Tyrosine-to-Cysteine Modification of Human α-Synuclein Enhances Protein Aggregation and Cellular Toxicity*

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The deposition of α-synuclein and other cellular proteins in Lewy bodies in midbrain dopamine neurons is a pathological hallmark of Parkinson’s disease. Nitrative and oxidative stress can induce α-synuclein protein aggregation, possibly initiated by the formation of stable cross-linking dimers. To determine whether enhanced dimer formation can accelerate protein aggregation and increase cellular toxicity, we have substituted cysteine for tyrosine at positions 39, 125, 133, and 136 in human wild-type (WT) α-synuclein, and in A53T and A30P mutants α-synuclein. To reduce the likelihood of cross-linking, phenylalanine was substituted for tyrosine at the same sites. We have found that overexpression of Y39C or Y125C mutant proteins leads to increased intracellular inclusions and apoptosis in a rat dopaminergic cell line (N27 cells) and in human embryonic kidney 293 cells. Expression of Y133C, Y136C, and all four Tyr-to-Cys mutations were not more cytotoxic than WT control. Exposure to oxidative stress increased Y39C and Y125C α-synuclein aggregation and toxicity. Dimers and oligomers were found in Triton X-100-soluble fractions from adenovirus-mediated overexpression of Y39C and Y125C in N27 cells. In contrast, WT β-synuclein and all four Tyr-to-Cys mutant β-synucleins did not cause protein aggregation and cell death. We conclude that cysteine substitution at critical positions in the α-synuclein molecule can increase dimer formation and accelerate protein aggregation and cellular toxicity of α-synuclein.

Parkinson’s disease (PD) is a common neurodegenerative disease affecting more than 1% of the population over age 65 (1, 2). Pathological examination shows a selective loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (3, 4). Some of the remaining DA neurons contain large intracellular inclusions, known as Lewy bodies (LBs) (5). The etiology of PD may involve a combination of environmental toxins, such as heavy metals and pesticides, and genetic factors (6). In recent years, mutations in the α-synuclein gene (A53T and A30P) have been found in some rare familial forms of PD (7, 8). Since that discovery, α-synuclein has been shown to be the major component of LBs in all patients with PD (9, 10). In adult brain, α-synuclein is mainly localized in presynaptic terminals (11, 12). The physiological function of α-synuclein is not completely known. Recent studies indicate that α-synuclein may regulate DA release, enhance dopamine transporter (DAT) function, and negatively regulate tyrosine hydroxylase activity (13–15). Biophysical studies have demonstrated that α-synuclein is capable of forming fibrils similar to those found in LBs (16). In fact, both A53T and A30P α-synuclein gene mutations significantly accelerate protein aggregation (17, 18). Transgenic flies and mice expressing human A53T mutant α-synuclein developed motor function deficits, α-synuclein–positive cytoplasmic inclusions, and neuronal degeneration (19–22). The data indicate that α-synuclein protein aggregation is important in the pathogenesis of PD.

The biochemical and biophysical factors that may initiate and propagate α-synuclein aggregation and promote its deposition into LBs are not completely understood. A growing body of evidence suggests that oxidative stress is fundamental. Widespread nitrination of α-synuclein has been found in LBs in PD and other synucleinopathies (23, 24). Both nitrating and oxidizing agents can induce α-synuclein aggregation in a cell model (25). In vitro, exposure of human recombinant α-synuclein protein to nitrating agents leads to the formation of stable dimers and oligomers through O-O’-dityrosine cross-linking (26). Recently, Carpenter and colleagues (27) demonstrated that oxidative dimer formation is the critical rate-limiting step for protein aggregation and fibrillogenesis. WT α-synuclein has four tyrosine residues at positions 39, 125, 133, 136, and lacks cysteine. Because cysteine can be easily oxidized to form stable cross-linked disulfide dimers, we have hypothesized that cysteine substitution for tyrosine should produce a form of α-synuclein that could more easily dimerize and subsequently polymerize. We have explored whether tyrosine-to-cysteine-modified α-synuclein accelerates protein aggregation and increases cellular toxicity. We have also generated control plasmids in which tyrosine is replaced by phenylalanine. In this study, we have found that cysteine substitution in the Y39C and Y125C positions but not the Y133C and Y136C positions significantly enhanced α-synuclein aggregation and toxicity. To test whether aggregation is the property only of α-synuclein, we have created a family of β-synucleins with similar Tyr-to-Cys substitutions and find no tendency for aggregation or toxicity.

