The mechanisms that govern the formation of α-synuclein (α-syn) aggregates are not well understood but are considered a central event in the pathogenesis of Parkinson’s disease (PD). A critically important modulator of α-syn aggregation in vitro is dopamine and other catechols, which can prevent the formation of α-syn aggregates in cell-free and cellular model systems. Despite the profound importance of this interaction for the pathogenesis of PD, the processes by which catechols alter α-syn aggregation are unclear. Molecular and biochemical approaches were employed to evaluate the mechanism of catechol-α-syn interactions and the effect on inclusion formation. The data show that the intracellular inhibition of α-syn aggregation requires the oxidation of catechols and the specific noncovalent interaction of the oxidized catechols with residues Y125F, E126A, and H9251 in the C-terminal region of the protein. Cell-free studies using novel near infrared fluorescence methodology for the detection of covalent protein−protein interactions and dopaminergic neurodegeneration.

α-Synuclein (α-syn)NACP, synelfin, a small, neuron-specific protein, was first linked to PD by genetic analysis of familial cases with autosomal dominant inheritance of the disease. Genetic analysis discovered a point mutation in the α-syn gene (SNCA), resulting in an amino acid conversion of Ala53 to Thr (1). Subsequently, α-syn protein was detected in Lewy bodies within the dopaminergic neurons of the substantia nigra pars compacta (2), the intracellular proteinaceous inclusions characteristic of PD and related disorders. Since this discovery, the process of α-syn aggregation has been proposed to underlie dopaminergic degeneration that occurs in PD. Therefore, delineating the mechanisms of α-syn aggregation and its pathophysiological role in neurodegeneration has been the focus of many investigations.

Although the in vivo factors that regulate α-syn aggregation are not well understood, mechanisms involving genetic and environmental factors have been proposed. Genetic analysis has uncovered two additional missense mutations in SNCA (A30P and E46K) (3, 4) as well as triplication of the SNCA genomic region (5). Mutations of α-syn or gene triplication may increase the rate of α-syn aggregation (6, 7), impair cellular degradation (8), or increase the amount of cytosolic α-syn beyond the critical concentration required to initiate polymerization (9). Most PD cases are sporadic and involve aggregation of wild type α-syn, indicating that other factors may influence disease progression. For example, slow, chronic administration of the selective dopaminergic toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine can induce the formation of α-syn aggregates in mice, putatively through mitochondrial damage and subsequent oxidative stress (10). Additionally, the pesticides rotenone and paraquat have been shown to induce or alter α-syn pathology in rodent models through complex I inhibition and oxidative injury (11, 12). Consistent with these findings, oxidative and nitritative modifications to α-syn have been documented in human PD brain (13) and can alter the aggregation rate of the protein through direct modifications or by impaired protein degradation (14, 15). Although α-syn has been clearly linked to PD pathogenesis, the mechanism by which a ubiquitously expressed neuronal protein causes accelerated cell death in circumscribed brain nuclei remains unknown.

