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Original Article

Macromolecular Crowding Enhances Interaction of α-synuclein with Vesicles

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α-synuclein (α-syn) is known to be implicated in the pathogenesis of Parkinson's disease and transiently bind to biological vesicles. In this study, we examined the effect of molecular crowding on the interaction of α-syn with biological vesicles by using inert polymers since the environment of proteins in cells are crowded with other macromolecules. The addition of different polymers including polyethylene glycol, dextran, and ficoll enhanced the binding of α-syn to vesicles in a concentration-dependent manner and the association of α-syn with vesicle was proportionally augmented by increased expression of α-syn. However, molecular crowding had a neglectable effect on the vesicle binding of α-syn mutants (A30P, TG6), which has been reported to show impaired vesicle binding capacity. These results suggest that transient interaction of α-syn with vesicles occurs more commonly in cells than expected implying interaction with vesicles may be one of the physiological processes in which α-syn is involved.

Key Words: Alpha-synuclein, Vesicles, Macromolecular crowding, Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder and is characterized by selective degeneration of dopaminergic neurons and the presence of Lewy bodies (LB) in remaining cells (Forno, 1996; Dauer and Przedborski, 2003). Although the cause of the disease is still unknown, α-synuclein (α-syn) has been known to be implicated in the development of PD (Hardy et al., 2003). Three missense mutations in α-syn gene (A53T, A30P, E46K) and triplications of the gene is known to be associated with PD (Polymeropoulos et al., 1997; Kruger et al., 1998; Singleton et al., 2003; Zarranz et al., 2004). There is a growing body of evidence that abnormal accumulation and aggregation of α-syn might play critical roles in pathogenesis of PD.

α-syn is a natively unfolded protein and highly expressed in neuron and localizes to nerve terminal (Iwai et al., 1995). α-syn consists of an N-terminal repeat region, a hydrophobic middle region, and an acidic C-terminal region. It has been proposed that α-syn is involved in synaptic vesicle recycling, storage and compartmentalization of neurotransmitters, leading to affecting synaptic transmission (Abeliovich et al., 2000; Goedert, 2001; Cabin et al., 2002; Liu et al., 2004; Yavich et al., 2004; Yavich et al., 2006). Although the precise mechanism of how α-syn functions in synaptic transmission is not clear, its function in lipid metabolism and vesicle trafficking may play a role. A genome-wide screening in yeast showed that nearly one-third of genes that enhance the toxicity of α-syn are functionally related to lipid metabolism and vesicle trafficking (Willingham et al., 2003). Expression profiling studies in transgenic flies

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show that expression of lipid and membrane transport genes were associated with α-syn expression (Scherzer et al., 2003). It was shown also that overexpression of α-syn in a neuronal cell line and homozygous deletions of α-syn in mice led to changes in cellular fatty acid uptake and metabolism, and membrane fluidity (Sharon et al., 2003b; Castagnet et al., 2005; Golovko et al., 2005).

α-syn interacts transiently with biological vesicles (Kim et al., 2006). This interaction is mediated by the N-terminal of the protein. Whereas the free form of α-syn is natively unfolded, the N-terminal region adopts an alpha-helical conformation upon binding to vesicles (Davidson et al., 1998). The binding of α-syn 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-syn with phspholipid membranes, fatty acids, or detergent micelles alters the kinetics of its aggregation (Perrin et al., 2001; Cole et al., 2002; Necula et al., 2003; Sharon et al., 2003a; Zhu and Fink, 2003; Zhu et al., 2003; Jo et al., 2004). Recently, we showed increased binding of α-syn to perturbed cellular membrane (Kim and Lee, 2011). In addition, it has been reported that α-syn modulates vesicle trafficking (Lee et al., 2011) and also that α-syn promotes SNARE-complex assembly *in vivo* and *in vitro* (Burre et al., 2010) and sequesters arachidonic acid to modulate SNARE-mediated exocytosis (Darios et al., 2010). There was an additional report showing that α-syn and polyunsaturated fatty acid promote clathrin-meidated endocytosis and synaptoic vesicle recycling (Ben Gedalya et al., 2009). These reports can support that interaction of α-syn with vesicle is one of the important intracellular processes.

