homogenates were crosslinked using DSG and then fractionated into cytosol and vesicles. α-Synuclein in the each fraction was identified by Western blotting with polyclonal anti-Myc antibody. Protein disulfide isomerase (PDI), a vesicular protein, was used as an internal control.

6. Aggregation of soluble α-synuclein is accelerated by α-synuclein bound to vesicles.

To examine the effect of α-synuclein bound to vesicles on the aggregation of α-synuclein, differentiated human neuroblastoma SH-SY5Y cells were infected adenovirus expressing α-synuclein (α-Syn) or α-synuclein tagged with Myc epitope (α-Syn-Myc). The homogenates (S2 fraction) of α-synuclein expressing cells were incubated in varied concentrations (0, 5, 10, 15, 20%) of PEG 8K and then crosslinked by addition of DSG. The crosslinked homogenates were fractionated into the cytosol and vesicles, then the vesicle fractions containing α-synuclein-bound vesicles were collected. On the other hand, the cytosolic fractions (S3 fraction) were obtained from the homogenates of α-Syn-Myc-expressing cells. Then, the solution of α-synuclein-bound vesicles were mixed with the cytosolic fraction containing soluble α-Syn-Myc and incubated at room temperature for 1 day, 3 days, and 5 days (Fig. 9). As shown in Fig. 10, the aggregation of soluble α-synuclein molecules tagged with Myc was increased proportionally to the added amount of α-synuclein bound to vesicles. This result suggests that aggregation of soluble, cytosolic α-synuclein is accelerated by the presence of α-synuclein bound to vesicles.

