UCH-L1 physically interacts with tubulin

To understand the molecular mechanism underlying toxic gain of function by UCH-L1, we attempted to identify UCH-L1^{I93M}-interacting proteins by coIP assay and subsequent LC-MS/MS analysis (Fig. 5A). A database search of the peptide sequences obtained identified α-tubulin as a UCH-L1^{I93M}-interacting protein (Supplementary Material, Table S1). The interaction between UCH-L1 and endogenous α-tubulin was confirmed with transiently expressed UCH-L1 (Fig. 5B and C). The interaction of UCH-L1^{I93M} with α-tubulin was increased compared with that of UCH-L1^{WT} (Fig. 5B). We detected the interaction of endogenous α-tubulin with endogenous UCH-L1 using Neuro2a cells (Fig. 5D). Tubulin is composed of a heterodimer of α- and β-tubulin, and we confirmed, using native-PAGE, that tubulin exists as a heterodimer in cell lysates in coIP experimental conditions (data not shown), indicating that UCH-L1 interacts with tubulin. Indeed, β-tubulin was also precipitated with UCH-L1 (Supplementary Material, Fig. S3). In contrast to tubulin, interaction of β-actin with UCH-L1 was not detected (Fig. 5C). To test whether UCH-L1 directly interacts with tubulin, we performed pull-down assay using recombinant UCH-L1 and purified tubulin. Direct interaction of UCH-L1 with tubulin was observed (Fig. 5E).

Since the interactions between UCH-L1 and proteins over 30 kDa are increased by carbonyl modification or I93M mutation of UCH-L1, we tested the effects of HAE on the interaction of UCH-L1 with tubulin. We found that HAE modification of UCH-L1 promotes interactions between UCH-L1 and tubulin (Fig. 5F, G and I). In addition, a coIP assay using C90S, C132S and C152S UCH-L1 mutants showed less binding of UCH-L1^{C90S} to tubulin than UCH-L1^{WT} did, when cells were treated with HNE or HHE (Fig. 5G–I), indicating that the increased interaction of UCH-L1 with tubulin is caused by the HAE modification of Cys-90 of UCH-L1. These results are consistent with the results showing that the HAE modification of Cys-90 of UCH-L1 promotes the interaction of UCH-L1 with multiple proteins. The I93M mutation and HNE modification of UCH-L1 also promote direct interactions between UCH-L1 and tubulin (data not shown). Thus, UCH-L1^{I93M} and HNE-UCH-L1 also exhibit common biochemical properties with respect to the interactions with tubulin.

Both UCH-L1^{I93M} and carbonyl-modified UCH-L1 aberrantly promote tubulin polymerization

Microtubules are dynamic polymers composed of tubulin that continuously grow and shorten through tubulin addition and loss at the microtubule ends. Microtubule-stabilizing agents such as paclitaxel, which promote tubulin polymerization and suppress microtubule dynamics, are effective chemotherapeutic agents for the treatment of many cancers. However, neuropathy is a major adverse effect of microtubule-stabilizing agents-based chemotherapy (35). Paclitaxel induces apoptosis in cortical neurons by a mechanism independent of its cell cycle effects, because postnatal cortical neurons are postmitotic (36). These findings indicate that tubulin polymerization must be tightly regulated for neurons to function and remain viable. Furthermore, abnormal microtubule dynamics and tubulin polymerization are associated with several neurodegenerative diseases including frontotemporal dementia and parkinsonism linked to chromosome 17 (37,38). Therefore, we examined the effects of UCH-L1^{WT}, UCH-L1^{I93M} and HNE-UCH-L1 on tubulin polymerization using an *in vitro* assay. Interestingly, both UCH-L1^{I93M} and HNE-UCH-L1 promote tubulin polymerization, although UCH-L1^{WT} had almost no effect on it (Fig. 6A and B). Promotion of tubulin polymerization may result in a stabilization of microtubules because of the dynamic instability of microtubules. To test whether abnormal UCH-L1 also promotes tubulin polymerization in mammalian cells, we analyzed the amounts of soluble, polymeric and total tubulin in cells expressing UCH-L1^{I93M}. Although transient expression of UCH-L1^{I93M} had no effect on the amount of total tubulin (Fig. 5B), cells stably expressing UCH-L1^{I93M} contained increased amount of total tubulin compared with control cells or cells expressing other UCH-L1 variants (Fig. 6C). Consistent with the *in vitro* polymerization assay, the amount of polymeric tubulin was increased in cells expressing UCH-L1^{I93M}, whereas the amount of soluble tubulin was not (~1.4 and 1.0-fold increase, respectively, compared with the amount of tubulin in cells expressing UCH-L1^{WT}) (Fig. 6D). The amount of β-actin was not affected by the expression of UCH-L1 variants (Fig. 6C and D), also consistent with the results showing that UCH-L1 does not interact with β-actin. We did not detect specific interaction of UCH-L1 with polymerized tubulin (Fig. 6E), indicating that UCH-L1 may not interact with microtubules, although the possibility is not excluded that they can interact under certain conditions or at a limited number of sites such as the microtubule ends.

