Ubiquitination of α-Synuclein†

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ABSTRACT: Filamentous α-synuclein depositions are the defining hallmarks of a subset of neurodegenerative diseases including Parkinson's disease (PD), dementia with Lewy bodies, and multiple system atrophy. We previously reported that α-synuclein in those brains are extensively phosphorylated at Ser129 [Fujiwara et al. (2002) *Nat. Cell Biol*. *4*, 160−164] and also partially ubiquitinated [Hasegawa et al. (2002) *J. Biol. Chem. 277*, 49071−49076]. Here, we investigate ubiquitination of α-synuclein *in vitro* and *in vivo* and report the ubiquitination sites and the effects of familial PD-linked mutations, phosphorylation, and fibril formation on ubiquitination. Protein-sequence analysis revealed that Lys21, Lys23, Lys32, and Lys34 within the repeats in the amino-terminal half are liable to ubiquitination *in vitro*. A site-directed mutagensis study confirmed that these are the major ubiquitination sites. A53T and A30P mutations had no significant effect on ubiquitination. Similarly, phosphorylation of α-synuclein at Ser129 did not affect ubiquitination. Notably, we show that assembled, filamentous α-synuclein is less ubiquitinated than the soluble form and that the major ubiquitination sites are localized to Lys6, Lys10, and Lys12 at the amino-terminal region of filamentous α-synuclein. Furthermore, we successfully detected ubiquitination of α-synuclein in 293T cells by cotransfection with α-synuclein and ubiquitin. The *in vivo* ubiquitination sites were found to be identical to those in filamentous α-synuclein. PD-linked mutations and phosphorylation at Ser129 had no effects on ubiquitination of α-synuclein *in vivo*. These data may have implications for the mechanisms of the formation of α-synuclein deposits in α-synucleinopathy brains.

Intracellular filamentous inclusion bodies comprised of distinct proteins in neurons and/or glial cells in affected brain regions are defining characteristics of many neurodegenerative diseases. α-Synuclein, an abundant presynaptic protein, has been identified as a causative gene of the familial form of Parkinson's disease (PD)[1] (*1*, *2*). Subsequently, it has been shown that α-synuclein is the major component of Lewy bodies (LBs) and Lewy neurites (*3*, *4*, *5*), which are the neuropathological hallmarks of PD and dementia with Lewy bodies (DLBs). Immunohistochemistry and biochemical studies using anti-α-synuclein antibodies revealed that α-synuclein is deposited in other types of inclusions including glial or neuronal cytoplasmic inclusions in multiple system atrophy (MSA) and Lewy body-like inclusions and dystrophic neurites in Hallervorden−Spatz disease (HSD) (*6*, *7*, *8*, *9*), leading to the collective nomenclature of "α-synucleinopathy" for these disorders.

LBs and other α-synuclein-positive inclusions in brains with these neurodegenerative diseases have been shown to be immunoreactive for ubiquitin (*10*), an essential molecule that triggers protein degradation by the ubiquitin-dependent proteasome system. Recent genetic studies have identified abnormalities in the genes encoding proteins related to the ubiquitin−proteasome pathway. Mutations in the *parkin* gene have been discovered in autosomal recessive juvenile parkinsonism (AR-JP) (*11*), and the gene product has been shown to be an E3 ubiquitin ligase (*12*). A mutation in ubiquitin C-terminal hydrolase (UCHL1) has also been reported (*13*). These findings, together with the presence of ubiquitin in intracellular inclusions in α-synucleinopathies, suggest that deposition of α-synuclein and impaired functions in the ubiquitin-dependent proteasome system may be involved in the pathogenesis of these neurodegenerative disorders.

We have analyzed α-synuclein deposited in DLB brains as well as other α-synucleinopathies by mass spectrometry and specific antibodies and have found that the deposited α-synuclein is highly phosphorylated at Ser129 (*14*). Quantitative immunoblot analyses have further shown that >90% of the insoluble α-synuclein in DLB brains is phosphorylated, whereas ∼4% of normal α-synuclein is phosphorylated at Ser129 (*14*), suggesting that the extensive phosphorylation

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[1] Abbreviations: PD, Parkinson's disease; DLBs, dementia with Lewy bodies; LBs, Lewy bodies; MSA, multiple system atrophy; HSD, Hallervorden−Spatz disease; HA, hemagglutinin.

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of α-synuclein is a pathological event. We also demonstrated immunochemically and by protein chemistry that the phosphorylated α-synuclein is partially ubiquitinated in those α-synucleinopathies and that the α-synuclein polypeptides undergo mono- and diubiquitination (*15*).

Recent reports indicate that a number of short-lived proteins are targeted for ubiquitination and degradation by phosphorylation at specific sites of substrate proteins (e.g., cyclin or *β*-catenin) (*16*, *17*). However, it is unknown whether the phosphorylation of α-synuclein at Ser129 may have some roles in ubiquitination and degradation of α-synuclein. It also remains unknown whether the two missense mutations of α-synuclein affect its ubiquitination. In addition, it is important to determine whether the filamentous form of α-synuclein can be ubiquitinated and whether the ubiquitin conjugation sites of the α-synuclein are identical to those in soluble α-synuclein. Although ubiquitin immunoreactivities are observed in filamentous inclusion bodies in many neurodegenerative diseases, it has not been determined whether ubiquitination occurs on filamentous or aggregated protein or, alternatively, whether ubiquitin is conjugated to soluble protein.