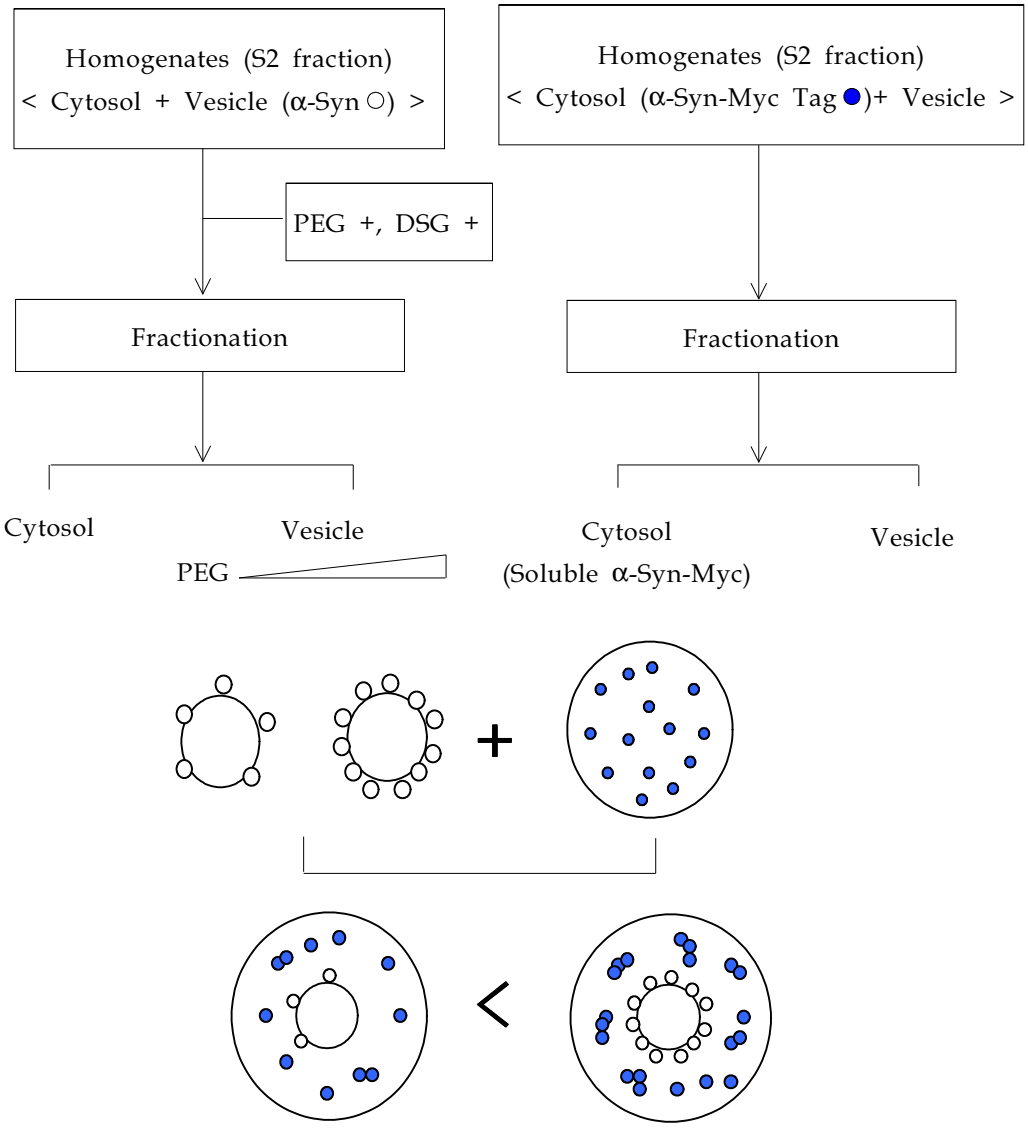


Figure 9. Experimental design for examining the effect of α-synuclein bound to vesicles on aggregation of soluble α-synuclein

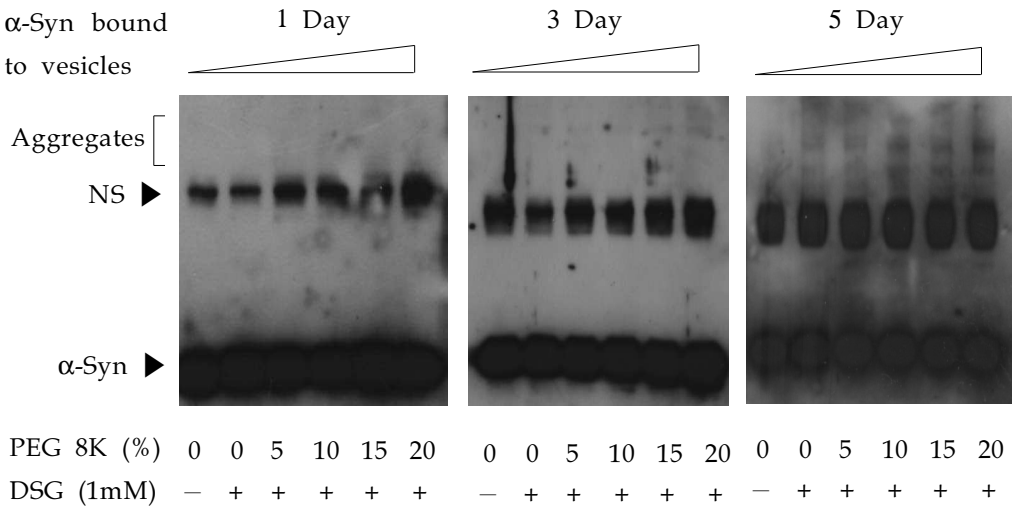


Figure 10. Aggregation of soluble α-synuclein by the presence of α-synuclein bound to vesicles. SH-SY5Y cells were infected adenovirus expressing α-synuclein or α-Syn-Myc. Varied concentrations of PEG 8K were added to the homogenates (S2 fraction) of cells expressing α-synuclein. The mixtures were incubated at room temperature for 30 min and then crosslinked by DSG. Cytosolic fractions (S3 fraction) were obtained from the cells expressing α-Syn-Myc. The solution containing α-synuclein-bound vesicles were mixed with the cytosolic fraction containing soluble α-Syn-Myc, which was then incubated at room temperature for 1, 3, or 5 days. α-Syn-Myc in the each fraction was identified by Western blotting with polyclonal anti-Myc antibody.