Catechols, such as dopamine, can be oxidized at physiological pH to generate reactive ortho-quinone (o-quinone) and aminochrome species. Electron-deficient o-quinones can directly modify protein structure and function through covalent attachment to cysteine or other nucleophilic residues (16, 17). In relation to PD pathophysiology, cell-free in vitro analysis has shown that oxidized catechols can inhibit the formation of α-syn aggregates by stabilizing soluble oli-
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gomeric intermediates (18–20). However, several critical questions relating to the mechanisms of dopamine-induced alterations in α-syn aggregation remain unanswered. Therefore, we employed a previously characterized cellular model (21) to explore the biochemical mechanisms that govern the interaction of α-syn with catechols. We utilized SH-SY5Y cells expressing a five-amino acid-mutated α-syn in the C-terminal region: Y125F, E126A, M127A, P128F, S129A (α-syn 125m) was generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described previously (20). The prk172 Δ71–82 α-syn mutant was generated with the ExSite PCR site-directed mutagenesis kit (Stratagene) using the following primers: 5′-Phos-ACA ACA GTG CCT GAG ACC AAA G-3′; 5′-Phos-GGC GGA TCG GAA TCC AAC CTT GTA CCC CAC CAT GGA TGT ATG CAT GAA AGG ACT TTC AAA GGC C-3′; 5′-Phos-GCC GGA TCG GAA TCC AAG CTT GTA CCC CAT GGA TGT ATG CAT GAA AGG ACT TTC AAA GGC C-3′. The PCR-generated fragment was subcloned into pcDNA3.0 at the 5′-KpnI and 3′-EcoRV sites. A53T-125m α-syn was generated with the ExSite PCR-based site-directed mutagenesis kit (Stratagene) as described previously (22). To generate both the Δ71–82 and 125m α-syn pCDNA3.0 constructs, the coding sequences were PCR-amplified from the prk172 vector to introduce a 5′ KpnI site followed by a Kozak consensus site. The following primers were used: 5′-Phos-GGG GTA CCC CAC CAT GGA TGT ATG CAT GAA AGG ACT TTC AAA GGC C-3′; 5′-Phos-GCC GGA TCG GAA TCC AAG CTT GTA CCC CAC CAT GGA TGT ATG CAT GAA AGG ACT TTC AAA GGC C-3′. The PCR-generated fragment was subcloned into pcDNA3.0 at the 5′-KpnI and 3′-EcoRV sites. A53T-125m α-syn was generated with the ExSite PCR-based site-directed mutagenesis kit (Stratagene) as described above and previously (21). Briefly, after viral transduction and RA differentiation, cells were washed in cold Dulbecco’s phosphate-buffered saline (D-PBS) and harvested in lysis buffer (1% (v/v) Triton X-100, 20 mM HEPES, 150 mM NaCl, 10% (v/v) glycerol, 1 mM EGTA, 1.5 mM MgCl₂, 1 mM phenylmethylsulfonfonyl fluoride, 10 mM sodium pyruvate, 50 mM NaF, 2 mM sodium orthovanadate, 1 μM lactacystin, and a protease inhibitor mixture (Sigma), pH 7.4). Homogenates were loaded directly onto 12% SDS-polyacrylamide gels in 1× sample buffer (20 mM Tris, 1% (v/v) glycerol, 180 mM β-mercaptoethanol, 0.003% (w/v) bromphenol blue, and 2% w/v SDS, pH 6.8) and transferred to polyvinylidene difluoride membranes. Blots were blocked in 20 mM Tris, pH 7.4, 150 mM NaCl, 0.2% (v/v) Tween 20 containing 5% (w/v) milk for 1 h followed by incubation with the following primary antibodies for 2 h at 25 °C: monoclonal Syn 204 (1:1000), monoclonal TH (1:5000; EMD Biosciences, La Jolla, CA), polyclonal NSE (1:2000; Polysciences, Warrington, PA). Primary antibodies were detected by a 1-h incubation with either goat anti-mouse IgG Alexa Fluor 680 (1:5000; Molecular Probes, Eugene, OR) or goat anti-rabbit IgGIRDye 800 (1:5000; Rockland, Gilbertsville, PA) conjugated secondary antibodies in blocking buffer and scanned at intensity level 2 with an Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE).

EXPERIMENTAL PROCEDURES

Generation of α-Syn-containing Plasmids—The bacterial expression vector prk172 encoding α-syn Y125F,E126A, M127A,P128F,S129A (α-syn 125m) was generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described previously (20). The prk172 Δ71–82 α-syn mutant was generated with the ExSite PCR site-directed mutagenesis kit (Stratagene) as described previously (22). To generate both the Δ71–82 and 125m α-syn pCDNA3.0 constructs, the coding sequences were PCR-amplified from the prk172 vector to introduce a 5′ KpnI site followed by a Kozak consensus site. The following primers were used: 5′-Phos-ACA ACA GTG CCT GAG ACC AAA G-3′; 5′-Phos-GGC GGA TCG GAA TCC AAG CTT GTA CCC CAC CAT GGA TGT ATG CAT GAA AGG ACT TTC AAA GGC C-3′; 5′-Phos-GCC GGA TCG GAA TCC AAG CTT GTA CCC CAC CAT GGA TGT ATG CAT GAA AGG ACT TTC AAA GGC C-3′. The PCR-generated fragment was subcloned into pcDNA3.0 at the 5′-KpnI and 3′-EcoRV sites. A53T-125m α-syn was generated with the ExSite PCR-based site-directed mutagenesis kit (Stratagene) as described above and previously (21).

CELL LINES—Generation of stably transfected C-terminal region: Y125F, E126A, M127A, P128F, S129A (α-syn 125m) α-syn-expressing SH-SY5Y Cell Lines—These procedures have been described in detail previously (21). Briefly, after viral transduction and RA differentiation, cells were washed in cold Dulbecco’s phosphate-buffered saline (D-PBS) and harvested in lysis buffer (1% (v/v) Triton X-100, 20 mM HEPES, 150 mM NaCl, 10% (v/v) glycerol, 1 mM EGTA, 1.5 mM MgCl₂, 1 mM phenylmethylsulfonfonyl fluoride, 10 mM sodium pyruvate, 50 mM NaF, 2 mM sodium orthovanadate, 1 μM lactacystin, and a protease inhibitor mixture (Sigma), pH 7.4). Homogenates were loaded directly onto 12% SDS-polyacrylamide gels in 1× sample buffer (20 mM Tris, 1% (v/v) glycerol, 180 mM β-mercaptoethanol, 0.003% (w/v) bromphenol blue, and 2% w/v SDS, pH 6.8) and transferred to polyvinylidene difluoride membranes. Blots were blocked in 20 mM Tris, pH 7.4, 150 mM NaCl, 0.2% (v/v) Tween 20 containing 5% (w/v) milk for 1 h followed by incubation with the following primary antibodies for 2 h at 25 °C: monoclonal Syn 204 (1:1000), monoclonal TH (1:5000; EMD Biosciences, La Jolla, CA), polyclonal NSE (1:2000; Polysciences, Warrington, PA). Primary antibodies were detected by a 1-h incubation with either goat anti-mouse IgG Alexa Fluor 680 (1:5000; Molecular Probes, Eugene, OR) or goat anti-rabbit IgGIRDye 800 (1:5000; Rockland, Gilbertsville, PA) conjugated secondary antibodies in blocking buffer and scanned at intensity level 2 with an Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE).