Since D30K and C90S mutations had no effect on the interaction of UCH-L1 and tubulin (Fig. 5B), we speculated that the tubulin-binding region of UCH-L1 is different from ubiquitin-binding region. To elucidate the amino acid residues of UCH-L1 involved in the interaction with tubulin and to show that modulation of tubulin polymerization is caused by the increased interaction of UCH-L1 with tubulin, we made a series of alanine substitutions of basic and acidic residues located on the surface of UCH-L1 and performed coIP assays using these mutants (Fig. 7A; Supplementary Material, Fig. S3). The R63A and H185A mutants displayed increased interactions with tubulin (Fig. 7A), indicating that Arg-63 and His-185, which are distinct from the ubiquitin-binding region (Fig. 7B), are involved in this interaction. The increased interactions of R63A and H185A UCH-L1 with tubulin may be caused by altered ionic interactions. In contrast to the I93M mutant or HNE-UCH-L1, the R63A mutant caused a decrease in tubulin polymerization (Fig. 7C). Although UCH-L1^{R63A} has opposite effects to the I93M mutant or HNE-UCH-L1, it also modulated tubulin polymerization. Thus, modulation of tubulin polymerization by UCH-L1 variants is caused by the abnormally increased interaction of UCH-L1 with tubulin.

From our results, we hypothesized that UCH-L1^{I93M}-associated neurodegeneration or PD is at least partly mediated by aberrant tubulin polymerization. Therefore, we tested the effects of UCH-L1^{I93M} and paclitaxel on neuronal cell death using differentiated Neuro2a cells, which