Here, we show that recombinant α-synuclein is ubiquitinated *in vitro* by reticulocyte-derived ubiquitin ligase mainly at Lys21, Lys23, Lys32, and Lys34, which are located within the imperfect tandem repeat sequences in the amino-terminal half. Phosphorylation of α-synuclein at Ser129, as well as the two familial PD-linked mutations (A53T and A30P), did not affect ubiquitination. Notably, we show here that assembled filamentous α-synuclein is less ubiquitinated than its soluble form and that the major ubiquitination sites are localized to Lys6, Lys10, and Lys12 that are more amino-terminally located compared to the ubiquitination sites of soluble α-synuclein. Furthermore, we detected the mono-ubiquitinated α-synuclein *in vivo* in transfected 293T cells and confirmed that Lys residues localized in the amino-terminal region of α-synuclein were ubiquitinated *in vivo*. This is the first report on the ubiquitination sites of α-synuclein *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Plasmids and Site-Directed Mutagenesis. Human α-synuclein cDNA in bacterial expression vector pRK172 was kindly provided by Dr. M. Goedert. cDNAs of human ubiquitin with a hemagglutinin (HA) tag were a generous gift from Dr. S. Hatakeyama. The open-reading frame of α-synuclein was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) for expression in cultured cells.

Site-directed mutagenesis was performed to substitute K6, K10, K12, K21, K23, K32, K34, K43, K45, K58, and K60 to arginine, A30 to proline, A53 to threonine, and S129 to alanine in the human α-synuclein by using the site-directed mutagenesis kit (Stratagene). All constructs were verified by DNA sequencing. Mutated α-synuclein proteins were expressed in *Escherichia coli* BL21 (DE3), purified, and ubiquitinated as described below.

Antibodies and Immunoblotting. An anti-α-synuclein antibody (mAb) Syn102 (epitope location on α-synuclein, residues 131−140) (*7*) and an anti-ubiquitin mAb 1510 (*18*) were used in this study. Immunoblotting was performed as follows. Aliquots of reaction mixtures were separated by SDS−PAGE, blotted onto polyvinylidene difluoride (PVDF) membrane (Millipore), blocked with 3% gelatin, and incubated with primary antibodies in 10% calf serum/phosphate-buffered saline (PBS) at a dilution of 1:∼3000−10 000, overnight at room temperature. After the blots were washed, they were incubated with a horseradish peroxidase-labeled secondary antibody (Bio-Rad) at a dilution of 1:10 000 in 10% calf serum/PBS for 1 h at room temperature. Signals were detected using a chemiluminescence reagent Immunostar (Wako).

Preparation of Recombinant α-Synuclein and in Vitro Ubiquitination. Recombinant human α-synuclein was expressed in *E. coli* BL21 (DE3) and purified by a boiling treatment and then Q-Sepharose ion-exchange chromatography followed by ammonium sulfate precipitation and dialysis against 30 mM Tris-HCl (pH 7.5). *In vitro* ubiquitination of α-synuclein was performed as described (*15*). Briefly, 20 *μ*g of recombinant α-synuclein and 2 *μ*g of methylated ubiquitin were incubated with a ubiquitin ligase fraction (fraction II) from rabbit reticulocytes (*19*) or rat-brain extracts at 37 °C for 2 h in a buffer containing 50 mM Tris-HCl (pH 9.0), 1 mM ATP, 5 mM $MgCl_2$, and 1 mM dithiothreitol (DTT).

Determination of Ubiquitination Sites of Soluble α-Synuclein. Recombinant α-synuclein was ubiquitinated by incubating with methylated ubiquitin, fraction II, and ATP for 2 h, and then the reaction was stopped by boiling and centrifuged at 15 000 rpm for 10 min. The supernatants were denatured by adding 6 M guanidine-HCl and fractionated on a TSK gel G3000SW column (4.6 × 300 mm, Tosoh) equilibrated with 6 M guanidine-HCl in 10 mM phosphate buffer (pH 6.0). The TSK fractions containing mono- and diubiquitinated α-synuclein were dialyzed with 30 mM Tris-HCl buffer (pH 7.5) and concentrated by lyophilization. The samples were resuspended in 50 mM Tris-HCl buffer (pH 9.0) and digested with lysylendopeptidase (*Achromobacter lyticus* protease I, AP1) at 37 °C for 14 h. The digests were mixed with 5× sample buffer, applied on 13.5% polyacrylamide Tris-tricine gel, and transferred to a PVDF membrane. The ubiquitin-positive ∼11-kDa band on the PVDF membrane were subjected to amino-terminal-sequence analysis with a model 492 Protein Sequencer (Applied Biosystems).

Ubiquitination of Filamentous α-Synuclein. Recombinant α-synuclein was prepared at a concentration of 10 mg/mL in 100 *μ*L of 30 mM Tris-HCl (pH 7.5) containing 0.01% NaN_3 and incubated at 37 °C for ∼48−72 h with shaking as described (*20*). Filamentous or aggregated α-synuclein was precipitated by ultracentrifugation at 50 000 rpm for 20 min. The pellet was resuspended in 500 *μ*L of 30 mM Tris-HCl (pH 7.5), ultracentrifuged again, and resuspended in 50 *μ*L of 30 mM Tris-HCl (pH 7.5). A part of the filamentous α-synuclein was solubilized in 6 M guanidine-HCl, and the concentration was measured by RP-HPLC. Equal amounts of soluble and filamentous α-synuclein were ubiquitinated *in vitro* and analyzed by immunoblotting as above. To determine the ubiquitination sites of filamentous α-synuclein, the reaction mixture was ultracentrifuged at 50 000 rpm for 20 min and the pellet was resuspended in 200 *μ*L of 6 M guanidine-HCl and fractionated on a TSK gel G3000SW column (4.6 × 300 mm, Tosoh). The TSK fractions containing mono- and diubiquitinated α-synuclein were

digested with lysylendopeptidase, separated on SDS−PAGE, and analyzed by the Protein Sequencer as described above.