Size Exclusion Chromatography—α-Syn was extracted as described above, and 0.5–1 mg of total Triton-soluble lysate was resolved on a Superdex 200 HR10/30 column (GE Healthcare) using 25 mM HEPES, 150 mM NaCl as the mobile phase at a flow rate of 0.3 ml/min. Fractions were collected (0.5 ml), concentrated with 5-kDa cut-off filters (Millipore), and loaded onto 12% SDS-polyacrylamide gels for Western blot analysis as described above and previously (21).

Quantification of Intracellular Catechols by High Pressure Liquid Chromatography—electrochemical detection (ECD)—After viral transduction and RA differentiation, cells (5 × 10⁵ cells/well) were washed in D-PBS and harvested in 100 μl of 0.1 M perchloric acid containing 100 μM dihydroxybenzylamine and lysed by sonication. Lysates were centrifuged at 16,000 × g for 10 min at 4 °C, and 50 μl of supernatant was injected on the high
pressure liquid chromatograph for analysis as described previously (21).

**Immunofluorescence Analysis**—Cells were cultured at 6 × 10⁴ in 12-well cluster dishes (Corning Glass). Cells were washed three times in ice-cold D-PBS and fixed in methanol followed by a 1:1 mix of methanol/acetone as described (20). The following primary antibodies for α-syn were used: affinity-purified polyclonal SNL-1 (1:1000), polyclonal SNL-4 (1:500), and monoclonal Syn 514 (1:400). Monoclonal or polyclonal antityrosine hydroxylase was also used (1:1000; EMD Biosciences, La Jolla, CA). Primary antibodies were incubated in blocking buffer (50 mM phosphate, 50 mM NaCl, pH 7.2, 0.3% (v/v) Triton (PBS-T), with 5% (w/v) bovine serum albumin and 10% (v/v) normal goat serum) at 4 °C overnight. The cells were washed three times in PBS-T followed by incubation of secondary antibodies for 1 h at 25 °C in blocking buffer (Cy3-conjugated anti-mouse or anti-rabbit IgG (1:100; Jackson ImmunoResearch, West Grove, PA); Alexa 488-conjugated anti-mouse or anti-rabbit IgG (1:300, Molecular Probes, Inc., Eugene, OR). Nuclei were visualized with 4',6-diamidino-2-phenylindole dihydrochloride staining at 200 ng/ml for 15 min. In some experiments, thioflavin S staining was done after secondary incubation using a 0.05% (w/v) solution dissolved in ethanol (21). Cells were imaged with an inverted Olympus IX70 microscope equipped with an IX-FLA fluorescence observation attachment (Olympus Optical Co., Tokyo, Japan). For quantification of cells containing aggregates, 10 fields of view were randomly selected per well, with at least 100 total cells counted per replicate. For SNL-1, total aggregates were scored (both large and small cytoplasmic punctated structures), whereas for SNL-4 and Syn 514, only juxtanuclear inclusions were counted. For thioflavin S, only inclusions that were juxtanuclear and co-localized with α-syn immunostaining were counted.

**Purification of Recombinant WT and Mutant α-Syn**—BL21-CodonPlus (DE3)-RIL-competent cells (Strategene) were transformed with expression plasmid prk172 WT or mutant α-syn and cultured to an optical density of 0.6–0.8 at 37 °C with shaking. Protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h. Bacterial cell pellets were homogenized with a pestle in high salt buffer (0.75 M NaCl, 10 mM Tris-Cl, pH 7.0, 1 mM EDTA) containing a mixture of protease inhibitors and 0.2 mM phenylmethanesulfonyl fluoride, heated to 100 °C for 10 min, and centrifuged at 20,000 × g for 30 min. Supernatant was dialyzed against 10 mM Tris containing 1 mM EDTA and 0.2 mM phenylmethanesulfonyl fluoride, pH 7.5, and applied to a Superdex 200 HR10/30 gel filtration column (GE Healthcare) in a mobile phase of 10 mM Tris, 150 mM NaCl, pH 7.5, at a flow rate of 0.4 ml/min. Fractions containing α-syn were collected, concentrated, and applied to a Resource Q column (GE Healthcare) and eluted with a 0–0.5 M NaCl gradient. Collected fractions containing α-syn were purified once more by gel filtration in D-PBS. Protein concentration was determined by the bicinchoninic acid protein assay (Pierce) using bovine serum albumin as a standard.