A common characteristic of the interior of all cells is the high concentration of macromolecule present. Approximately 40% of neuronal cytoplasmic volume may be occupied by macromolecules, predominantly RNA and proteins (Zimmerman and Trach, 1991; Zimmerman and Minton, 1993; Minton, 2000; Ellis, 2001a). In this crowded environment, several cellular events including interaction of molecules may be affected. Crowded condition is different from that in the typical dilute solution used to study proteins *in vitro* (Ellis, 2001b). Therefore, to mimic intracellular environment, crowding by cytoplasmic macromolecules have been experimently modeled with inert polymers such as polyethylene glycol, detran, ficoll (Cuneo et al., 1992; Cole and Ralston, 1994; Lindner and Ralston, 1995; Martin and Hartl, 1997; Rivas et al., 1999; van den Berg et al., 1999; van den Berg et al., 2000; Rivas et al., 2001). The level of nonspecific crowding in a mammalian CHO cell cytoplasm has been approximated by solutions containing 20% polyethylene glycol (PEG) 8,000, 20% dextran 11,000 (Swaminathan et al., 1997; LiCata and Allewell, 1998; Elowitz et al., 1999).

In the present study, the effect of molecular crowding on interaction of alpha-syn with vesicles has been examined. Molecular crowding influenced the binding of α-syn to vesicles. Also, expression level of α-syn affected the interaction with vesicle. These results suggest that significant amount of α-syn binds transiently to vesicles in cells and that vesicle binding of α-syn may be a critical process by which α-syn play a physiological or pathophysiological role.

MATERIALS AND METHODS

Materials

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-syn (Syn-1) and protein disulfide isomerase were purchased from BD Biosicences (SanDiego, CA). Disuccinimidyl glutarate (DSG) were purchased from Pierce Biotechnology Inc. (Rockford, IL). Lipofectamine 2000 is a product of Invitrogen Corp. (Carlsbad, CA).

Cell culture and α-syn expression

SH-SY5Y cells were grown in DMEM supplemented with 10% FBS and penicillin-streptomycin, maintained at 37℃ in humidified atmosphere with 5% CO_2, and differentiated as described previously (Yavich et al., 2004). Briefly, the cells were split to about 10% confluency and the next day, induced to differentiate with 50 mM all-trans retinoic acid (RA). On day 5 of differentiation, cells were

infected with appropriate adenoviral vectors containing cDNA for human α-syn (adeno/α-syn), C-terminal tagged human α-syn WT (adeno/α-syn-MycHis), A53T (adeno/A53T-MycHis), A30P (adeno/A30P-MycHis), or empty vector at a multiplicity of infection (m.o.i.) of 10. (Yavich et al., 2006). The next day, the infection medium was replaced with fresh medium with RA. After an additional 24 h, cells were homogenized and subjected to the cross-linking reaction. For transfection, COS-7 cells were split to about 45% confluency and incubated for 24 h. The cells were grown in 100 mm culture dishes were transfected with 5 mg of plasmids (MycHis-tagged α-syn WT and TG6 mutant) using 15 μl of lipofectamine 2000. 2 days after transfection, cells were homogenized and subjected to the crosslinking reaction.

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. Cells were disrupted using the Dounce homogenizer. The extract was then centrifuged at 10,000 x g for 10 min (Fig. 1A) and the supernatant was collected (we defined this supernatant as S2 fraction). The S2 fraction was mixed with 60% iodixanol to obtain a final concentration of 40% and then 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 x 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.

Treatment of polymer and crosslinking

For cell-free crosslinking (Fig. 1B), cell homogenates (S2 fractions) were incubated with inert polymer such as polyethylene glycol, dextran, ficoll at room temperature for 30 mins. Then the mixture were crosslinked with 1 mM DSG at room temperature for 30 mins and the reaction was stopped by adding 1 M glycine (pH 8.0) to a final concentration of 100 mM.