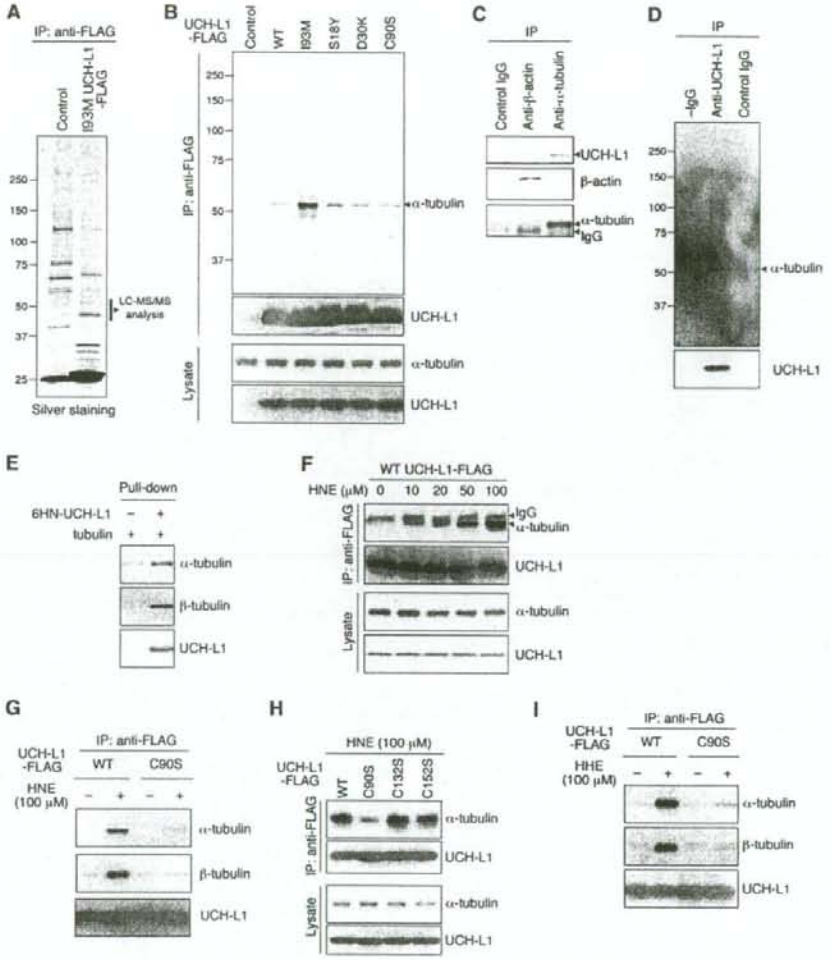

Figure 5. Physical interactions of UCH-L1 with tubulin. (**A**) Lysates of HeLa cells transfected with the indicated constructs (control: GFP) were immunoprecipitated with anti-FLAG antibody and analyzed by silver staining. Proteins ~50 kDa in size were subjected to LC-MS/MS analysis. (**B**) Lysates of COS-7 cells transfected with the indicated constructs (control: empty vector) were immunoprecipitated with anti-FLAG antibody and analyzed by immunoblotting. (**C**) Lysates of NIH-3T3 cells stably expressing FLAG-HA-tagged UCH-L1 were immunoprecipitated with the indicated antibodies and analyzed by immunoblotting. (**D**) Lysates of Neuro2a cells were immunoprecipitated with control IgG or anti-UCH-L1 antibody and analyzed by immunoblotting. –IgG, without IgG. (**E**) A pull-down assay was performed using the indicated purified proteins. [(**F**)–(**I**)] COS-7 cells transfected with the indicated constructs were treated with the indicated concentrations of HNE. Lysates were immunoprecipitated with anti-FLAG antibody and analyzed by immunoblotting.

have been used to assess the toxicity of mutant proteins linked to neurodegenerative diseases (17,39,40). We confirmed that paclitaxel does not interfere with the interaction between UCH-L1 and tubulin (data not shown). Treatment of cells with 5 μM paclitaxel slightly but significantly elevated cell death in cells expressing UCH-L1^{I93M}, but had no effect in cells expressing UCH-L1^{WT} (Fig. 6F). This indicated that the toxicity of UCH-L1^{I93M} may be at least in part mediated by aberrant microtubule dynamics or tubulin polymerization. Given that tightly regulated tubulin polymerization is necessary for neurons to be viable, our findings strongly suggest that aberrant tubulin polymerization caused by UCH-L1^{I93M} partly underlies the toxic gain of function of mutant UCH-L1, and that carbonyl-modified UCH-L1 also functions as a toxic protein in neurons. We propose that interactions of mutant or carbonyl-modified UCH-L1 with other proteins, including tubulin, constitute one of the causes of not only familial PD, but also sporadic PD (Fig. 7D).