MATERIALS AND METHODS

Cloning and Mutagenesis—The cloning of cDNAs containing WT, A53T, and A30P human α-synuclein has been described previously (28, 29). Human wild-type β-synuclein was cloned from an expressed sequence tag clone and verified by DNA sequencing. In this study, α- and β-synuclein coding sequences were subcloned into pcDNA3.1 vector

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The abbreviations used are: PD, Parkinson’s disease; DA, dopamine; LB, Lewy body; DAT, dopamine transporter; WT, wild type; HEK, human embryonic kidney; Ad, adenovirus; GFP, green fluorescent protein; Syn, synuclein; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling.

1 The abbreviations used are: PD, Parkinson’s disease; DA, dopamine; LB, Lewy body; DAT, dopamine transporter; WT, wild type; HNK, human embryonic kidney; Ad, adenovirus; GFP, green fluorescent protein; Syn, synuclein; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling.
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(Invitrogen). We generated α-synuclein mutations (Y39, Y125, Y133, Y136) and β-synuclein mutations (Y39, Y119, Y127, Y130) with tyrosine to either cysteine or phenylalanine using a PCR-based mutagenesis method (30). All the constructs were confirmed by DNA sequencing. For recombinant protein expression, human α-synuclein coding sequences were cloned into pRSET vector (Invitrogen).

**Cell Culture, Transfection, and Oxidative Stress Treatment—**Rat dopaminergic N27 cells (31) and human embryonic kidney (HEK) 293 cells were used for the transfection study. N27 cells were plated at a density of 1 × 10⁴ cells/cm² on polyethyleneimine (1 mg/ml; Sigma) coated 24-well plates in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and 2 mM t-glutamine. Effective reagent was used for transfection of N27 cells according to the supplier’s instruction (QIAGEN, Valencia, CA). HEK 293 cells were cultured on 12-well plates in a medium of Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and 2 mM t-glutamine. We used the calcium phosphate method to transfect 293 cells. For oxidative stress treatment, transfected cells were exposed to 50 µM dopamine for 4 h and analyzed 24 h later.

**Recombinant Adenovirus and Cell Transduction—**Recombinant adenoviruses expressing green fluorescent protein (Ad-GFP), WT, A53T, and A30P α-synuclein (Ad-WT-Syn, Ad-A53T-Syn, and Ad-A30P-Syn) have been described previously (28, 29). Adenovirus expressing Y39C and Y125C human α-synuclein (Ad-Y39C-Syn and Ad-Y125C-Syn) were constructed similarly. N27 cells were cultured on 24-well plates and exposed to adenoviruses at the dose of 200 plaque-forming units/cell.

**Immunocytochemistry—**N27 cells and HEK 293 cells were fixed in 4% paraformaldehyde for 30 min and processed for immunostaining as described previously (28, 29). Antibodies used include mouse anti-α-synuclein antibody (synuclein-1, 1:400; BD Transduction Laboratories, Lexington, KY); rabbit anti-ubiquitin antibody (1:200; Chemicon); rabbit anti-phospho-serine 129 α-synuclein (1:200; a kind gift from Dr. Takeshi Izutsu, University of Tokyo).

**Cell Viability, Apoptosis, and Protein Aggregation—**Three days after adenovirus transduction, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium was added to N27 cell cultures (0.4 mg/ml), and cell viability was measured as described previously (28). After α-synuclein immunostaining, cells were counterstained with Hoechst dye 33258 (1 mg/ml; Sigma) for 10 min. We examined α-synuclein-positive cells (i.e. transfected cells) for protein inclusions and apoptosis. Apoptotic cells were identified by a condensed, fragmented nucleus. We also used terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay (in situ apoptosis detection kit; Roche) to confirm apoptosis. To determine whether α-synuclein protein inclusion contained amyloid, thioflavin S (0.01%; Sigma) staining was used after α-synuclein immunostaining.