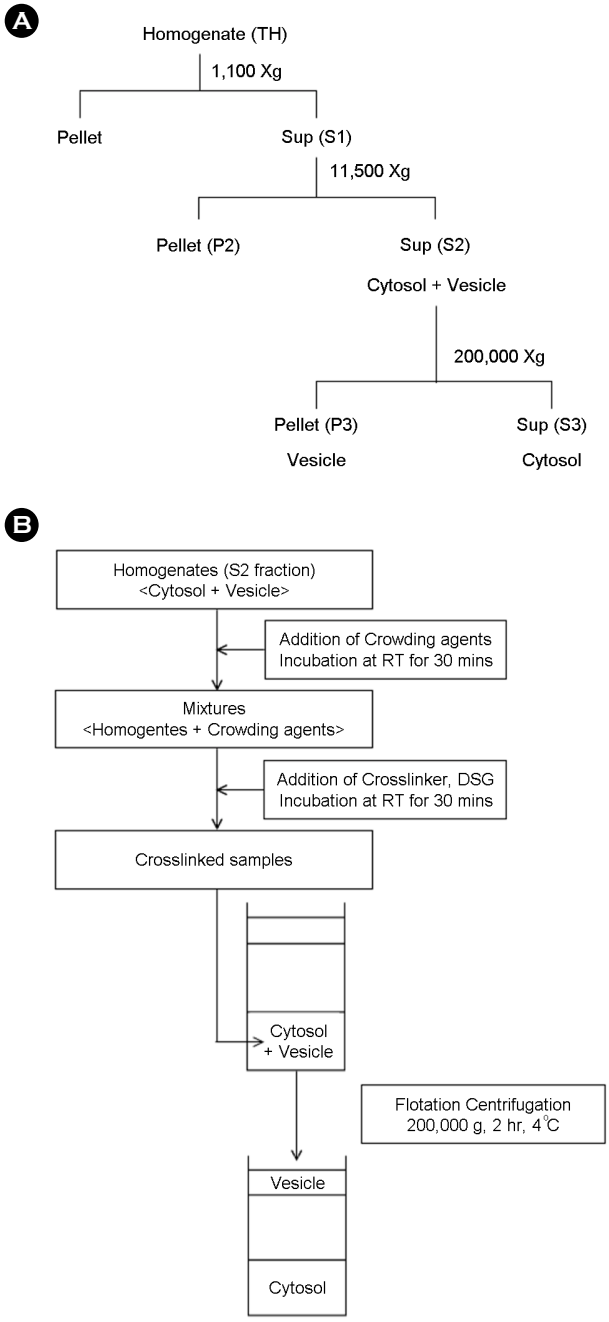

Fig. 1. Schematic diagram of procedure for (A) cell extract fractionation and (B) cross-linking and vesicle fractionation.

Western blot analysis

Cytosolic and vesicle fraction were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was transferred to a nitrocellulose filters. The blots were blocked by 10 ml of tris-buffered saline Tween 20 (TBST, Tris 0.05 M, NaCl 0.15 M, Tween

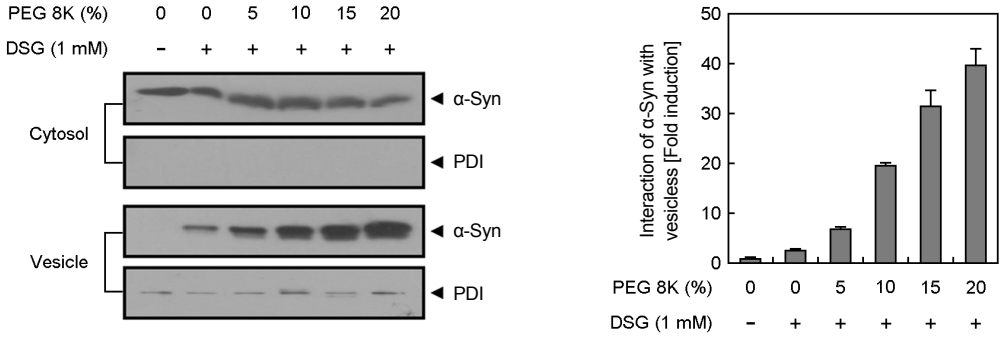

Fig. 2. The interaction of α-syn with vesicles is increased by the PEG-induced molecular crowding. Differentiated SH-SY5Y cells expressing α-Syn were homogenized first, and the cell homogenates were incubated with the indicated concentration of PEG 8K at room temperature for 30 min and crosslinked using DSG Then, crosslinked mixtures were fractionated into the cytosol and vesicles by flotation centrifugation. Samples were separated on 15% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. α-syn was detected by Western blotting with anti-α-syn antibody (left panel). The vesicular protein, protein disulfide isomerase (PDI) was used as an internal control. Densitometric quantification of the Western blotting results (right panel). Data are expressed as mean ± SD and results from three independent experiments.