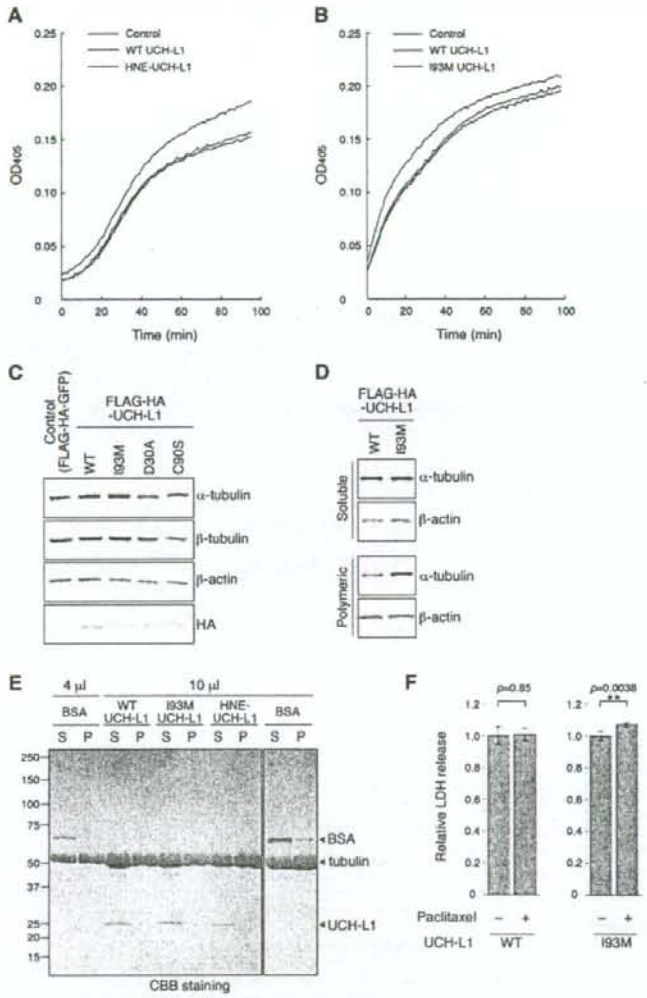

Figure 6. Effects of the I93M mutation and HNE modification of UCH-L1 on tubulin polymerization. [(**A**) and (**B**)] A tubulin polymerization assay was performed in the absence (control) or in the presence of recombinant UCH-L1. The assays were performed at least three times; representative results are shown. [(**C**) and (**D**)] Total lysates (C), soluble tubulin fractions and polymeric tubulin fractions (D) of NIH-3T3 cells stably expressing FLAG-HA-tagged UCH-L1 were analyzed by immunoblotting. (**E**) Interactions of proteins with microtubules. After the tubulin polymerization assay, the polymerized tubulin was pelleted by centrifugation. The indicated volumes of samples from the supernatants (S) and the pellets (P) were analyzed by CBB staining. BSA was used as a control that does not specifically interact with microtubules. The amount of BSA detected in the pellet fraction was approximately one-twelfth of the amount detected in the supernatant fraction. UCH-L1 levels in the pellet fraction were below detectable levels. (**F**) Differentiated Neuro2a cells transfected with the indicated constructs were incubated with or without 5 μM paclitaxel for 24 h. Cell death was assessed by a lactate dehydrogenase release assay. Data are expressed as the means ± SD ($n = 4$). $^{**}P < 0.01$ (t-test).

DISCUSSION

Our previous study using CD suggests that the I93M mutation increases the β-sheet content, but reduces the α-helix content of UCH-L1 (9). We have also shown, using small-angle neutron scattering, that UCH-L1^{WT} has an ellipsoidal shape, whereas UCH-L1^{I93M} has a more globular shape in an aqueous solution (10). However, the biochemical and molecular properties of UCH-L1^{I93M} in mammalian cells, as well as the molecular mechanisms that underlie UCH-L1^{I93M}-associated PD, have not been elucidated. In this study, we have shown that, compared with UCH-L1^{WT}, UCH-L1^{I93M} displays