Detection of Ubiquitinated α-Synuclein in Vivo. Human kidney-derived 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical) supplemented with 10% (v/v) fetal calf serum. 293T cells were transfected with expression vectors (total of 4 μg) using FuGENE6 (Roche) or LipofectAMINE 2000 reagent (Invitrogen) according to the instructions of the manufacturer. A total of 30 h after transfection, the cells were collected and lysed in 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM phenylmethanesulfonyl fluoride, and 5 mM ethylenediaminetetraacetic acid with protease inhibitors. Lysates were boiled for 5 min, and heat-stable fractions including ubiquitinated α-synuclein and unreacted α-synuclein monomer were recovered by centrifugation at 15 000 rpm for 15 min. Ubiquitinated α-synuclein were detected by immunoblot analysis using anti-α-synuclein (Syn102).

RESULTS

Determination of in Vitro Ubiquitination Sites on α-Synuclein. We previously reported that α-synuclein is effectively ubiquitinated *in vitro* by rabbit reticulocyte lysates using methylated ubiquitin and that the ubiquitinated α-synuclein comigrated with the bands of mono- and diubiquitinated α-synuclein from Sarkosyl-insoluble fraction from DLB, MSA, and HSD brains on immunoblot analysis (*15*). To determine the ubiquitination sites of α-synuclein, we performed protein chemical analysis of *in vitro* ubiquitinated α-synuclein. Mono- and diubiquitinated α-synuclein were separated on gel-filtration HPLC using a TSK G3000SW column in the presence of 6 M guanidine-HCl as judged by immunoblot analysis (Figure 1A). Most of the unconjugated ubiquitin monomer was separated from the reaction mixture by this step. The TSK fractions containing ubiquitinated α-synuclein were collected, desalted by dialysis, and digested with lysylendopeptidase AP1. Although AP1 efficiently cleaves Lys-X sequences, the enzyme does not cleave the carboxyl terminus of modified Lys residues. Namely, AP1 digests unubiquitinated α-synuclein, but it does not cleave ubiquitinated Lys-X sites nor the methylubiquitin moiety of ubiquitinated α-synuclein. Therefore, α-synuclein monomer was eliminated by the digestion, and the α-synuclein fragments conjugated with full-length methylubiquitin could be separated by SDS−PAGE. Immunoblot analysis of digested mixtures with anti-ubiquitin antibody detected a ~11-kDa band (Figure 1B), which may represent the ubiquitinated α-synuclein fragments. We then analyzed this band by protein sequencing to identify the ubiquitination sites of α-synuclein. Ubiquitinated Lys does not give any signal of PTH amino acid on the Protein Sequencer because of its modification with ubiquitin. Therefore, the Lys residue without a significant signal is expected to be the ubiquitination site. Protein-sequence analysis of the ~11-kDa band revealed that this band contains at least four peptides (Figure 1C). Major sequences were EGVVAAAExTK and TxQGVAEAAGK, suggesting that ubiquitin is conjugated to Lys21 and Lys23, respectively. Minor sequences of QGVAEAAGxTK and TxEGVLYVGSK were also detected, which suggested that ubiquitin is also attached to Lys32 and Lys34 of α-synuclein, respectively. α-Synuclein has five imperfect tandem repeat sequences, KTKEGV, in the amino-terminal half (Figure 1D). Interestingly, all four Lys residues identified as the ubiquitination sites were located within these repeat sequences.

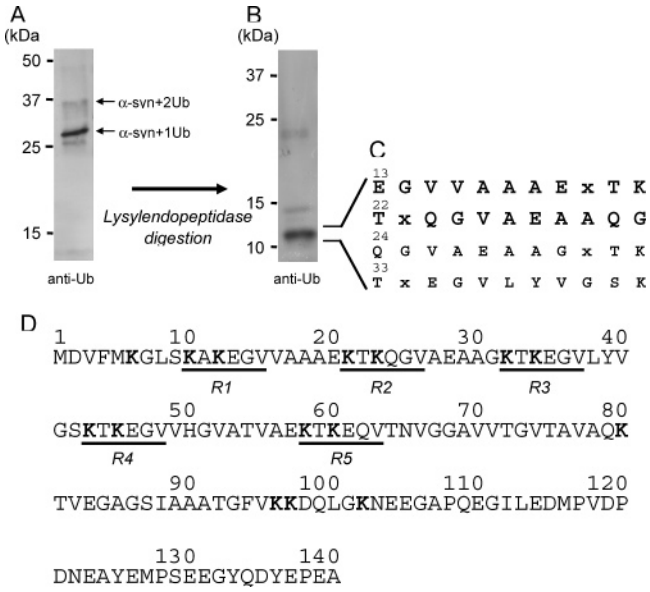

FIGURE 1: Protein-sequencing analysis of ubiquitinated α-synuclein. (A) Immunoblot of the TSK fraction of ubiquitinated α-synuclein with anti-ubiquitin antibody 1510 (anti-Ub). Mono- (α-syn + 1 Ub) and di- (α-syn + 2 Ub) ubiquitinated α-synuclein are indicated. (B) Immunoblot of methylubiquitin-conjugated α-synuclein after lysylendopeptidase digestion. (C) Ubiquitin-positive ~11-kDa band on the membrane were subjected to amino-terminal sequence analysis by the Protein Sequencer. Two major and two minor sequences were detected. Signals for lysine residues indicated by the letter x were undetectable and suggested to be the ubiquitination sites. (D) Amino acid sequences of human α-synuclein. Lysine residues are indicated in bold letters. Imperfect five repeat sequences (*R1*−*R5*) are underlined.