**Quantification and Statistics—**For quantification, three wells were examined in each condition (about 350–400 transfected cells from each well), and independent experiments were repeated at least three times. The fraction of transfected cells with inclusions was calculated as the ratio of the number of inclusion-containing cells to the total number of transfected cells. The fraction of transfected cells that were apoptotic was calculated in a similar way. Nuclear morphology or TUNEL-positive cells defined apoptotic cells. The data were compared by analysis of variance, and a post hoc Neuman test was performed.

**Western Blotting—**N27 cells and HEK293 cells were cultured on 6-well plates and transfected as described above. Adenovirus-transduced N27 cells were also used. Three days later, cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin (Sigma). After homogenizing with a Dounce homogenizer, cells were centrifuged at 14,000 rpm for 10 min, and the pellet (P1) and supernatant (S1) were collected. The Triton X-100-insoluble pellets were further dissolved in lysis buffer containing 1% SDS, centrifuged for 10 min, and the resulting supernatant (S2) was used for Western blotting. Protein concentration was measured by the BCA method (Pierce). Ten micrograms (for adenovirus experiments) or 50 µg (for transfection experiments) of total protein from S1 and S2 were separated on a 12% SDS-PAGE gel and transferred to nitrocellulose membrane (MSI, Westboro, MA). The blot was probed with mouse anti-α-synuclein antibody (synuclein-1, 1:1000; BD Transduction Laboratories) and detected with ECL reagents (PerkinElmer Life and Analytical Sciences).

**Recombinant Protein Purification and in Vitro Aggregation Assay—**The WT and mutant human α-synuclein proteins were expressed in Escherichia coli BL21 (DE3) cells (Invitrogen). Recombinant proteins were purified with Talon metal affinity resin beads (BD Biosciences Clontech). The protein concentration was determined by spectrophotometry. For protein aggregation assays, 100 µl of recombinant protein (0.2 mg/ml in PBS, pH 7.4) was incubated with 50 µM H₂O₂ for 4 h at room temperature. At the end of the experiment, 2 µl of protein solution was separated in 12% SDS-PAGE gel and transferred to nitrocellulose membrane. The Western blot staining was processed as described above. The protein band density was determined by densitometry.

**RESULTS**

Y39C and Y125C Mutations Increased α-Synuclein-Positive Inclusions and Apoptosis in N27 Cells and HEK293 Cells—To examine whether tyrosine-to-cysteine/phenylalanine modifica-
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Fig. 2. Expression of tyrosine-to-cysteine mutants of α-synuclein caused an increase in apoptosis and protein aggregation in N27 cells (left column) and 293 cells (right column). All four tyrosines were replaced by cysteine or phenylalanine in each of three different human α-synuclein: WT (A and E), A53T (B and F), and A30P (C and G). Human WT β-synuclein and its four tyrosine-to-cysteine mutations were used as controls (D and H). The percentage of transfected cells that were apoptotic and the percentage that had α-synuclein inclusions were determined for each mutant. Cells transfected with Y39C and Y125C mutant α-synuclein had significantly more apoptosis and more inclusions compared with WT α-synuclein (A and E, n = 12; *, p < 0.05). All other mutations did not differ significantly from WT control. Mutations based on A53T (B and F, n = 12; *, p < 0.05) and A30P (C and G, n = 12; *, p < 0.05) α-synuclein produced results similar to those seen in WT. By contrast, WT α-synuclein and all four Tyr-to-Cys mutant β-synucleins had no toxicity compared with vector-only control (D and H, n = 9, p > 0.1). Simultaneous with the β-synuclein experiments, a series of α-synuclein experiments with WT, Y39C, and Y125C were performed as positive controls. All forms showed significant increases in inclusions and apoptosis compared with β-synuclein (D and H, n = 9; *, p < 0.05; #, p < 0.01).

tions of human α-synuclein can change protein aggregation and toxicity, we generated three sets of mutations based on human WT, A53T, and A30P α-synuclein genes. As controls, human β-synuclein and its four tyrosine-to-cysteine mutations were constructed. We expressed these constructs in both N27 cells and HEK 293 cells by transient transfection. N27 cells are from rat embryonic day 12 midbrain and immortalized with T-antigen (31). N27 cells have a dopaminergic phenotype that includes the expression of tyrosine hydroxylase and DAT (28, 31). N27 cells were further confirmed by TUNEL-positive staining (Fig. 1, L and M). In contrast, WT β-synuclein (Fig. 1K) and its four Tyr-to-Cys mutations (data not shown) showed no protein aggregation. We have found that α-synuclein-positive inclusions and cytoplasm were also positive for thioflavin S staining (Fig. 1, N and O), indicating the presence of α-synuclein fibrils. These α-synuclein protein inclusions were negative for ubiquitin and phospho-Ser129 α-synuclein staining (data not shown).