20 0.05%, pH 7.4) solution containing 5% skim milk for 1 hour at room temperature. It was then washed with TBST solution 3 times for 10 minutes and then submerged into 10 ml of TBST solution containing mouse anti-α-syn monoclonal antibody (1:2,000 dilution) for 1 hour. Then, the blots were also washed with TBST solution 3 times for each 10 minutes. The secondary antibody (10 ml of TBST solution containing horse radish peroxidase conjugated goat anti-mouse IgG, 1:3,000 dilution) was incubated with the blots for 40 minutes. After washing with TBST solution 3 times for each 10 minutes, the blots were submerged in the enhanced chemiluminescence solution using a PicoEPD™ (Enhanced Peroxidase Detection) Western blot detection kit (Elpis Biotech, Rep. of Korea). For the quantitative analysis, Western results were quantified by computer-assisted densitometry using ImageQuaNT software (Molecular Dynamics) under equal light and power settings.

RESULTS

PEG-induced molecular crowding increases the interaction of α-syn with vesicles

To investigate the effect of molecular crowding on the interaction of α-syn with vesicles *in vitro*, differentiated human neuroblastoma SH-SY5Y cells were infected adenovirus expressing alpha-syn and homogenized. The homogenates (S2 fraction) were added with different concentrations of PEG 8K, one of crowding agents (the inert polymers) and incubated at room temperature for 30 min. Then, the mixture was crosslinked with DSG for stabilizing transient binding of α-syn to vesicles and fractionated into cytosol and vesicles. Binding of α-syn to vesicles were detected by Western blotting using anti-alpha-syn antibody. As shown in Fig. 2, DSG-induced crosslinking stabilized the interaction of α-syn with vesicles (compare lane 1 and 2) and the interaction was increased by PEG 8K-induced molecular crowding in a dose-dependent manner. This result shows molecular crowding in cells influences the interaction of α-syn with vesicles.

The interaction of α-syn with vesicles are also augmented by addition of other inert polymers

To confirm that molecular crowding affects the binding of α-syn to vesicles, other polymers, which have chemical properties different form PEG, were used to mimic crowded environment. α-syn-expressed SH-SY5Y cells were homogenized and the homogenates (S2 fraction) were incubated with different concentrations of dextran or Ficoll. Then, the mixtures were crosslinked with DSG and were fractionated by flotation centrifugation. As expected, dextran or Ficoll-induced molecular crowding increased the interaction of α-syn with vesicles (Fig. 3). The effect of dextran or Ficoll was similar to the effect of PEG. This result suggests increased interaction of α-syn with vesicles are not resulted