In Vitro Ubiquitination of Arg Mutant α-Synuclein. To confirm the observations by protein-sequencing analysis, we used site-directed mutagenesis to replace the lysine residues with arginines and performed the *in vitro* ubiquitination assay. We systematically mutated lysine residues of α-synuclein to arginines: α-synuclein harbors 15 lysine residues, 11 of which are located in the amino-terminal half, where the imperfect five KTKEGV repeat sequences are present. We generated the following mutants with arginine substitution in the repeat sequences: mR1 (K6R, K10R, and K12R), mR2 (K21R and K23R), mR3 (K32R and K34R), mR4 (K43R and K45R), mR5 (K58R and K60R), mR23 (K21R, K23R, K32R, and K34R), mR24 (K21R, K23R, K43R, and K45R), mR34 (K32R, K34R, K43R, and K45R), and mR234 (K21R, K23R, K32R, K34R, K43R, and K45R) and performed *in vitro* ubiquitination. As shown in Figure 2A, levels of ubiquitination of mR2 were ~60% lower than that of the wild type, whereas the levels of ubiquitination of other mR1, mR3, mR4, and mR5 were ~20−30% lower than that of the wild type. Furthermore, ubiquitination was negligible in the mR23 or mR234 mutants (parts A and B of Figure 2). These results suggest that Lys21 and Lys23 are the major ubiquitin conjugation sites, whereas the Lys residues in other repeat sequences are minor ones. These results confirmed the observations by protein chemical analysis and showed that Lys21, Lys23, Lys32, and Lys34 of soluble α-synuclein are the major conjugation sites of

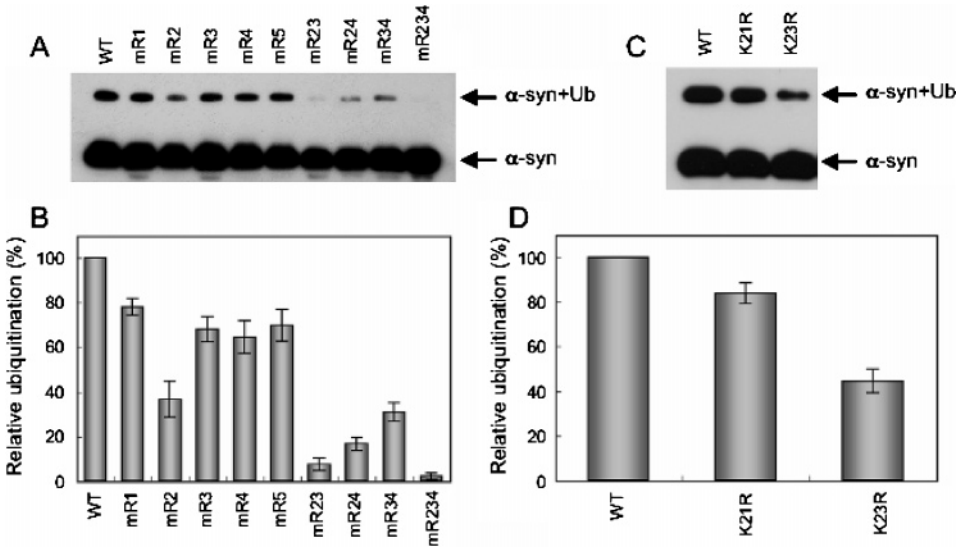

FIGURE 2: Ubiquitination of wild-type and mutant α-synuclein proteins harboring lysine to arginine substitutions. (A) Immunoblot analysis of ubiquitination of wild-type α-synuclein and the mR mutant series with anti-α-synuclein antibody (Syn102): mR1 (K6R, K10R, and K12R), mR2 (K21R and K23R), mR3 (K32R and K34R), mR4 (K43R and K45R), mR5 (K58R and K60R), mR23 (K21R, K23R, K32R, and K34R), mR24 (K21R, K23R, K43R, and K45R), mR34 (K32R, K34R, K43R, and K45R), and mR234 (K21R, K23R, K32R, K34R, K43R, and K45R) with rabbit reticulocytes lysate fraction II. α-Synuclein monomer (α-syn) and monoubiquitinated α-synuclein (α-syn + Ub) are indicated. (B) Quantitative analysis of the monoubiquitinated α-synuclein bands. The results were expressed as means ± standard deviation (SD) ($n = 3$). (C) Immunoblot analysis of ubiquitination of wild-type α-synuclein and K21R and K23R mutants using anti-α-synuclein antibody (Syn102). α-Synuclein monomer (α-syn) and monoubiquitinated α-synuclein (α-syn + Ub) are indicated. (D) Quantitative analysis of the monoubiquitinated α-synuclein bands.

ubiquitin. In the analysis of ubiquitination of mutant α-synuclein with single substitution (K21R and K23R), K21R and K23R gave 20 and 60% lower levels of ubiquitination compared to that of the wild-type (parts C and D of Figure 2). These data, together with the results of protein chemical analysis, strongly suggested that Lys23 is the major ubiquitin conjugation site of soluble α-synuclein, although multiple Lys residues in the repeats of α-synuclein are ubiquitinated.