The percentage of apoptotic cells and the percentage of cells with α-synuclein inclusions are shown in Fig. 2. In both N27 and 293 cells, we found that cells transfected with Y39C and Y125C mutants resulted in significantly more apoptotic cell death and significantly more inclusions (Fig. 2A, E, n = 12; *, p < 0.05) compared with WT, whereas Y133C and Y136C substitutions did not differ significantly from the WT control. The effect of cysteine substitution was also tested in the non-tyrosine position. Cysteine substitution at A53 (A53C) had no effect on apoptosis or inclusion formation (Fig. 2, A and E, n =
In four Tyr-to-Phe \( \alpha \)-synuclein mutations, there were no changes in protein inclusions and apoptosis compared with WT protein (Fig. 2, A and E, \( n = 12, p > 0.1 \)). In the A53T and A30P series of mutant genes, the effects of tyrosine-to-cysteine/phenylalanine substitutions were similar to those seen in the WT human \( \alpha \)-synuclein (Fig. 2, B, C, F, and G). In both series, only Y39C and Y125C mutants caused significantly more apoptosis and \( \alpha \)-synuclein inclusions than controls (Fig. 2, B, C, F, and G, \( n = 12; *, p < 0.05 \)). No other mutations differed significantly from control (\( p > 0.1 \)). In contrast, no inclusions were found in cells transfected with WT \( \beta \)-synuclein and four Tyr-to-Cys mutations (Fig. 2, D and H, \( n = 9 \)). The apoptotic rates for all five \( \beta \)-synuclein genes were similar to vector-only control (Fig. 2, D and H, \( n = 9, p > 0.1 \)). A positive control run simultaneously with the \( \alpha \)-synuclein experiments, an additional series of \( \alpha \)-synuclein experiments using WT, Y39C, and Y125C all showed significantly more apoptotic cell death and protein inclusions than \( \beta \)-synuclein (Fig. 2, D and H, \( n = 9; *, p < 0.05 \); #, \( p < 0.01 \)).

\( \alpha \)-Synuclein Inclusion Associated with Apoptosis—We asked whether the formation of \( \alpha \)-synuclein-positive inclusions could cause apoptotic cell death. We further analyzed transfected cells and divided them into two cell populations: with and without \( \alpha \)-synuclein inclusions. The rate of apoptosis in the two cell populations is shown in Fig. 3. Both N27 and 293 cells with inclusions had a significant higher rate of apoptosis than cells without inclusions (Fig. 3, A and B, \( n = 12, p < 0.01 \)). Furthermore, cells with Y39C and Y125C mutations had significant more inclusions and a higher apoptotic rate than cells with WT \( \alpha \)-synuclein (Fig. 3, A and B, \( n = 12, p < 0.05 \)). Similar data were found in the mutants based on A53T or A30P \( \alpha \)-synuclein (\( n = 12 \); data not shown). The results indicate that the apoptosis is highly associated with \( \alpha \)-synuclein inclusions.

Oxidative Stress Promotes Y39C and Y125C Toxicity in N27 Cells—We observed higher toxicity of Y39C and Y125C \( \alpha \)-synuclein in N27 cells than 293 cells, which could be a result of the endogenous production of DA in N27 cells (31). We tested whether additional oxidative stress would further enhance protein aggregation and cellular toxicity in N27 cells. Two days after transfection, cells were exposed to 50 \( \mu \)M DA. We found apoptosis was significantly enhanced in cells transfected with Y39C and Y125C \( \alpha \)-synuclein compared with WT control (Fig. 4A, \( n = 9; *, p < 0.05 \)). The DA treatment also significantly increased \( \alpha \)-synuclein-positive inclusions in Y39C and Y125C transfected cells compared with WT (Fig. 4B, \( n = 9; *, p < 0.05 \)). Unlike N27 cells, treatment with 50 \( \mu \)M DA had no effect.
on apoptosis or inclusion formation in 293 cells, possibly because these cells lack DAT (n = 9; data not shown). The results indicate that oxidative stress can potentiate the neurotoxicity of cysteine-substituted α-synuclein in dopamine neurons.