**In Vitro Analysis of α-Syn Aggregation**—α-Syn was incubated in conditions previously described for promoting fibril forma-

**Mass Spectrometry of Catechol-modified α-Syn**—Recombinant α-syn was incubated with constant shaking in D-PBS, 37 °C for 48 h as described above. Following incubation, the sample was centrifuged at 100,000 × g at 4 °C for 30 min, and 2 μg of total soluble protein was injected into the mass spectrometer. Mass spectrometry was performed on an Agilent 1100 series quadrupole mass spectrometer equipped with an electrospray ion source in a mobile phase of 0.1% (v/v) formic acid. The spectrometer was operated in positive ion mode.

**In Vitro Aggregation of Fibrinogen, Alcohol Dehydrogenase, and γ-Crystallin**—Aggregation for all three proteins was determined by light scattering at 360 nm and followed as a function...
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FIGURE 1. Stable expression of A53T-125m α-syn in differentiated SH-SY5Y cells induces the formation of inclusions. a, Western blot analysis of α-syn-expressing cell lysates using monoclonal Ab Syn 204 was used to monitor expression levels, and NSE was used as a loading control. b, selected stably transfected cell lines expressing ΔT1–82 (line 40) and A53T-125m α-syn (line 34) were incubated in the presence of 20 μM RA or dimethyl sulfoxide (vehicle (Veh)) for 8 days to differentiate SH-SY5Y cells into a neuron-like phenotype. c, immunofluorescence analysis of α-syn-expressing cell lines using polyclonal Ab SNL-1 (red) after 8 days of RA (×30 magnification). Mature, juxtanuclear (JN) aggregates co-localized with the amyloid binding dye thioflavin S (Thio S; shown yellow in the merged image). Nuclei were visualized with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) (blue). d, quantification of cells containing total (small and large juxtanuclear aggregates) and larger, thioflavin S-positive inclusions (n = 3; values are the mean ± S.E.).

Statistical Analysis—Data were analyzed using Sigmasat software version 2.03 (SPSS Inc., Chicago, IL) and are expressed as the mean ± S.E. One-way analysis of variance followed by Tukey’s post hoc test was utilized to determine if groups were statistically different, with p values of <0.05 considered significant.

RESULTS

To determine the cellular mechanism of catechol-α-syn interactions and the effect on inclusion formation, stably transfected α-syn 125m SH-SY5Y cell lines were generated. Because expression of α-syn 125m did not result in a substantial amount of cells containing inclusions (Fig. S1, a and b), the A53T mutation was introduced into the 125m construct to generate α-syn A53T-125m. Several cell lines were generated and analyzed for A53T-125m expression levels by Western blot analysis (Fig. 1, a and b). Based on this analysis, line 34 was selected for subsequent experiments. Immunofluorescence analysis of RA-differentiated A53T-125m cells revealed the presence of cytoplasmic, punctated aggregates as well as larger, juxtanuclear inclusions that co-localized with thioflavin S, indicating the presence of amyloid inclusions (Fig. 1, c and d). The number of A53T-125m cells containing punctated and juxtanuclear inclusions that co-localized with thioflavin S was comparable with cells expressing A53T α-syn. As a control, cells expressing ΔT1–82 α-syn, a mutant α-syn protein that is incapable of polymerizing in vitro (22) and in vivo (26), were used. Immunofluorescence analysis showed a diffuse, cytoplasmic staining pattern with only a small percentage of cells (<1%) that contained small punctated α-syn aggregates (Fig. 1, c and d).

The transfected SH-SY5Y cell lines used for these experiments do not contain detectable amounts of catechols or the rate-limiting synthesizing enzyme, tyrosine hydroxylase (TH) (vector condition; Fig. 2, a and b). Therefore, to determine the effect of increasing catechol levels on α-syn aggregation in A53T-125m cells, TH was expressed by transduction with a lentiviral vector encoding for human TH-1. To achieve elevated levels of cytotoxic catechols, a mutant form of TH was utilized, TH RR-EE, which contains an altered catecholamine feedback inhibition site (residues 37 and 38 converted from Arg to Glu), allowing the enzyme to produce higher catechol levels compared with wild type TH (21, 27). After RA differentiation, the TH RR-EE-transduced A53T-125m cells contained higher levels of catechols compared with empty vector-transduced cells (Fig. 2b). As shown previously, commensurate to increasing catechol levels, the number of cells containing α-syn aggregates in A53T expressing cells declined (Fig. 3, a and b). However, the same treatment in A53T-125m cells had no effect on α-syn cytoplasmic distribution (Fig. 3, a and b). Co-staining for α-syn and TH showed that a significantly higher amount of TH-positive A53T-125m cells contained α-syn aggregates compared with A53T cells (Fig. 3, a and b). These findings were further verified by immunostaining A53T-125m cells with polyclonal...
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Ab SNL-4, which recognizes an N-terminal region of α-syn, and monoclonal Ab Syn 514, which preferentially detects mature, juxtanuclear α-syn inclusions. Both SNL-4 and Syn 514 revealed a higher proportion of TH-positive A53T-125m cells containing α-syn inclusions compared with TH-positive A53T cells (Figs. 3, a and b, and S2, a and b). These results indicated that the 125YEMPS129 region of α-syn is required for catechol-mediated inhibition of α-syn aggregation in SH-SY5Y cells.