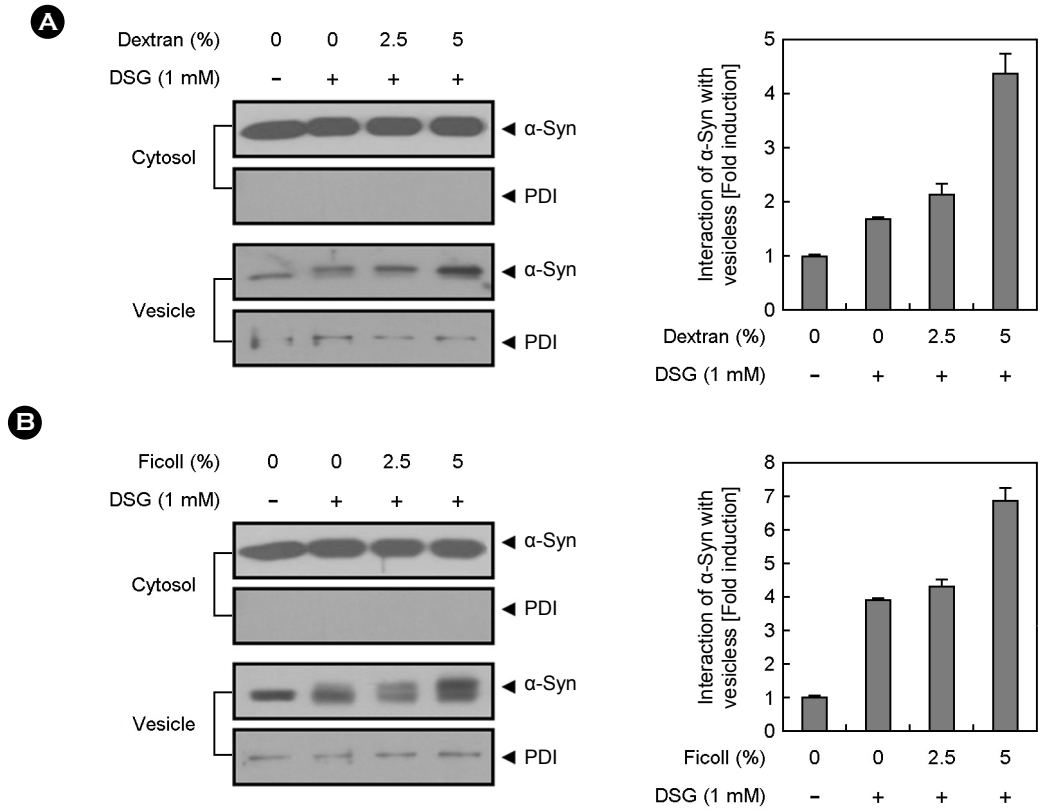

Fig. 3. The binding of α-syn to vesicles is augmented by other polymers-induced molecular crowding. Differentiated SH-SY5Y cells expressing α-syn were homogenized and the homogenates were incubated in the indicated concentration of dextran (A) or (B) Ficoll at room temperatrue for 30 mins. The mixtures were crosslinked with DSG (1 mM) and fractionated into the cytosol and vesicles. Samples were subjected to Western blotting using anti-α-syn antibody (left panel). The vesicular protein, protein disulfide isomerase (PDI) was used as an internal control. Densitometric quantification of the Western blotting results (right panel).

from the unique chemical properties of used polymers but resulted from the effect of molecular crowding.

The interaction of α-syn with vesicles is increased by the increased expression of α-syn

Molecular crowding causes the local concentration of given macromolecules in cells. Thus, addition of crowding agents such as PEG, dextran, and Ficoll results in increase of local concentration of α-syn. Therefore, to examine whether concentration of α-syn protein influences the interaction with vesicles, SH-SY5Y cells were infected with different amounts of adenovirus expressing α-syn and homogenized. Then, the homogenates were crosslinked with DSG at room temperature for 30 mins and fractionated into cytosolic and vesicle fraction. As shown in Fig. 4, the binding of α-syn to vesicles was proportionally augmented by increase of α-syn expression level. It is possible that molecular crowding increases the local concentration of α-syn and this increased local concentration causes to augmentation of interaction with vesicles.

Molecular crowding has less effect on the binding of A30P mutant to vesicles

It has been reported that A30P mutant among the PD-linked α-syn mutation has a reduced capability for interaction with vesicles. Therefore, we examined the effect of molecular crowding on the vesicle interaction of PD-linked mutants. SH-SY5Y cells were transfected with plasmids expressing wild-type, A30P mutant, and A53T mutant of alpha-syn respectively. The transfected cells were homogenized and incubated in the absence or presence of PEG 8K. Then the mixtures were crosslinked and fractionated. The binding of A53T mutant to vesicles were increased by addition of crowding agents (Fig. 5). This increased inter-