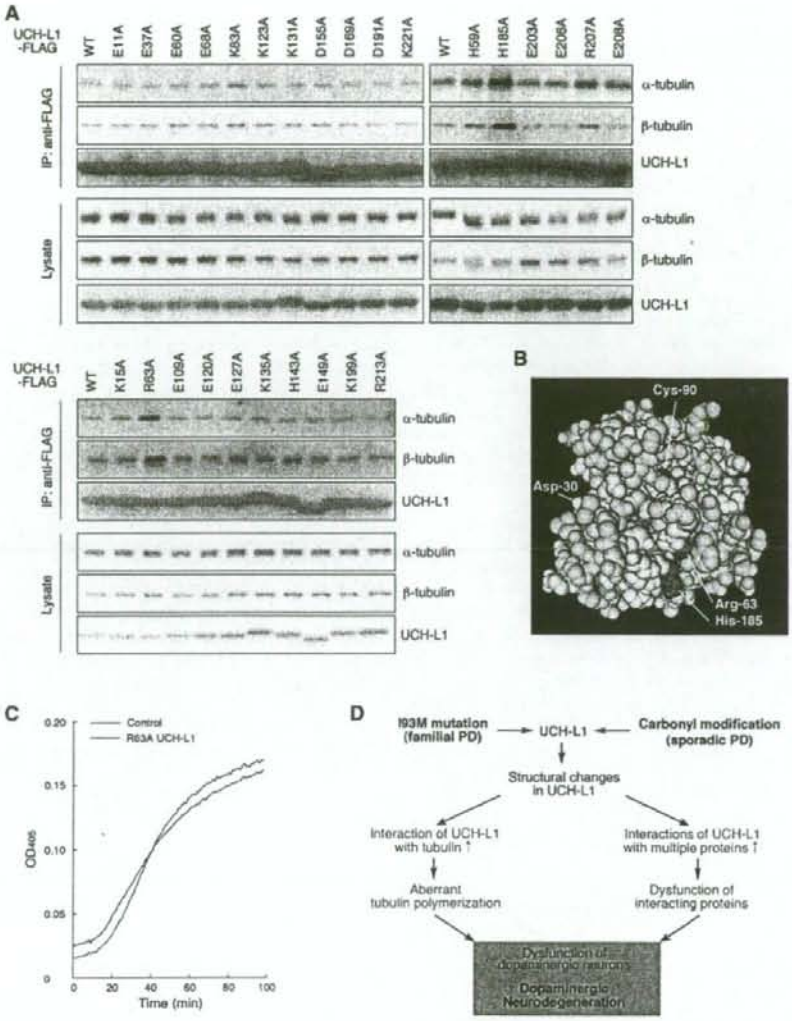

Figure 7. Amino acid residues of UCH-L1 involved in the interaction with tubulin. (**A**) Alanine-scanning mutagenesis of UCH-L1. Lysates of COS-7 cells transfected with the indicated constructs were immunoprecipitated with anti-FLAG antibody and analyzed by immunoblotting. (**B**) Structural model for human UCH-L1. Cys-90 is shown in blue, Arg-63 and His-185 are in magenta and basic and acidic amino acid residues that had no effect on tubulin interaction (Figs 5B and7A) are shown in white, using NCBI's structural model (mmdbId:38174). (**C**) A tubulin polymerization assay was performed in the absence (control) or in the presence of recombinant UCH-L1. (**D**) Schematic representation of a model for the roles of UCH-L1^{I93M} and carbonyl-modified UCH-L1 in PD. The I93M mutation (as occurs in familial PD associated with UCH-L1^{I93M}) and carbonyl modification (as occurs in sporadic PD) cause conformational changes in UCH-L1. Owing to the excess of oxidative stresses including HNE (in the case of sporadic PD) and the abundant expression of UCH-L1 in dopaminergic neurons, abnormal UCH-L1 proteins are overproduced in dopaminergic neurons. Abnormal UCH-L1 interacts with tubulin and aberrantly modulates tubulin polymerization. The aberrant interactions of UCH-L1 variants with multiple proteins may also cause dysfunctions of interacting proteins. The deregulations of abnormal UCH-L1-interacting proteins, including tubulin, result in dysfunction of dopaminergic neurons, leading to neurodegeneration.

increased insolubility, which is characteristic of several neurodegenerative disease-linked mutants, aberrantly elevated interactions with multiple proteins over 30 kDa and decreased interaction with monoubiquitin (Fig. 1). Taken together, our new and previous findings indicate that the I93M mutation in UCH-L1 alters its conformation, resulting in changes in the biochemical properties of UCH-L1.

Similar to UCH-L1^{I93M}, other dominantly inherited neurodegenerative disease-linked mutants, such as mutant SOD1 and mutant α-synuclein, cause neurodegeneration, presumably