Effects of Familial PD-Linked Mutations and Phosphorylation of α-Synuclein on Ubiquitination. Pathogenic mutations of α-synuclein, A30P and A53T, have been identified in rare familial cases of PD. Because ubiquitination sites identified here are located close to the mutations, we investigated whether these mutations affect ubiquitination. We also examined the effect of phosphorylation of α-synuclein at Ser129 on ubiquitination, because a fraction of phosphorylated α-synuclein has been shown to be ubiquitinated in the brains of patients with α-synucleinopathies. α-Synuclein with single amino acid substitutions of either A30P, A53T, or S129A were ubiquitinated *in vitro* using rabbit reticulocytes fraction II and analyzed by immunoblotting. As shown in Figure 3, A30P, A53T, or S129A mutants were similarly ubiquitinated and no significant difference was detected in the levels of ubiquitination compared to that of the wild type. Phosphorylation of α-synuclein by casein kinase II, which is shown to phosphorylate Ser129 of α-synuclein *in vitro*, did not promote ubiquitination of α-synuclein (data not shown). These results suggest that A30P and A53T mutations and phosphorylation of Ser129 may not affect ubiquitination *in vitro*.

Ubiquitination of α-Synuclein with Rat-Brain Extracts. To investigate the physiological relevance of the observations with the rabbit reticulocytes fraction II, we examined ubiquitin ligase activities of α-synuclein in rat brain. Six-week-old rat brain was homogenized in an equal volume of

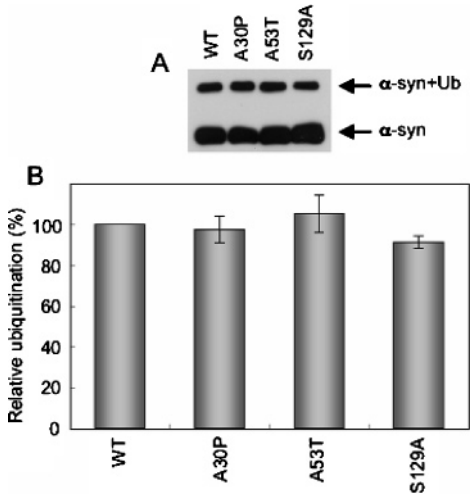

FIGURE 3: Effects familial PD-linked mutations and phosphorylation of α-synuclein on its ubiquitination. (A) Immunoblot analysis of ubiquitination of wild-type α-synuclein and A53T, A30P, or S129A mutants ubiquitinated by fraction II using anti-α-synuclein antibody (Syn102). α-Synuclein monomer (α-syn) and monoubiquitinated α-synuclein (α-syn + Ub) are indicated. (B) Quantitative analysis of the monoubiquitinated α-synuclein bands. The results were expressed as means ± SD ($n = 3$).

10 mM Tris-HCl buffer (pH 7.4) containing 5 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 2 mM DTT, and 1 mM PMSF and ultracentrifuged at 80 000 rpm for 20 min. The supernatants were used as an ubiquitin ligase fraction of α-synuclein instead of the fraction II. When the brain extracts were added to the reaction mixture, bands of ubiquitinated α-synuclein similar to those treated with fraction II were detected, showing that α-synuclein ubiquitin ligase activities are present in the brain extracts (Figure 4). Using the arginines mutants, we investigated the ubiquitination sites of α-synuclein. As shown in Figure 4, reduced

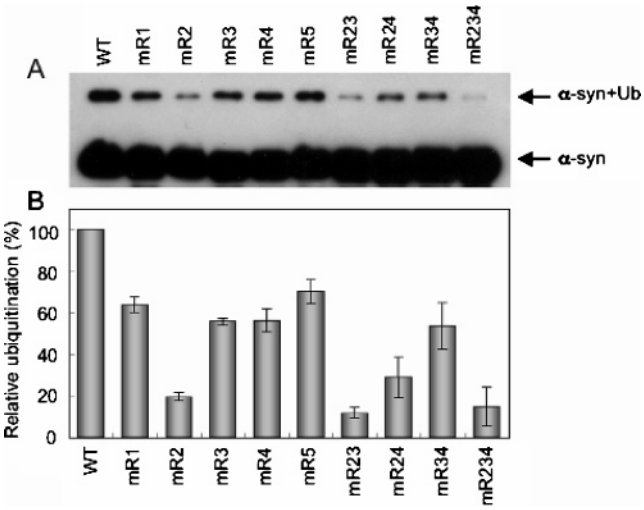

FIGURE 4: Ubiquitination of wild-type and mutant α-synuclein with arginine substitutions by rat-brain extracts (A) Immunoblot analysis of ubiquitination of wild-type α-synuclein and the mR mutant series: mR1, mR2, mR3, mR4, mR5, mR23, mR24, mR34, and mR234 with rat-brain extracts using anti-α-synuclein antibody (Syn102). α-Synuclein monomer (α-syn) and monoubiquitinated α-synuclein (α-syn + Ub) are indicated. (B) Quantitative analysis of the monoubiquitinated α-synuclein bands. The results were expressed as means ± SD ($n = 3$).