Previous analysis revealed that cytosolic catechols inhibit the formation of Triton-insoluble α-syn with a concomitant increase of soluble oligomers in A53T α-syn-expressing cell lines (21). To determine if this process requires catechol oxidation, cells were transduced to express TH RR-EE and then differentiated with RA in the presence of NAC. Formation of Triton-insoluble α-syn-expressing cells were analyzed to determine if oxidized catechols have the ability to interact with monomeric α-syn to induce the formation of soluble α-syn oligomers. Increasing catechols did not change the elution profile of Δ71–82 α-syn (Fig. 4c). Taken together, these results imply that oxidized catechols interact with the 125YEMPS129 motif of α-syn oligomers that are in the process of forming polymers.

To gain further insight into the mechanism of catechol-induced inhibition of α-syn aggregation, cell-free in vitro systems were utilized. Purified recombinant α-syn was incubated in the presence of the deaminated metabolite of DA, DOPAC, for 48 h (1:1.5 molar ratio of protein/catechol), and aggregation was assessed by centrifugal sedimentation analysis followed by SDS-PAGE, which detects aggregated α-syn in the pellet fraction of the reaction mixture. Similar to the previously described effects of L-DOPA and DA, DOPAC also inhibited the formation of pelletable α-syn (Fig. 5a). The soluble protein was composed of the monomer (18 kDa) and SDS/heat-stable dimers (36 kDa) and trimers (54 kDa). As anticipated, this effect was reversed by the addition of the antioxidant NAC, indicating a requirement for catechol oxidation to form o-quinone (Fig. 5a).

These results were further corroborated by quantification of thioflavin T binding (Fig. 5b). L-DOPA, DA, and DOPAC effectively inhibited α-syn aggregation (percentage inhibition: 91 ± 1, 90 ± 1, and 85 ± 1, respectively). Because DOPAC lacks a reactive amine required to form the cyclic aminochrome structure, these data suggest that inhibition of α-syn aggregation by catechols occurs by a general mechanism, through interactions with o-quinone-containing compounds.

Although electron-deficient o-quinone-containing compounds have been shown to covalently attach to proteins by reacting with nucleophilic amino acid residues, previous analysis determined that incubation of α-syn with DA does not form significant amounts of covalent protein adducts (19, 20). This effect was presumed to be due to both the absence of cysteine residues in α-syn and the relative rapid in vitro polymerization rate of oxidized DA, which consumes the reactive o-quinone into cyclic aminochrome structures. However, we reasoned that incubation with DOPAC would probably result in relatively higher amounts of catechol-protein adducts, due to the extended half-life of the reactive DOPAC-o-quinone, which is not consumed into polymers as rapidly as DA under physiological conditions. Moreover, previous studies in rodent models have shown that intrastral DA injections result in nearly 6-fold higher protein-bound DOPAC compared with DA adducts (28), indicating that intracellular DOPAC-o-quinone reacts more readily with proteins compared with DA. Therefore, we developed a new method for in-gel detection of quinone-modified proteins based on nIRF, which takes advantage of the specific excitation-emission spectra of oxidized catechols. nIRF analysis revealed the presence of o-quinone-containing α-syn monomers (18 kDa) and dimers (36 kDa) in DOPAC-incubated conditions (Fig. 5c) but not with L-DOPA or DA (Fig. S3a). Detection of a DOPAC-α-syn adduct was corroborated with a well established method for detecting qui-
none-modified proteins using redox-cycling staining to reduce tetrazolium to formazan (23) (Fig. 5c). Finally, to verify the presence of the α-syn-DOPAC adduct, electrospray ionization-mass spectrometry was utilized. After a 48-h incubation, mass shifts (multiples of 16 atomic mass units) were observed in both control and DA-treated samples, probably indicating the pres-
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FIGURE 4. Stabilization of catechol-induced soluble α-syn oligomers is dependent on amino acids in the C-terminal region and occurs through oxidative dependent events. a, α-Syn-expressing cells were infected with TH RR-EE-containing lentiviral vector and differentiated with RA for 8 days. Triton-soluble lysates were separated by size exclusion chromatography, and collected fractions were analyzed by SDS-PAGE and Western blot using monoclonal Ab Syn 204. The protein syn is in A, whereas the vertical marker indicates size in kDa. NSE was used to monitor column loading and performance. NAC at 200 μM was added 24 h prior to lentiviral infection and during RA treatment. b and c, A53T-125m- and Δ71–82 α-syn-expressing cells were analyzed as in a.