IV. DISCUSSION

The binding of α-synuclein to vesicles has been a subject of intense investigation. Recent studies showed that α-synuclein interacts with biological vesicles and these interactions are transient and reversible[20]. However, there remain yet unsolved questions; (1) how much amount of α-synuclein binds to biological vesicles in cells? and (2) what is the physiological and (or) pathophysiological meaning of this interaction? In this study, inert polymers were used as a crowding agent to mimic intracellular environment. Proteins and vesicles in cells are crowded with other macromolecules. Thus, macromolecular association such as binding of proteins to vesicles may be affected by molecular crowding. The amount of α-synuclein bound to vesicles in the cells may be different from that obtained from an *in vitro* experiment using a diluted solution. In this study, I demonstrated the followings: (1) molecular crowding increased the interaction of α-synuclein with intracellular vesicles, (2) binding of α-synuclein to vesicles was enhanced by increased α-synuclein expression, (3) molecular crowding little influenced vesicle interaction of A30P and TG6 α-synuclein mutants that have reduced vesicle binding capacity, (4) aggregation of soluble α-synuclein was accelerated by α-synuclein bound to vesicles.

To elucidate the effect of molecular crowding on the interaction of α-synuclein with intracellular vesicles, the extent of its association with vesicles was examined in the presence of various inert polymers including polyethylene glycol, dextran, and ficoll. Regardless of polymer types, addition of these polymers was able to enhance the interaction of α-synuclein with vesicles in a dose-dependent manner. This implies that the enhanced interaction is resulted from simple molecular crowding provided by the polymers, not from a specific

environment provided by any particular polymers. Also, this may imply that significant amount of α-synuclein transiently binds to vesicles in cells.

It has been proposed that fibrillation or aggregation of α-synuclein is accelerated by molecular crowding. It has been also suggested that biological vesicles induces aggregation of α-synuclein[11]. Thus, it is possible to speculate that molecular crowding may enhance the interaction of α-synuclein with vesicles and the increased interaction may accelerate induction of α-synuclein aggregation. Those observations and speculation indirectly suggest that the binding of α-synuclein to intracellular vesicles may be involved in α-synuclein aggregation which is a major cause of Parkinson's disease development.

According to the experimental results in this study, increased α-synuclein expression in the cell induced higher augmentation of α-synuclein binding to vesicles. In addition, it has known that the local concentration of intracellular proteins increases under molecular crowding conditions[21-26]. Therefore, it is reasonable to speculate that the increased crowdness by polymers may elevate the local concentration of α-synuclein and result in augmentation of α-synuclein binding to vesicles. It has been also known that increase of α-synuclein expression induces α-synuclein aggregation in the cell and transgenic animal models[40]. Thus, it is possible to state that the increased local concentration of α-synuclein by molecular crowding leads to enhance α-synuclein binding to intracellular vesicles and result in augmentation of α-synuclein aggregation.

It has been reported that PD-linked A30P α-synuclein mutant has a diminished vesicle-binding capability[40]. In this study, molecular crowding by polymers had a negligible effect on the interaction of the A30P mutant with vesicles. This suggests that vesicle-binding capability between the wild-type and A30P mutant is different from each other in the cell, more than expected previously[7]. Also, it has been known that α-helix formation in N-terminal

region of α-synuclein is critical for its binding capability to vesicles. The data in this study also confirmed that the TG6 mutant, incapable of forming α-helix conformation in its N-terminal region, was not able to augment its interaction with vesicles even under the molecular crowding condition. This result suggests that the α-helix formation in N-terminus may be also critical for α-synuclein binding to vesicles in the cytoplasm.

To determine the role of α-synuclein bound to vesicles in aggregation of α-synuclein proteins solublized in the cytoplasm, aggregation of free α-synuclein was examined by mixing α-synuclein bound to vesicles with free α-synuclein (α-Syn-Myc). Aggregation of free α-synuclein in the cytosolic fractions was clearly accelerated by the incubation with α-synuclein bound to vesicles under the PEG-induced molecular crowding condition. This is concordant with previous reports[10-19] that the interaction of α-synuclein with phospholipid membranes, fatty acids, or detergent micelles alters the kinetics of its aggregation. Based on these observations, it is reasonable to claim that abnormal accumulation and stabilization of interaction of α-synuclein with vesicle has an important role in seeding or nucleation of aberrant α-synuclein aggregation.

In summary, this study showed that the environment of molecular crowding augmented the interaction of α-synuclein with cytoplasmic vesicles. This suggests that more amount of α-synuclein can bind transiently and reversibly to vesicles in cells than expected previously. These results imply that α-synuclein interaction with vesicles may be an important physiological and (or) pathophysiological event in normal cells and PD-associated cells.

V. REFERENCES

1. Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. Neuron. 2003; 39:889-909.