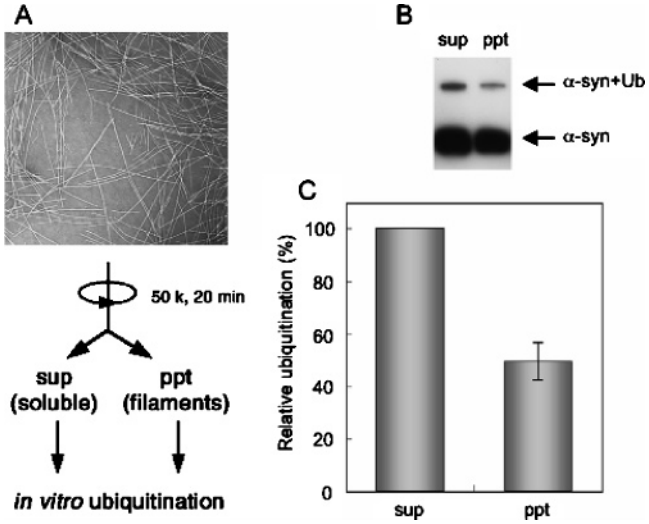

FIGURE 5: Ubiquitination of soluble or filamentous α-synuclein proteins. (A) Electron microscope view of the assembled α-synuclein and procedure of the experiment. Recombinant α-synuclein was incubated at 37 °C for 72 h and observed by negative staining. After ultracentrifugation, the supernatant (soluble) and pellet (filamentous insoluble α-synuclein) were ubiquitinated with rabbit reticulocytes lysate fraction II. (B) Immunoblot analysis of ubiquitination of soluble and insoluble α-synuclein using anti-α-synuclein antibody (Syn102). α-Synuclein monomer (α-syn) and monoubiquitinated α-synuclein (α-syn + Ub) are indicated. (C) Quantitative analysis of the monoubiquitinated α-synuclein bands. The results were expressed as means ± SD ($n = 3$).

levels of ubiquitination by 80% compared to the control were detected in mR2 (K21R and K23R) mutants and ~40% reduction was detected in the mR1, mR3, mR4, or mR5 mutants. These results indicate that ubiquitin ligase activities are present in rat brain and that the ubiquitin conjugation sites of α-synuclein are similar to that by rabbit reticulocytes fraction II.

Ubiquitination of Filamentous α-Synuclein. Ubiquitinated α-synuclein was identified in Sarkosyl-insoluble fractions of brains of patients with α-synucleinopathies, whereas it has never been detected in the soluble fractions from any other brain preparations. We then investigated the possibilities of ubiquitination of filamentous insoluble α-synuclein. When recombinant α-synuclein is incubated at a high concentration with shaking, α-synuclein forms filaments that morphologically and biochemically resemble to those seen in α-synucleinopathy brains (Figure 5A). We isolated the filamentous α-synuclein by ultracentrifugation and performed *in vitro* ubiquitination. As shown in Figure 5B, filamentous α-synuclein was ubiquitinated. However, the level of ubiquitination of insoluble α-synuclein was ~45% of that using soluble α-synuclein as a substrate (Figure 5C).

To further determine the conjugation sites of ubiquitin on the filamentous α-synuclein, we recovered ubiquitinated filamentous α-synuclein by ultracentrifugation and solubilized them in 6 M guanidine-HCl. Mono- and diubiquitinated α-synuclein were fractionated by TSK gel filtration, desalted by dialysis, and digested with lysylendopeptidase. Methylated-ubiquitin-conjugated fragments of α-synuclein were separated on SDS−PAGE. Protein-sequence analysis of the ~11-kDa band revealed that the band contains some peptides. Very interestingly, three major sequences, MDVFMxGLSK (residues 1−10), GLSxAK (residues 7−12), and AxEGVVAAAEK (residues 11−21), were detected, confirming that ubiquitin is attached at Lys6, Lys10, and Lys12 of α-synuclein (see Figure 1D and Figure 7). Minor signals of EGVVAAAExTK (residues 13−23) and TxQGVAEAAGK (residues 22−32) were also detected, although the levels of these signals were much lower than those of the former three residues. These results demonstrate that insoluble filamentous α-synuclein can serve as a substrate for ubiquitination and that the ubiquitination sites are different from those of soluble α-synuclein.

Ubiquitination of α-Synuclein in Vivo. Although we identified the *in vitro* ubiquitination sites of α-synuclein, there remained a major question as to whether these sites are actually ubiquitinated *in vivo*. To address this issue, we tried to detect ubiquitination of α-synuclein *in vivo* using cotransfection of cDNAs encoding α-synuclein and ubiquitin in human-kidney-derived 293T cells. Nontagged α-synuclein were coexpressed with HA-tagged ubiquitin in 293T cells. The lysates were boiled and analyzed by immunoblotting. As shown in Figure 6A, the ~28-kDa band was found when both α-synuclein and HA ubiquitin were cotransfected, while neither more ubiquitinated forms of α-synuclein nor high-molecular-weight smear bands were detected, indicating that α-synuclein is mainly monoubiquitinated in 293T cells under our experimental conditions. The ~28-kD band was also immunoreactive with anti-ubiquitin antibody or anti-HA antibody (data not shown). No such band was detected by single transfection with α-synuclein nor with HA ubiquitin. To investigate the *in vivo* ubiquitination sites of α-synuclein, we transfected several mutants with arginine substitution in the repeat sequences in 293T cells. A marked reduction of ubiquitinated α-synuclein was observed when mR1, mR12, and mR23 were expressed, and a significant reduction was detected when the mutants mR2 or mR3 were expressed, indicating that lysine residues in the amino-terminal region are liable to ubiquitination in 293T cells (parts B and C of Figure 6). This result showed that the *in vivo* ubiquitination sites of α-synuclein in 293T cells are almost identical to those