Adenovirus-mediated Overexpression of Y39C and Y125C α-Synuclein Caused Apoptotic Cell Death—To overcome the low transfection efficiency in N27 cells, we generated adenoviruses expressing Y39C and Y125C α-synuclein. We had previously shown that adenovirus could achieve 90% transduction efficiency (28). N27 cells were transduced with adenovirus at a multiplicity of infection of 200. In Ad-Y39C-Syn and Ad-Y125C-Syn transduced cells, we found a significant decrease in total cell viability (Fig. 5A, n = 9; *, p < 0.05; #, p < 0.01), increase in apoptosis (Fig. 5B, n = 9; *, p < 0.01), and increase in the rate of cells with inclusions (Fig. 5C, n = 9; #, p < 0.01) compared with Ad-WT-Syn, Ad-GFP, or mock control. Cells transduced with Ad-A53T-Syn and Ad-A30P-Syn also showed significantly less viability (Fig. 5A, n = 9; *, p < 0.05; #, p < 0.01), increase in apoptosis (Fig. 5B, n = 9; #, p < 0.01), and increase in the rate of inclusion (Fig. 5C, n = 9; #, p < 0.01) compared with WT and controls. The Ad-Y39C-Syn and Ad-Y125C-Syn did not cause significantly more cell death and apoptosis than Ad-A53T-Syn, but Ad-Y39C-Syn did have significantly more cell death and apoptosis than Ad-A30P-Syn (n = 9; *, p < 0.05).

Oxidative Stress and Adenovirus-mediated α-Synuclein Overexpression Enhanced Dimer and Oligomer Formation in N27 Cells—To investigate whether cysteine substitution increases dimer or oligomer formation, we analyzed α-synuclein protein using Western blotting. Under basal conditions, only monomer α-synuclein was observed in both Triton X-100-soluble and insoluble fractions (data not shown). After treatment with 50 μM DA for 24 h before harvesting, α-synuclein dimers were found in Y39C and Y125C α-synuclein in Triton X-100-soluble fractions (Fig. 6A). In Triton X-100-insoluble fractions, only α-synuclein monomer was identified (Fig. 6B). Unlike N27 cells, there were no dimers or oligomers detected in 293 cells at the basal conditions or with the 50 μM DA treatment (data not shown). In N27 cells transduced with adenovirus, we found dimers and oligomers in Ad-Y39C-Syn and Ad-Y125C-Syn-treated cells in Triton X-100-soluble fractions (Fig. 6C), as well as high molecular weight α-synuclein species in Triton X-100-insoluble fractions (Fig. 6D). In contrast, Ad-WT-Syn, Ad-A53T-Syn, and Ad-A30P-Syn did not produce dimers or oligomers. Recombinant Y39C Proteins Form More Dimer and Oligomer after Oxidative Stress in Solution—We investigated whether recombinant proteins with cysteine substitutions can form dimers and oligomers under mild oxidizing conditions in solution. Purified recombinant proteins were incubated with 50 μM...
In this study, we found that the expression level of α-synuclein overexpression promote dimer formation in N27 cells. A and B, transfected cells were treated with 50 μM dopamine for 4 h and analyzed 24 h later. Western blots (50 μg of total protein per lane) detected dimer formation in the Triton X-100-soluble fraction recovered from Y39C and Y125C transfected cells (A). There were no dimers or oligomers recovery from Triton X-100-insoluble fractions (B). C and D, adenovirus-transduced N27 cells were analyzed in Western blots (10 μg of total protein per lane). Dimers and oligomers were found in Ad-Y39C-Syn- and Ad-Y125C-Syn-transduced cells in Triton X-100-soluble fractions (C) as well as high molecular weight oligomers in Triton X-100-insoluble fractions (D). The asterisk in D indicates the stacking and resolving gel interface. Experiments were repeated three times with similar results.