To further explore if oxidized catechols specifically interact with a YEMPS motif during the process of fibril formation, the effect of catechols on the aggregation of other proteins was assessed in cell-free systems. Similar to α-syn, the polymerization of human fibrinogen involves a transition through proteofibrillar intermediates to form fibrils (29). The reaction is initiated by incubation with thrombin, which sequentially cleaves two fibrinopeptides from the A and B chains. Incubation of fibrinogen with a 1:2 molar excess of fresh DA, autoxidized DA, DOPAC, or autoxidized DOPAC did not prevent fibril formation (Fig. S4c). The lack of inhibition may relate to the relatively large size of fibrinogen molecules as compared with α-syn or the lack of the interacting motif YEMPS in fibrinogen. Similarly, a 1:2 molar excess of fresh DA, oxidized DA, DOPAC, or oxidized DOPAC did not prevent the thermal or acid-induced aggregation of alcohol dehydrogenase or bovine lens γ-crystallin (Fig. S4, a and b). Bovine lens γ-crystallin is a 175-amino acid protein with an apparent molecular mass of 20,000 Da and contains a YEMPS motif (residues 135–139) in the C terminus. Unlike α-syn polymerization, thermal or acid protein aggregation of these proteins does not involve the formation of fibrils (30). Therefore, these results suggest that catechols and oxidized derivatives are not capable of preventing protein aggregation that does not undergo organized conformational changes to proteofibrils and fibrils, even when a YEMPS sequence is present.

**DISCUSSION**

The discovery of insoluble, α-syn fibrils in Lewy body inclusions of PD brain has suggested that aberrant conformational changes that induce α-syn aggregation are an important pathological event (2). Although the in vivo factors governing the formation of α-syn aggregates are not entirely understood, recent studies have suggested that oxidative modifications by dopamine may be critically involved. Cell-free in vitro studies revealed that dopamine and other catecholamines have the ability to inhibit the formation of α-syn fibrils through stabilization of soluble α-syn oligomers (18), providing a possible explanation into how accelerated dopaminergic cell death might occur in PD. The cell-free data have been further vali-
dated in human neuroblastoma cell lines, which employed molecular and biochemical approaches to show that cytosolic catechols influence \( \alpha \)-syn aggregation (21). Despite these advances, the biochemical mechanisms that mediate the intracellular effects of catechols on \( \alpha \)-syn inclusion formation remained unclear. The current work revealed the importance of the 125YEMPS129 C-terminal region of \( \alpha \)-syn in the intracellular interaction with catechols by showing that inclusion formation in A53T-125m cells is not affected by the increase in intracellular catechol levels as compared with A53T \( \alpha \)-syn. The data also showed that intracellular oxidation of dopamine and the noncovalent interaction of oxidized catechols with the 125YEMPS129 prevented the formation of \( \alpha \)-syn inclusions. Collectively, these data suggest that dopamine and other catechols have the ability to modulate the progression of PD through C-terminal interactions with \( \alpha \)-syn. Aberrant \( \alpha \)-syn C-terminal processing may lead to selective vulnerability of dopaminergic neurons by accelerating the formation of insoluble \( \alpha \)-syn inclusions.

Previous studies have shown that the negatively charged C terminus of \( \alpha \)-syn is an important regulator of fibril formation in vitro, since removal of this region accelerates the rate of \( \alpha \)-syn polymerization (31–33). Enrichment of C-terminal truncated \( \alpha \)-syn has been documented in regions of PD brain, including the substantia nigra, implicating the forma-
degeneration despite dramatic effects on altering the formation of insoluble aggregates (38, 39), whereas others have shown a strong correlation between inclusion formation, disease onset, and neurodegeneration (35–37). In SH-SY5Y cells, expression of truncated α-syn (1–120) induces the formation of inclusions in these regions (37). Therefore, in human PD, C-terminal cleavage may act synergistically with a decline in intracellular catechol levels, eventually leading to the formation of insoluble α-syn inclusions.