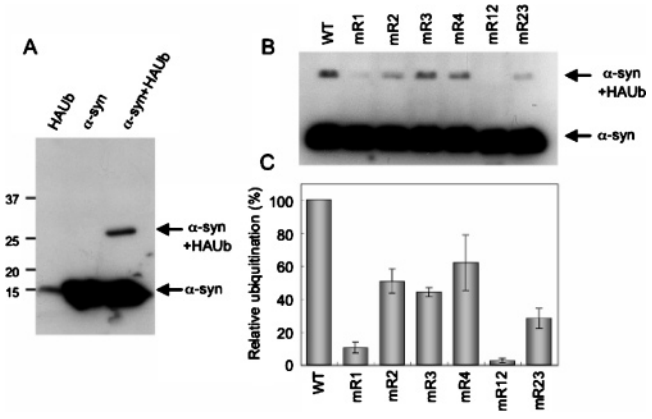

FIGURE 6: *In vivo* ubiquitination of α-synuclein. (A) Immunoblot analysis of lysates from 293T cells transfected with α-synuclein and/or HA-tagged ubiquitin using anti-α-synuclein antibody (Syn102). α-Synuclein (α-syn) and monoubiquitinated α-synuclein (α-syn + HAUb) were indicated. (B) Lysates from 293T cells transfected with HA-tagged ubiquitin and α-synuclein or its mutants with arginine substitution, mR1, mR2, mR3, mR4, mR12, and mR23, were subjected to Western-blotting analysis with anti-α-synuclein antibody (Syn102). α-Synuclein (α-syn) and monoubiquitinated α-synuclein (α-syn + HAUb) were indicated. (C) Quantitative analysis of the monoubiquitinated α-synuclein bands. The results were expressed as means ± SD ($n = 3$).

found in the case of *in vitro* ubiquitination of α-synuclein filaments (see further discussion below).

We also examined whether two pathogenic mutations of familial PD and phosphorylation at Ser129 affect the *in vivo* ubiquitination. Wild-type or A30P, A53T, or S129A mutant α-synuclein were transfected with HA ubiquitin, and lysates were prepared as described in the Experimental Procedures. Immunoblot analysis showed that all mutant α-synucleins were ubiquitinated at comparable levels (data not shown), suggesting that the two pathogenic mutations and phosphorylation at Ser129 have no effect on ubiquitination of α-synuclein *in vivo*.

DISCUSSION

In this study, we investigated the ubiquitination sites of α-synuclein *in vitro* as well as *in vivo* and the effects of phosphorylation, familial PD-linked mutations, and filament formation on its ubiquitination. Protein chemical analysis and mutagenesis study on ubiquitination of α-synuclein with rabbit reticulocytes fraction II identified that multiple lysine residues in the KTKEGV repeats of the amino-terminal half of α-synuclein are the possible ubiquitination sites. The major *in vitro* ubiquitination site was located at Lys23 in the second repeat (Figure 7). To date, no consensus sequence that determines the specificity of the Lys residues serving as ubiquitin target sites have been identified. In some cases, distinct lysine residues are required for ubiquitination, whereas there is little or no specificity in the others: yeast iso-2-cytochrome *c* undergoes polyubiquitination almost exclusively on a single lysine (*21*). The proto-oncogene product Mos has also been shown to require a single lysine residue for ubiquitination and degradation (*22*). In contrast, ubiquitination of the ζ chain of the T cell receptor is independent of any particular Lys residues (*23*). Similarly, no single specific lysine residue is required for ubiquitination of c-Jun (*24*). In the case of MyoD, the NH_2 group of the

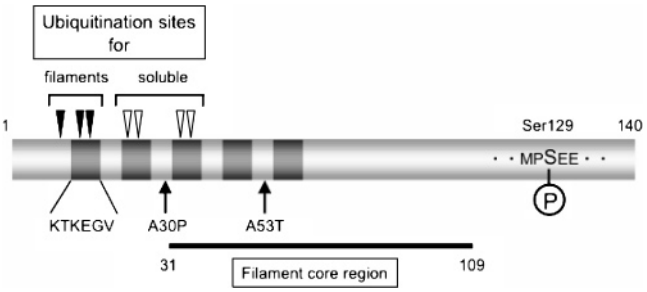

FIGURE 7: Ubiquitination sites of α-synuclein. Schematic diagram of α-synuclein and ubiquitination sites identified in soluble (white arrowheads) or insoluble (black arrowheads) filamentous forms of α-synuclein in the cell-free system. Ubiquitination sites determined in 293T cells cotransfected with α-synuclein and HA ubiquitin are identical to those of filamentous α-synuclein *in vitro*.

amino-terminal residue, rather than internal lysine, serves as an essential and sufficient conjugation site for subsequent degradation of the protein (*25*).

We found that ubiquitin ligase activities that conjugate ubiquitin to α-synuclein are present in rat-brain extracts. Interestingly, the ubiquitin-attached sites of α-synuclein by the ligase activities are similar to those conjugated by fraction II from rabbit reticulocyte lysates. This result indicates that major ligase activities in the brain exhibit a similar substrate specificity to that present in fraction II, suggesting that ubiquitination sites of α-synuclein are substrate-dependent.

Ubiquitination of aggregated or filamentous proteins has been implicated in the pathogenesis of many neurodegenerative diseases. Intracellular inclusion bodies such as nuclear inclusion bodies (*26*), composed of expanded polyglutamine repeats in many polyglutamine diseases, neurofibrillary tangles (*27*) comprised of hyperphosphorylated τ in Alzheimer's disease, LBs, and related inclusions made of hyperphosphorylated α-synuclein in α-synucleinopathies (*15*) have been shown to be ubiquitin-positive. Genetic studies have also demonstrated that dysfunction of proteins related in the ubiquitin−proteasome pathway cause neurodegeneration (*12, 13*). Most of the inclusions are composed of ordered filaments with diameters of ∼5−20 nm. Therefore, it is important to determine whether proteins in filamentous structures can be ubiquitinated as effectively as the soluble form and whether the ubiquitination sites are identical. To address this issue, we performed *in vitro* ubiquitination of filamentous α-synuclein, determined the ubiquitin conjugation sites, and compared them with those of the soluble form. Interestingly, filamentous α-synuclein was ubiquitinated, although the efficiency was lower than that of the soluble form. Furthermore, the major ubiquitin conjugation sites of filamentous α-synuclein were localized to Lys6, Lys10, and Lys12 in the amino-terminal region, which are different from those of soluble α-synuclein (Figure 7). This result permits two interpretations: one possibility is that ubiquitin ligase(s) recognized some conformational changes of α-synuclein and altered its ubiquitination sites. The other is that normal ubiquitination sites (e.g., Lys23) become inaccessible to ubiquitin ligases when α-synuclein forms filaments, whereas the minor ubiquitination sites, Lys6, Lys10, and Lys12, are exposed and ubiquitinated as the major sites. We have previously analyzed protenase-K-resistant α-synuclein filaments and identified the core region of the filament to be residues 31−109 (*20*). Because the ubiquitination sites of