**DISCUSSION**

In cell culture, we have shown that mutant forms of human α-synuclein with tyrosine-to-cysteine substitutions can enhance α-synuclein protein aggregation and increase cell toxicity. We have seen that the position of the tyrosine-to-cysteine substitution is critical; the Y39C and Y125C enhance protein aggregation and toxicity whereas Y133C and Y136C substitutions have no effect. In addition, the substitution of tyrosine with phenylalanine had no effect on intracellular inclusions and cytotoxicity. The changes in toxicity were specific for α-synuclein; Y39C and Y125C mutations in β-synuclein did not cause protein toxicity.

In this study, we found that the expression level of α-synuclein protein influenced the aggregation and toxicity of Y39C and Y125C. Using adenovirus-mediated overexpression, we found that the level of α-synuclein overexpression is five times greater than that of transient transfection (as seen in Fig. 6, compare α-synuclein seen from loading 10 μg of total protein for adenovirus versus 50 μg for transfection). Because higher concentrations of α-synuclein were produced, we were able to detect the dimers and oligomers in Triton X-100-soluble fractions even under basal conditions. In transfection experiments, because only a small number of cells (3–5%) contained α-synuclein inclusions, dimers were not seen in N27 cells or 293 cells under basal conditions. Exposure to 50 μM DA greatly enhanced α-synuclein inclusions in the N27 cells transfected with Y39C and Y125C mutant α-synuclein, and dimers were then detected. When cells were transfected with A53T and A30P α-synuclein, the mutant proteins caused no toxicity compared with WT α-synuclein. However, the enhanced gene expression produced by adenovirus led to significant toxicity of A53T and A30P mutant forms of α-synuclein. These results indicate that A53T and A30P α-synuclein require relatively high levels of protein to show toxicity.

The cysteine-substituted α-synuclein we have developed offers a new insight into the importance of Y39 and Y125 in α-synuclein toxicity. The effect of cysteine substitution is spe-
cific for \( \alpha \)-synuclein, not for \( \beta \)-synuclein. This difference may reflect the non-amyloidogenic nature of \( \beta \)-synuclein. The reason why only Y39C and Y125C, but not Y133 and Y136, have these effects on \( \alpha \)-synuclein is not known currently. Previous study indicates nitrating and oxidizing agents can attack specific amino acids in the target proteins (32). \( \alpha \)-Synuclein is such a target protein in which nitration occurs on specific tyrosines in synucleinopathies (23, 24). Exposure of cells to nitrating agents can produce aggregations of \( \alpha \)-synuclein that contain nitrated tyrosine (25). We hypothesized that if nitrating and oxidizing agents can recognize specific tyrosine sites in the \( \alpha \)-synuclein molecule, then cysteine substitution at the same positions might also facilitate disulfide dimer formation at these sites. Because of the ease of oxidation of cysteine to a disulfide, we also reasoned that oxidation should occur at a lower oxidative stress level and that the disulfide dimers formed would be more chemically stable. Previous study has shown that the Y125 is the most important site for in vitro dityrosine cross-linking by nitration (33). Recently, Norris et al. (34) found that all four tyrosines are potential targets for protein nitration as well as dityrosine cross-linking. We can only speculate on the mechanism for selective aggregation of Y39C and Y125C \( \alpha \)-synuclein compared with other \( \alpha \)- or \( \beta \)-synucleins. Inside the cell, \( \alpha \)-synuclein could bind to membrane and other proteins, which may lead to conformational changes that increase dimer formation. It may be possible that Y39C and Y125C are more exposed to oxidative agents than Y133C and Y136C.