Data from cell-free in vitro studies provided further insights into the nature of the catechol-α-syn interaction. We show that oxidation of DOPAC, the deaminated metabolite of DA, also has the ability to inhibit α-syn aggregation. This suggests that α-quinone structures, in addition to cyclized aminochromes, interact with α-syn. Other analysis revealed that covalent modification of α-syn by α-quinones does not mediate α-syn aggregation. This conclusion was based on the following observations: 1) although α-DOPA, DA, and DOPAC are almost equally effective at inhibiting fibril formation, only DOPAC has the ability to covalently modify α-syn, as shown by nIRF analysis and electrospray ionization-mass spectrometry; 2) DOPAC α-quinone was found to covalently modify 125m α-syn to the same extent as WT α-syn, irrespective of the effect on fibril formation. Collectively, these data indicate that noncovalent interaction of oxidized catechols with the C-terminal motif of α-syn oligomers governs the ability to prevent the incorporation into fibrils.

Although implications for the involvement of α-syn in neurodegeneration are clear, the specific form of α-syn (i.e., monomer, oligomer, or fibril) responsible for toxicity has not been determined. Studies in animal models have shown that elevated soluble forms of α-syn may cause toxicity without the formation of insoluble aggregates (38, 39), whereas others have shown a strong correlation between inclusion formation, disease onset, and neurodegeneration (35–37). In SH-SY5Y cells, although elevated levels of A53T-α-syn induced toxicity, variation of intracellular catechol levels did not affect cellular degeneration despite dramatic effects on altering α-syn aggregation (21). Similarly, increasing catechol levels in A53T-125m expressing cells had no effect on cell viability (data not shown). Whereas oxidation of cytosolic catechols is relatively well tolerated, as evident by the presence of neuromelanin in apparently healthy nigral neurons, excessive cytosolic oxidation of catechols can be neurotoxic, as documented in cell culture and rodent models (28, 40, 41). Overall, the data indicate that oxidized catechols influence α-syn polymerization, which in turn may control the toxicity of α-synuclein.