via an acquired toxicity. Studies of the mutants strongly suggest that abnormally increased interactions of these mutant proteins with other proteins constitute a cause of disease (22–25). Therefore we screened for UCH-L1-interacting proteins using a coIP assay and subsequent LC-MS/MS analysis. We found that tubulin is a novel UCH-L1-interacting protein, and that the interactions of UCH-L1^{I93M} with these proteins are increased compared with those of UCH-L1^{WT} (Fig. 5B). We have also shown that UCH-L1^{I93M} promotes tubulin polymerization and stabilizes microtubules (Fig. 6B–D). UCH-L1^{I93M} and paclitaxel coordinately induced neuronal cell death (Fig. 6F). Together with the fact that tightly regulated tubulin polymerization is essential for neurons to function and remain viable, and that abnormal microtubule dynamics and tubulin polymerization are associated with several neurodegenerative diseases (37,38), our results strongly suggest that aberrant tubulin polymerization caused by mutant UCH-L1 at least partly constitutes a toxic function of mutant UCH-L1. Other than tubulin, mutant UCH-L1 interacts with multiple proteins (Figs 1F and 5A). These other interactors may also be involved in the mechanism of UCH-L1-mediated neurodegeneration (Fig. 7D). We have identified some of these interactors (T.K. and K.W., unpublished data), and these proteins are currently under investigation.

It is known that the majority of PD cases occur sporadically, and that oxidative/carbonyl stresses are elevated in PD brains (12,13). However, the molecular mechanisms underlying the causes of sporadic PD have remained largely unknown. Choi *et al.* (12) have shown that UCH-L1 is a major target of carbonyl damage associated with sporadic PD, implying that carbonyl-modified UCH-L1 is involved in the cause of sporadic PD. In the present study, we found that carbonyl-modified UCH-L1 and UCH-L1^{I93M} share molecular and functional properties. Importantly, both UCH-L1s display shared properties in all of the experiments we performed (Supplementary Material, Table S2). These results strongly suggest that carbonyl-modified UCH-L1 is also toxic to neurons and constitutes one of the causes of sporadic PD. Considering that UCH-L1 is abundant in the brain (5), and that UCH-L1 is a major target of carbonyl damage in PD brains (12), it is possible that carbonyl-modified UCH-L1 is the major cause of the disease.

It has been reported that UCH-L1 mRNA is expressed abundantly in dopaminergic neurons in the human brain (41). Abundant expression of UCH-L1 protein in dopaminergic neurons was also observed in mouse brains (Supplementary Material, Fig. S4 and S5). Dopaminergic neurons are particularly exposed to oxidative and carbonyl stresses because dopamine can auto-oxidize into toxic dopamine quinone, superoxide radicals and hydrogen peroxide (42). In addition, it has been reported that oxidative stresses in dopaminergic neurons in sporadic PD brains are higher than the stresses in control brains (30). Thus, in PD, UCH-L1^{I93M} or oxidative/carbonyl-modified UCH-L1 is possibly overproduced in dopaminergic neurons, leading to the selective loss of dopaminergic neurons (Fig. 7D).

Oxidatively modified UCH-L1 has also been found in the brains of both familial and sporadic Alzheimer's disease (AD) patients (12,43,44). AD is characterized pathologically by deposition of the amyloid β-protein in the form of amyloid plaques in the brain, and the deposition of the amyloid β is thought to be a major cause of both familial and sporadic AD (20). Thus, although it is possible that toxicity of carbonyl-modified UCH-L1 is involved in amyloid β-mediated neurodegeneration in AD, carbonyl-modified UCH-L1 may not be the primary cause of AD. A recent report has shown that brains from patients with sporadic PD and AD contain decreased levels of UCH-L1 (30 and 50% decrease, respectively) (12). Gong *et al.* (45) showed that the introduction of exogenous UCH-L1 rescued the synaptic and cognitive functions of AD model mice, which exhibit decreased levels of UCH-L1 in their hippocampi. We have also shown that mice deficient in UCH-L1 exhibit memory dysfunction (46). These findings indicate that a reduction in the levels of functional UCH-L1 may contribute to the pathogenesis of AD. Oxidative modification of several proteins, including antioxidant proteins, is found in mice deficient in UCH-L1 (47), suggesting involvement of these proteins in AD. Since diminution of the proteasome activity may lead to neurodegeneration (48), it is also possible that decreased UCH-L1 function leads to dysfunction of the ubiquitin-proteasome system and this dysfunction contributes to neurodegeneration in AD. On the contrary, mice deficient in UCH-L1 do not exhibit obvious dopaminergic cell loss, indicating that a loss or decrease in the level of UCH-L1 is not the main cause of PD. Investigation of the relationship between the specificity of brain areas that is affected by oxidative stress and genetic or environmental factors should generate further insights into the mechanism of oxidative stress in the pathogenesis of sporadic PD and AD.