\( \alpha \)-Synuclein protein plays a role in oxidative stress and is in turn influenced by it. Overexpression of WT, A53T, and A30P mutant \( \alpha \)-synuclein leads to increased reactive oxygen species, enhanced catalysis of hydrogen peroxide, and mitochondrial deficits (35–37). Overexpression of WT and mutant \( \alpha \)-synuclein can increase the susceptibility of cells to oxidative stress (28–29, 38–41). In N27 cells, low levels of DA and the DAT can enhance oxidative stress because of metabolism of DA itself (31, 42). We found that overexpression of Y39C and Y125C produced greater protein aggregation and apoptosis in N27 cells compared with 293 cells, perhaps because N27 cells are a rat dopaminergic cell line, whereas 293 cells neither synthesize DA nor have the DAT to bring DA into the cell. With additional oxidative stress, we found more protein aggregation and cellular toxicity in N27 cells. Biophysical studies indicate the dimer forming process is the rate-limiting step in \( \alpha \)-synuclein aggregation (27). Once the accumulation of dimer reaches a critical threshold, protein aggregation progresses rapidly. Eventually, dimer concentrations diminish as dimers are converted to polymers and fibrils (27). Our results suggest that elevated oxidative stress can induce dimer and fibril formation, eventually leading to cellular toxicity.

We have found some dissociation between dimer formation and cellular toxicity. In the recombinant protein solution chemistry experiment (Fig. 7), only Y39C showed increased dimer formation. This result suggests that Y39C and Y125C proteins may have different sensitivities to oxidative challenge. We used very mild oxidative stress (50 \( \mu \)M \( \text{H}_2\text{O}_2 \)) to induce dimer formation, because this concentration is comparable with the 50 \( \mu \)M DA we used in tissue culture. With high oxidative stress (1.2 \( \mu \)M \( \text{H}_2\text{O}_2 \), Uversky et al. (43) have found extensive oxidation of all methionine in \( \alpha \)-synuclein protein. In experiments with Ad-A53T-Syn and Ad-A30P-Syn, no dimers were found in Western blots, but both mutations caused toxicity and inclusions. It is possible that dimer formation was not needed for protein aggregation. Cell toxicity could be caused by conformational changes and self-assembly by the protofibril and fibril. In Y39C and Y125C mutations, the increased dimer formation could stabilize and accelerate oligomer formation, which could lead to toxic protofibril formation and fibrillogenesis. Thus, the toxicity induced by A53T and A30P mutations might have a different mechanism than the Y39C and Y125C mutations. Recently, Norris et al. (34) reported that nitrative and oxidative stress can lead to different mechanisms for \( \alpha \)-synuclein fibrillogenesis.

The importance of the site of cysteine substitution to cause \( \alpha \)-synuclein toxicity was further shown by the fact that the A53C mutant had no toxicity. The differential toxicity of Y39C and A53C may be related to their positions relative to the 11-mer repeats (XKTKEGVXXXX consensus motif), because Y39 is inside the third repeat, whereas A53 is just outside the fourth repeat (44, 45). These 11-mer repeats have been proposed to form an \( \alpha \)-helix when binding to the membrane (46). It is possible the Y39C mutation may disrupt the \( \alpha \)-helix structure and render the protein more hydrophobic, which could lead to protein aggregation, whereas A53C substitution might not change \( \alpha \)-helix structure. On the other hand, the cysteine substitution may cause \( \alpha \)-synuclein protein conformational changes by stabilizing the formation of \( \beta \)-sheet structures or promote the misfolding of the protein.

In our cell culture model of \( \alpha \)-synuclein toxicity, we have found that inclusions contain \( \alpha \)-synuclein fibril, because they are positive for thioflavine S staining. Because ubiquitin is not present in \( \alpha \)-synuclein-positive inclusions and Ser-129 is not phosphorylated, the inclusions are likely to be composed of \( \alpha \)-synuclein protein aggregates, without Lewy body-like structure. We have observed a high correlation between protein aggregation and apoptotic cell death. Cell death was enhanced by added oxidative stress and was probably caused by toxic protofibril formation in the protein aggregation process (47), or by deficits in the ubiquitin-proteasome system (6, 48).

Although the tyrosine-to-cysteine mutations we have generated are an artificial system not found in patients with PD, this family of mutant proteins provides a molecular model that we have shown is more sensitive to oxidative stress than the native form of \( \alpha \)-synuclein. Because stable cysteine dimer can be formed from these proteins, these mutants may be useful for structural assessment by NMR and other chemical methods. We are currently developing and evaluating the Y39C transgenic mouse. We have shown that these novel mutants of \( \alpha \)-synuclein produce site-specific cell toxicity as well as dimerization under oxidative stress that leads to protein aggregation and cell death.

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