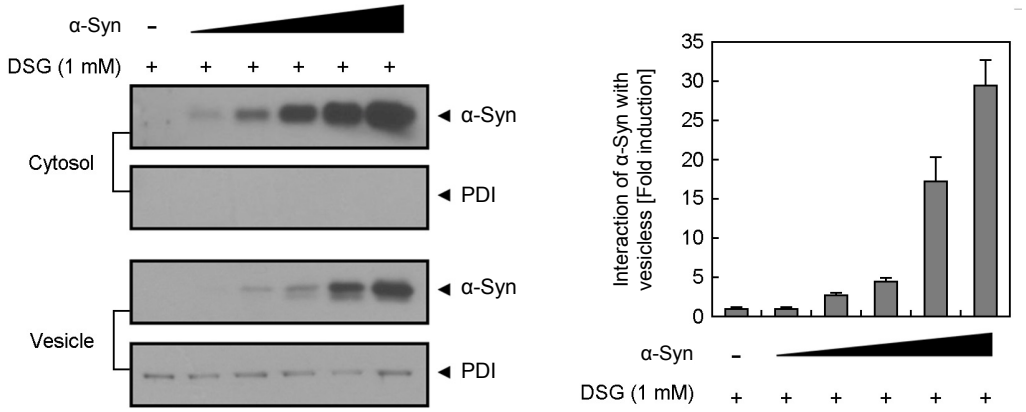

Fig. 4. The interaction of α-syn with vesicles is increased by the increased expression of α-syn. Differentiated SH-SY5Y cells were infected with different concentrations of adenoviral vectors expressing for human α-syn and then, homogenates were prepared. The homogenates were crosslinked with DSG (1 mM) and fractionated into the cytosol and vesicles by flotation centrifugation. Samples were resolved by 12% DSD/polyacrylamide gels and transferred to nitrocellulose membrane. Binding of α-syn to vesicles was detected by Western blotting with anti-α-syn antibody (left panel). Densitometric quantification of the Western blotting results (right panel).

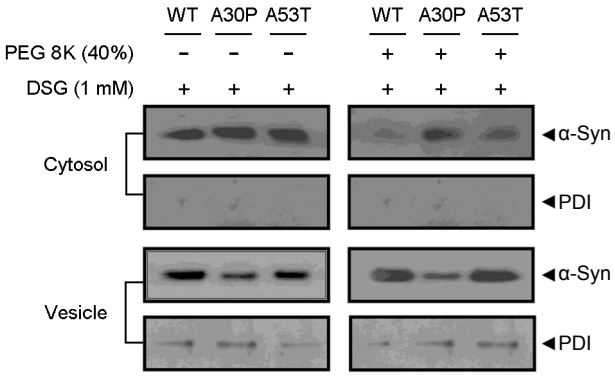

Fig. 5. The effect of molecular crowding on the interaction of PD-linked α-syn mutants with vesicles. SH-SY5Y cells were infected with adenoviral vectors for wild-type (WT), A30P, or A53T mutants of α-syn and homogenized. The homogenates were incubated with or without PEG 8K (20%) at room temperature for 30 mins, then cross-linked with DSG (1 mM). After fractionation, cytosolic (upper panel) and vesicle fraction (lower panel) were analyzed by 15% polyacrylamide gels. Immunoblotting was performed using monoclonal anti-α-syn antibody. The vesicular protein, protein disulfide isomerase (PDI) was used as an internal control.

action was similar to the increase of binding of wild-type to vesicles. On the other hand, molecular crowding had less effect on the interaction of A30P mutant with vesicles. This result suggests that molecular crowding doesn't enhance vesicle binding of α-syn mutant which has originally impaired binding capacity.

Molecular crowding does not affect the interaction of TG6 mutant with vesicles

It has been known that the interaction of α-syn with vesicles requires the formation of an alpha-helix in its N-terminal repeat region. We examined whether molecular crowding affects the binding of TG6 mutant to vesicles. TG6 mutant of α-syn is a mutant that has six threonine residues replaced with glycine in its N-terminal region. Because glycine does not favor alpha-helix formation, these Thr to Gly substitutions can interfere with the helix formation in alpha-synuclein. COS-7 cells were transfected with expression vectors for wild-type and TG6 mutant of α-syn respectively. The cells were homogenized and the homogenates were incubated with or without PEG. Then, the mixtures were subjected to the crosslinking and fractionation by flotation ultracentrifugation. As shown in Fig. 6, the interaction of TG6 with vesicles was completely abolished and addition of crowding agent did not lead to binding of TG6 to vesicle. This implies that molecular crowding doesn't cause to vesicle binding of α-syn mutant which originally doesn't have vesicle binding capacity.