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REFERENCES

1. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S. C., Athanasiadiou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) Science 276, 2045–2047
2. Spillantini, M. G., Schmid, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997) Nature 388, 839–840
3. Kruger, R., Kuhn, W., Muller, T., Woiwold, T., Graeber, M., Kosel, S., Pritz, E., Epplen, J. T., Schols, L., and Riess, O. (1998) Nat. Genet. 18, 106–108
4. Zarranz, J. J., Alegre, J., Gomez-Esteban, J. C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoennick, J., Rodriguez, O., Atares, B., Llorens, V., Gomez Tortosa, E., del Ser, T., Munoz, D. G., and de Yebenes, J. G. (2004) Ann. Neurol. 55, 164–173
5. Singleton, A. B., Farrer, M., Johnson, S., Singleton, A., Hugos, J., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., Lincoln, S., Crawley, A., Hanson, M., Maragakos, D., O’Don, C., Cookson, M. R., Muerter, M., Baptista, M., Miller, D., Blancto, J., Hardy, J., and Gwinn-Hardy, K. (2003) Science 302, 841
6. Giasson, B. I., Uryu, K., Trojanowski, J. Q., and Lee, V. M. (1999) J. Biol. Chem. 274, 7619–7622
7. Greenbaum, E. A., Graves, C. L., Mishizen-Eberz, A. J., Lopuli, M. A., Lynch, D. R., Englander, S. W., Axelsen, P. H., and Giasson, B. I. (2005) J. Biol. Chem. 279, 7800–7807
8. Li, W., Lesuisse, C., Xu, Y., Troncoso, J. C., Price, D. L., and Lee, M. K. (2004) J. Neurosci. 24, 7409–7409
9. Singleton, A., and Gwinn-Hardy, K. (2004) Lancet Neurol. 3, 1105–1107
10. Fornai, F., Schluter, O. M., Lenzi, P., Gess, M., Ruffoli, R., Ferrucci, M., Lazzari, G., Busceti, C. L., Pontarelli, F., Battaglia, G., Pellegrini, A., Nicolletti, F., Ruggieri, S., Paparelli, A., and Sudhof, T. C. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 3413–3418
11. Betarbet, R., Sherer, T. B., Mackenzie, G., Garcia-Ortiz, M., Panov, A. V., and Greenamyre, J. T. (2000) Nat. Neurosci. 3, 1301–1306
12. Manning-Bog, A. B., McCormack, A. L., Li, J., Uversky, V. N., Fink, A. L., and Di Monte, D. A. (2002) J. Biol. Chem. 277, 1641–1644
13. Giasson, B. I., Duda, J. E., Murray, I. V., Chen, Q., Souza, J. M., Hurtig, H. I., Ichihara, H., Trojanowski, J. Q., and Lee, V. M. (2000) Science 290, 985–989
14. Hodara, N., Norris, E. H., Giasson, B. I., Mishizen-Eberz, A. J., Lynch, D. R., Lee, V. M., and Ichihara, H. (2004) J. Biol. Chem. 279, 47746–47753
15. Norris, E. H., Giasson, B. I., Ichihara, H., and Lee, V. M. (2003) J. Biol. Chem. 278, 27230–27240
16. LaVoie, M. J., and Hastings, T. G. (1999) J. Neurosci. 19, 1484–1491
17. LaVoie, M. J., Ostaszewski, B. L., Weihofen, A., Schlossmacher, M. G., and Selkoe, D. J. (2005) Nat. Med. 11, 1214–1221
18. Conway, K. A., Rochet, J. C., Bieganski, R. M., and Lansbury, P. T., Jr. (2001) Science 294, 1346–1349
19. Li, J., Zhu, M., Manning-Bog, A. B., Di Monte, D. A., and Fink, A. L. (2004) FASEB J. 18, 962–964
20. Norris, E. H., Giasson, B. I., Hodara, R., Xu, S., Trojanowski, J. Q., Ichihara, H., and Lee, V. M. (2005) J. Biol. Chem. 280, 21212–21219
21. Muzzalli, J. R., Mishizen, A. J., Giasson, B. I., Lynch, D. R., Thomas, S. A., Nakashima, A., Nagatsu, T., Ota, A., and Ichihara, H. (2006) J. Neurosci. 26, 10068–10078
22. Giasson, B. I., Murray, I. V., Trojanowski, J. Q., and Lee, V. M. (2001) J. Biol. Chem. 276, 2380–2386
23. Paz, M. A., Fluckiger, R., Boak, A., Kagan, H. M., and Gallop, P. M. (1991) J. Biol. Chem. 266, 689–692
24. Vadseth, C., Souza, J. M., Thomson, L., Seagraves, A., Nagaswami, C., Scheiner, T., Torbet, J., Vilaire, G., Bennett, J. S., Murciano, J. C., Muzny, J., Penn, M. S., Hazen, S. L., Weisel, J. W., and Ichihara, H. (2004) J. Biol. Chem. 279, 8820–8826
25. Horwith, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10449–10453
26. Periquet, M., Fulga, T., Myllykangas, L., Schlossmacher, M. G., and Feany, M. B. (2007) J. Neurosci. 27, 3338–3346
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27. Nakashima, A., Kaneko, Y. S., Mori, K., Fujiwara, K., Tsugu, T., Suzuki, T., Nagatsu, T., and Ota, A. (2002) J. Neurochem. 82, 202–206
28. Hastings, T. G., Lewis, D. A., and Zigmond, M. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1956–1961
29. Weisel, J. W., Stauffacher, C. V., Bullitt, E., and Cohen, C. (1985) Science 230, 1388–1391
30. Flaugh, S. L., Mills, I. A., and King, I. (2006) J. Biol. Chem. 281, 30782–30793
31. Li, W., West, N., Colla, E., Pletnikova, O., Troncoso, J. C., Marsh, L., Dawson, T. M., Jakala, P., Hartmann, T., Price, D. L., and Lee, M. K. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 2162–2167
32. Murray, I. V., Giasson, B. I., Quinn, S. M., Koppaka, V., Axelsen, P. H., Ischiropoulos, H., Trojanowski, J. Q., and Lee, V. M. (2003) Biochemistry 42, 8530–8540
33. Serpell, L. C., Berriman, J., Jakes, R., Goedert, M., and Crowther, R. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4897–4902
34. Liu, C. W., Giasson, B. I., Lewis, K. A., Lee, V. M., Demartino, G. N., and Thomas, P. J. (2005) J. Biol. Chem. 280, 22670–22678
35. Giasson, B. I., Duda, J. E., Quinn, S. M., Zhang, B., Trojanowski, J. Q., and Lee, V. M. (2002) Neuron 34, 521–533
36. Lee, M. K., Stirling, W., Xu, Y., Xu, X., Qui, D., Mandir, A. S., Dawson, T. M., Copeland, N. G., Jenkins, N. A., and Price, D. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 8968–8973
37. Tofaris, G. K., Garcia Reitbock, P., Humby, T., Lambourne, S. L., O’Connell, M., Ghetti, B., Gossage, H., Emson, P. C., Wilkinson, L. S., Goedert, M., and Spillantini, M. G. (2006) J. Neurosci. 26, 3942–3950
38. Chen, L., and Feany, M. B. (2005) Nat. Neurosci. 8, 657–663
39. Gispert, S., Del Turco, D., Garrett, L., Chen, A., Bernard, D. J., Hamm-Clement, J., Korf, H. W., Deller, T., Braak, H., Auberger, G., and Nussbaum, R. L. (2003) Mol. Cell Neurosci. 24, 419–429
40. Caudle, M. W., Richardson, J. R., Wang, M. Z., Taylor, T. N., Guillot, T. S., McCormack, A. L., Colebrooke, R. E., Di Monte, D. A., Emson, P. C., and Miller, G. W. (2007) J. Neurosci. 27, 8138–8148
41. Rosenberg, P. A. (1988) J. Neurosci. 8, 2887–2894