2. Forno LS. Neuropathology of Parkinson's disease. J Neuropathol. Exp. Neurol. 1996; 55:259-272.

3. Hardy J, Cookson MR, Singleton A. Genes and parkinsonism. Lancet Neurol. 2003; 2:221-228.

4. Krüger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, Riess O. Ala30 Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet. 1998; 18:106-108.

5. Zarranz JJ, Alegre J, Gómez-Esteban JC, Lezcano E, Ros R, Ampuero I, Vidal L, Hoenicka J, Rodriguez O, Atarés B, Llorens V, Gomez Tortosa E, del Ser T, Muñoz DG, de Yebenes JG. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann Neurol. 2004; 55:164-173.

6. Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, Hulihan M, Peuralinna T, Dutra A, Nussbaum R, Lincoln S, Crawley A, Hanson M, Maraganore D, Adler C, Cookson MR, Muenter M, Baptista M, Miller D, Blancato J, Hardy J, Gwinn-Hardy K. alpha-Synuclein locus triplication causes Parkinson's disease. Science 2003; 302:841.

7. Abeliovich A, Schmitz Y, Fariñas I, Choi-Lundberg D, Ho WH, Castillo PE, Shinsky N, Verdugo JM, Armanini M, Ryan A, Hynes M, Phillips H, Sulzer D, Rosenthal A. Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. Neuron. 2000; 25:239-252.

8. Iwai A, Masliah E, Yoshimoto M, Ge N, Flanagan L, de Silva HA, Kittel A. The precursor protein of non-A beta component of Alzheimer's disease

amyloid is a presynaptic protein of the central nervous system. Neuron. 1995; 14:467-475.

9. Goedert M. Alpha-synuclein and neurodegenerative diseases. Nat Rev Neurosci. 2001; 2:492-501.

10. Liu S, Ninan I, Antonova I, Battaglia F, Trinchese F, Narasanna A, Kolodilov N, Dauer W, Hawkins RD, Arancio O. alpha-Synuclein produces a long-lasting increase in neurotransmitter release. EMBO J. 2004; 23: 4506-4516.

11. Cabin DE, Shimazu K, Murphy D, Cole NB, Gottschalk W, McIlwain KL, Orrison B, Chen A, Ellis CE, Paylor R, Lu B, Nussbaum RL. Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein. J Neurosci. 2002; 22: 8797-8807.

12. Yavich L, Tanila H, Vepsalainen S, Jakala P. Role of alpha-synuclein in presynaptic dopamine recruitment. J Neurosci. 2004; 24:11165-11170.

13. Yavich L, Jakala P, Tanila H. Abnormal compartmentalization of norepinephrine in mouse dentate gyrus in alpha-synuclein knockout and A30P transgenic mice. J Neurochem. 2006; 99:724-732.

14. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL. Mutation in the alpha-synuclein gene identified in families with Parkinson''s disease. Science. 1997; 276: 2045-2047.

15. Willingham S, Outeiro TF, DeVit MJ, Lindquist SL, Muchowski PJ. Yeast genes that enhance the toxicity of a mutant huntingtin fragment or alpha-synuclein. Science. 2003; 302:1769-1772.

16. Scherzer CR, Jensen RV, Gullans SR, Feany MB. Gene expression changes presage neurodegeneration in a Drosophila model of Parkinson's disease. Hum Mol Genet. 2003; 12:2457-2466.

17. Sharon R, Bar-Joseph I, Mirick GE, Serhan CN, Selkoe DJ. Altered fatty acid composition of dopaminergic neurons expressing alpha-synuclein and human brains with alpha-synucleinopathies. J Biol Chem. 2003; 278: 49874-49881.

18. Golovko MY, Faergeman NJ, Cole NB, Castagnet PI, Nussbaum RL, Murphy EJ. Alpha-synuclein gene deletion decreases brain palmitate uptake and alters the palmitate metabolism in the absence of alpha-synuclein palmitate binding. Biochemistry. 2005; 44:8251-8259.

19. Castagnet PI, Golovko MY, Barcelo-Coblijn GC, Nussbaum RL, Murphy EJ. Fatty acid incorporation is decreased in astrocytes cultured from alpha-synuclein gene-ablated mice. J Neurochem. 2005; 94: 839-849.