DISCUSSION

The binding of α-syn to vesicles has been a subject of

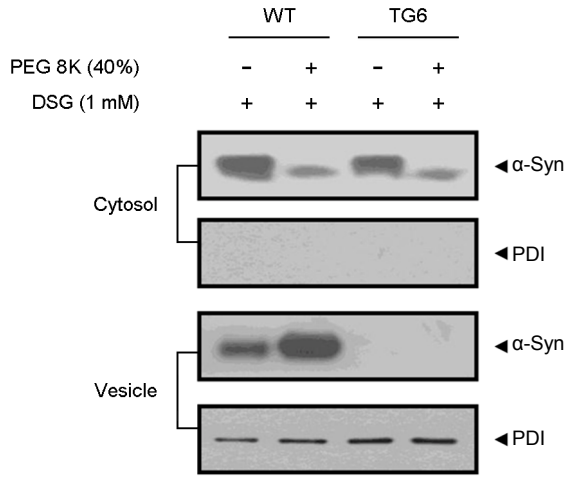

Fig. 6. The effect of molecular crowding on the binding of α-syn mutant (TG6) to vesicles. COS-7 cells were transfected with plasmids expressing either wild-type (WT) α-syn or the TG6 mutant (TG6), both of which are tagged with myc epitope and homogenized. The homogenates were treated with or without PEG 8K (20%) at room temperature for 30 mins, then were crosslinked with DSG (1 mM). The mixtures were fractionated into cytosol (upper panel) and vesicle fraction (lower panel) and the fraction was separated by 15% polyacrylamide gels. Immunoblotting was performed using polyclonal anti-myc antibody. The vesicular protein, protein disulfide isomerase (PDI) was used as an internal control.

intense investigation. Recent studies showed that α-syn interacts with biological vesicles and these interactions are transient and reversible. However, there remains yet unsolved questions; How much amount of α-syn binds to biological vesicles in cells? What is the physiological and (or) pathophysiological meaning of this interaction? Proteins and vesicles in cells are crowded with other macromolecules. Thus, intracellular processes such as binding of proteins to vesicles may be affected by molecular crowding. Here, inert polymers were used as crowding agents to mimic intracellular environment and we demonstrated the following: 1) molecular crowding increases the interaction of α-syn with vesicles. 2) Binding of α-syn to vesicle is augmented by increased expression of α-syn. 3) Molecular crowding did not influence vesicle interaction of A30P and TG6 mutant of α-syn, which is known to show reduced or impaired vesicle binding capacity.

To elucidate the effect of molecular crowding on the interaction of α-syn with vesicles, interaction with vesicles was examined in the presence of different inert polymers including polyetheylene glycol, detran, and Ficoll. Addition of these polymers increased interaction of α-syn with vesicles in a dose-dependent manner. Since polymers tested have different chemical properties, this effect doesn't seems to be results from the unique chemical effect of polymers themselves, but seems to be results from the effect of molecular crowding induced by polymers.

It has been proposed that aggregation of α-syn is accelerated by molecular crowding and also that vesicles induce aggregation of α-syn. Thus it is possible that molecular crowding enhances the interaction of α-syn with vesicles and this increased vesicle binding accelerates induction of α-syn aggregation. Binding of α-syn to vesicles may be important for aggregation of α-syn causing development of Parkinson's disease.

We also found that increased expression of α-syn induces the augmentation of binding of α-syn to vesicles. Molecular crowding has an effect for increase of local concentration of proteins. Thus, it is possible that addition of polymers enhances molecular crowding, then this increased crowding causes the increase of local concentration of α-syn, resulting in augmentation of α-syn binding to vesicles.

It has been known that PD-linked A30P mutation causes reduced capability for vesicle binding. Our data showed that molecular crowding have a neglectable effect on the interaction of this mutant with vesicles. Alpha-helix formation in N-terminal region is known to be critical for the binding of α-syn to vesicles. We also showed that the vesicle interaction of TG6 mutant, which is incapable of forming alpha-helix conformation in its N-terminal region, is not augmented by molecular crowding. This implies that these mutants have impaired binding capacity in cells.

Our present study showed that molecular crowding increases the interaction of α-syn with vesicles. This suggests that more amount of α-syn can bind transiently and reversibly to vesicles in cells than expected previously. Further studies seem to be needed to elucidate a physiological and (or) pathophysiological meaning of α-syn interaction with vesicles in cells.

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