soluble α-synuclein are located close to the core region, it is reasonable to speculate that Lys21, Lys23, Lys32, and Lys34 may be partially buried or masked, whereas the Lys6, Lys10, and Lys12 in the amino-terminal franking region become more accessible to the ligase when α-synuclein formed a filamentous structure. In either case, these results suggest that ubiquitination sites can be altered depending on the structural state of the substrate. These results provide initial evidence that the aggregated filamentous form of protein can be ubiquitinated *in vitro* and that the ubiquitination sites can be altered by the conformational change of the substrate.

To examine whether the *in vitro* ubiquitination sites of α-synuclein determined by protein chemical analysis are actually ubiquitinated *in vivo*, we performed cotransfection of α-synuclein and ubiquitin into 293T cells. We successfully detected monoubiquitinated α-synuclein *in vivo* for the first time. Although Chung et al. reported that ubiquitination of α-synuclein was not seen in HEK293 cells cotransfected with α-synuclein and HA ubiquitin (*28*), we could clearly detect monoubiquitinated α-synuclein after the boiling treatment of cell lysates. The boiling treatment may be effective in detecting monoubiquitinated α-synuclein in transfected 293T cells, because in our immunoprecipitation experiments, monoubiquitinated α-synuclein was also found but in lesser levels compared to that in boiling treatment experiments (data not shown). Furthermore, expression of mutant α-synuclein with arginine substitution unequivocally showed that the *in vivo* ubiquitination sites of α-synuclein are almost identical to those found *in vitro*. Interestingly, the major *in vivo* ubiquitination sites were localized to the mR1 sites (Lys6, Lys10, and Lys12) and identified in the filamentous α-synuclein rather than the mR2 sites (Lys21 and Lys23) in the soluble form of α-synuclein. It is possible that *in vivo* ubiquitination sites of α-synuclein are slightly different from those *in vitro*, for example, because of interactions with other molecules. However, as described above, α-synuclein is ubiquitinated mainly at the mR1 sites (Lys6, Lys10, and Lys12) *in vitro* when it forms a filamentous conformation. A small portion of overexpressed α-synuclein may be oligomerized, change its conformation to a similar state to the filamentous one, and serve as a substrate for ubiquitination in 293T cells, although we could not detect any high-molecular-weight forms of α-synuclein in the RIPA-soluble or insoluble fraction from transfected 293T cells (data not shown). Alternatively, only a minor fraction of α-synuclein with some conformational change may be ubiquitinated in 293T cells.

Two point mutations, A30P and A53T, had no significant effect on ubiquitination *in vitro* and *in vivo*. Substitution of the phosphorylation site Ser129 to alanine did not affect ubiquitination, too. It has been frequently reported that protein phosphorylation at specific site(s) serves as recognition signals for ubiquitin ligase and promotes ubiquitination and degradation of the short-lived proteins by the proteasome system (*16*, *17*). Cotransfection of α-synuclein and ubiquitin into 293T cells showed that α-synuclein can be ubiquitinated in cultured cells. However, despite the very high expression of α-synuclein, only a weak band of monoubiquitinated α-synuclein was detected and no polyubiquitinated form of α-synuclein was observed in the cells. In addition, the total pools of α-synuclein were not affected by coexpression of ubiquitin. Furthermore, no accumulation of α-synuclein was detected, even when mutant α-synuclein lacking the ubiquitination sites was overexpressed (data not shown). These observations suggest that α-synuclein may not be normally degraded by the ubiquitin-dependent proteasome pathway. Recent studies have reported that natively unfolded proteins are degraded by the ubiquitin-independent proteasome pathway (*29*, *30*). α-Synuclein is known to be a natively unfolded protein, which could be degraded by this pathway in transfected culture cells (*31*).

Alzheimer paired helical filaments have been shown to be ubiquitinated (*27*), and we have previously identified a couple of ubiquitination sites within the microtubule-binding domain of τ from N-terminal-processed τ in the Sarkosyl-insoluble fraction (*32*). Because *in vitro* ubiquitination sites of τ have not been determined, it also remains to be elucidated whether any conformational changes or modifications of τ, such as phosphorylation or insoluble filament formation, affect its ubiquitination sites. However, because of a number of similarities between τ and α-synuclein, it is possible to speculate that ubiquitination of those protein inclusions is executed by similar mechanisms: Lys residues within or in the flanking positions of the core structure of insoluble filaments are prone to undergo ubiquitination by some specific ubiquitin ligation system that recognizes abnormally fibrillized proteins, although an effective ubiquitination is interfered by its conformational change, allowing the progressive deposition of ubiquitinated proteins that are less effectively targeted to proteasome degradation.

The exact location of the ubiquitin conjugation sites of α-synuclein deposited in the brains with α-synucleinopathies remains unknown. However, the present results, together with other reports, suggest that ubiquitination sites of α-synuclein in α-synucleinopathy brains may also be located in the amino-terminal region of the molecule and that ubiquitination occurs after the formation of α-synuclein filaments.

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