20. Kim YS, Laurine E, Woods W, Lee SJ. A Novel Mechanism of Interaction between α-Synuclein and Biological Membranes. J Mol Biol. 2006; 360: 386-397.

21. Davidson WS, Jonas A, Clayton DF, George JM. Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes. J Biol Chem. 1998; 273:9443-9449.

22. Necula M, Chirita CN, Kuret J. Rapid anionic micelle-mediated alpha-synuclein fibrillization in vitro. J Biol Chem. 2003; 278: 46674-46680.

23. Zhu M, Fink AL. Lipid binding inhibits alpha-synuclein fibril formation. J Biol Chem. 2003; 278:16873-16877.

24. Zhu M, Li J, Fink AL. The association of alpha-synuclein with membranes affects bilayer structure, stability, and fibril formation. J Biol Chem. 2003;

278:40186-40197.

25. Perrin RJ, Woods WS, Clayton DF, George JM. Exposure to long-chain polyunsaturated fatty acids triggers rapid multimerization of synucleins. J Biol Chem. 2001; 276:41958-41962.

26. Cole NB, Murphy DD, Grider T, Rueter S, Brasaemle D, Nussbaum RL. Lipid droplet binding and oligomerization properties of the Parkinson's disease protein alpha-synuclein. J Biol Chem. 2002; 277:6344-6352.

27. Sharon R, Bar-Joseph I, Frosch MP, Walsh DM, Hamilton JA, Selkoe DJ. The formation of highly soluble oligomers of alpha-synuclein is regulated by fatty acids and enhanced in Parkinson's disease. Neuron. 2003; 37: 583-595.

28. Jo E, Darabie AA, Han K, Tandon A, Fraser PE, McLaurin J. alpha-Synuclein-synaptosomal membrane interactions: implications for fibrillogenesis. Eur J Biochem. 2004; 271:3180-3189.

29. Herzfeld J. Entropically Driven Order in Crowded Solutions: From Liquid Crystals to Cell Biology. Acc Chem Res. 1996; 29:31-37.

30. Minton AP. Implications of macromolecular crowding for protein assembly. Curr Opin Struct Biol. 2000; 10:34-39.

31. Ellis RJ. Macromolecular crowding: an important but neglected aspect of the intracellular environment. Curr Opin Struct Biol. 2001; 11:114-119.

32. Zimmerman SB, Minton AP. Macromolecular crowding: biochemical, biophysical, and physiological consequences. Annu Rev Biophys Biomol Struct. 1993; 22:27-65.

33. Zimmerman SB, Trach SO. Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of Escherichia coli. J Mol Biol.

1991; 222:599-620.

34. Ellis RJ. Macromolecular crowding: obvious but underappreciated. Trends Biochem Sci. 2001; 26:597-604.

35. Rivas G, Fernandez JA, Minton AP. Direct observation of the enhancement of noncooperative protein self-assembly by macromolecular crowding: indefinite linear self-association of bacterial cell division protein FtsZ. Proc Natl Acad Sci USA. 2001; 98:3150-3155.

36. Rivas G, Fernandez JA, Minton AP. Direct observation of the self-association of dilute proteins in the presence of inert macromolecules at high concentration via tracer sedimentation equilibrium: theory, experiment, and biological significance. Biochemistry. 1999; 38: 9379-9388.

37. Cole N, Ralston GB. Int. J. Enhancement of self-association of human spectrin by polyethylene glycol. Int J Biochem. 1994; 26:799-804.

38. Lindner R, Ralston G. Effects of dextran on the self-association of human spectrin. Biophys Chem. 1995; 57:15-25.

39. Cuneo P, Magri E, Verzola A, Grazi E. 'Macromolecular crowding' is a primary factor in the organization of the cytoskeleton. Biochem J. 1992; 281: 507-512.

40. Martin J, Hartl FU. The effect of macromolecular crowding on chaperonin-mediated protein folding. Proc Natl Acad Sci USA. 1997; 94:1107-1112.

41. van den Berg B, Ellis RJ, Dobson CM. Effects of macromolecular crowding on protein folding and aggregation. EMBO J. 1999; 18:6927-6933.

42. van den Berg B, Wain R, Dobson CM, Ellis RJ. Macromolecular crowding perturbs protein refolding kinetics: implications for folding inside the cell. EMBO J. 2000; 19:3870-3875.

43. LiCata VJ, Allewell NM. Measuring hydration changes of proteins in solution: applications of osmotic stress and structure-based calculations. Methods Enzymol. 1998; 295:42-62.

44. Swaminathan R, Hoang CP, Verkman AS. Photobleaching recovery and anisotropy decay of green fluorescent protein GFP-S65T in solution and cells: cytoplasmic viscosity probed by green fluorescent protein translational and rotational diffusion. Biophys J. 1997; 72:1900-1907.

45. Elowitz MB, Surette MG, Wolf PE, Stock JB, Leibler S. Protein mobility in the cytoplasm of Escherichia coli. J Bacteriol. 1999; 181:197-203.

46. Lee HJ, Khoshaghideh F, Patel S, Lee SJ. Clearance of alpha-synuclein oligomeric intermediates via the lysosomal degradation pathway. J Neurosci. 2004; 24:1888-1896.

47. Lee HJ, Shin SY, Choi C, Lee YH, Lee SJ. Formation and removal of alpha-synuclein aggregates in cells exposed to mitochondrial inhibitors. J Biol Chem. 2002; 277:5411-5417.

국문요약

Molecular crowding이 alpha-synuclein과 세포막의 상호작용에 미치는 영향

파킨슨병은 뇌의 흑색질에서 신경 세포가 파괴됨으로써 신경 전달 물질인 도파민의 결핍에 의해 생기는 질환으로, 이 질환에 관련된 주요 인자중 하나인 α-synuclein은 세포내에서 대부분 세포질에 존재하고 있으며, 일부가 vesicle과 결합하는 것으로 알려져 있다. 현재까지 발표된 연구 결과들에 의하면 α-synuclein이 vesicle간의 결합이 가역적이고, 일시적이며, 결합시 N말단 부위가 α 나선 구조를 이루는 것으로 밝혀졌다. 한편, 세포 내에는 높은 농도의 RNA, DNA, 단백질 등의 거대 분자가 존재 하는데, 특히 신경 세포 내에는 세포 부피의 40 % 이상이 이러한 거대 분자로 채워져 있다. 이런 세포내 환경을 molecular crowding이라 하며, 이는 분자 간 결합 등의 여러 화학 반응에 영향을 미친다고 알려져 있다. 이에 따라, *in vitro* 실험에서 세포 내의 환경과 비슷한 조건을 만들기 위하여 다양한 불활성 중합체를 이용해 세포내와 유사한 crowding환경을 만드는 여러 모델들이 제시되어왔다. 본 실험에서는 polyethylene glycol, dextran, ficoll 등의 여러 가지 중합체를 이용한 molecular crowding 조건에서 α-synuclein과 vesicle간의 결합 정도를 연구하였으며, crosslinker인 DSG를 이용하여 가역적이고 일시적인 결합을 안정화하여 실험하였다. 실험 결과 α-synuclein과 vesicle간의 결합이 중합체의 농도 의존적으로 증가됨을 확인하였으며, α-synuclein 발현 증가에 따라서 vesicle과의 결합이 증가함을 관찰하였다. 그러나 이러한 molecular crowding이 vesicle 결합능이 떨어지는 것으로 알려진 α-synuclein 돌연변이 A30P (30번 자리에 alanine이 proline으로 바뀐 돌연변이)와 vesicle과의 결합능이 없는 α- synuclein 돌연변이 TG6 (22번, 33번, 44번, 59번, 81번, 92번 자리에 threonine이 glycine으로 바뀐 돌연변이)의 vesicle 결합에는 크게 영향을 주지 않은 것을 확인하였다. 또한, vesicle에 결합한 α-synuclein이 세포질에 존재하는 수용성 α-synuclein의 응집에

영향을 미쳐 파킨슨병의 대표적 병리학적 특성인 α-synuclein 침전물이 형성됨을 관찰하였다. 결과적으로, 세포내에서 α- synuclein과 vesicle의 결합은 기존의 연구 결과에서 예측되었던 것보다 훨씬 빈번하게 일어나고 있으며, 이는 α-synuclein과 vesicle간의 결합이 α-synuclein의 세포내 기능에 매우 중요함을 간접적으로 제시하고 있다.

핵심어 : 파킨슨병, alpha-Synuclein, vesicle, Molecular crowding, 불활성 중합체