In conclusion, familial PD-associated UCH-L1^{I93M} and carbonyl-modified UCH-L1, which is associated with sporadic PD, display common aberrant properties. Thus, UCH-L1^{I93M} would be a useful tool for studying the molecular mechanism underlying sporadic PD. We propose that the abnormal interactions of UCH-L1 variants with other proteins including tubulin constitute one of the causes of not only familial PD associated with UCH-L1^{I93M}, but also sporadic PD, and can be therapeutic targets for these diseases and possibly for other neurodegenerative diseases.

MATERIALS AND METHODS

Plasmids

pCI-neo-hUCH-L1 plasmids containing human WT UCH-L1 and UCH-L1 variants with or without FLAG tag were prepared as described previously (49) or generated using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The expression plasmid pCR3-hα-synuclein containing FLAG-tagged human α-synuclein was kindly donated by Ryosuke Takahashi (Kyoto University, Kyoto, Japan) and Yuzuru Imai (Tohoku University, Miyagi, Japan) (50). The pcDNA3-hSOD1 expression plasmids containing WT, A4V, G85R or G93A mutant SOD1, and pCI-hα-synuclein expression plasmids containing WT, A30P or A53T mutant α-synuclein were prepared as described previously (17). The expression plasmid pEF-hUCH-L1 containing WT UCH-L1 was constructed by ligating the cDNA

encoding UCH-L1 into pEF-BOS vector (51). The bacterial expression plasmid pPROTetE-hUCH-L1 containing 6HN-tagged UCH-L1 was prepared as described previously (9). pGEX-hUCH-L1 bacterial expression plasmids containing WT, I93M or R63A UCH-L1 with a GST-tag were constructed by ligating the cDNA encoding each UCH-L1 into pGEX-6P-1 vector (GE Healthcare UK Ltd, Buckinghamshire HP7 9NA, UK).

Cell culture and transfection

Neuro2a, SH-SY5Y, COS-7 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA). NIH-3T3 cells stably expressing human UCH-L1 with a FLAG-HA double-tag at the N terminus were cultured as described previously (49). Transient transfection of Neuro2a, SH-SY5Y and COS-7 cells with each vector was performed using the FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA), TransFectin Lipid Reagent (Bio-Rad, Hercules, CA, USA) and Lipofectamine Reagent (Invitrogen, Carlsbad, CA, USA), respectively. For the experiments investigating the carbonyl modification of UCH-L1, cells were incubated at 37°C for 90 min with each carbonyl compound or H_2O_2 in PBS containing 5 mM glucose, 0.3 mM $CaCl_2$ and 0.62 mM $MgCl_2$.

Immunoblotting

SDS–PAGE was performed under reducing conditions. Immunoblotting was performed according to standard procedures. The preparation of detergent (1% Triton X-100)-soluble and -insoluble fractions was performed as described previously (17). Mouse anti-α-tubulin and anti-β-tubulin antibodies were purchased from Sigma. Rabbit anti-α-tubulin and anti-β-tubulin antibodies were from Cell Signaling (Danvers, MA, USA). Mouse anti-HNE and rabbit anti-HNE antibodies were from Oxis (Portland, OR, USA) and Alpha Diagnostic (San Antonio, TX, USA), respectively. Antibodies against SOD1, UCH-L1 and reduced-HNE were purchased from Stressgen Bioreagents (Victoria, BC, Canada), UltraClone (England, UK) and Calbiochem (Darmstadt, Germany), respectively. Anti-β-actin, ubiquitin and FLAG antibodies were from Sigma. The antibody against α-synuclein was from Chemicon (Temecula, CA, USA). For immunoblotting with anti-reduced HNE antibody, the proteins on a PVDF membrane were reduced with 10 mM $NaBH_4$ in Tris-buffered saline for 30 min at room temperature before being reacted with anti-reduced HNE antibody. Carbonyl modification of proteins was detected using an OxyBlot Protein Oxidation Detection Kit (Chemicon) containing an anti-DNP antibody.

Immunoprecipitation

Immunoprecipitation was performed as previously described (52). Cells were harvested by cold immunoprecipitation buffer (15 mM Tris pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM EGTA, 2 mM EDTA, 0.5% Triton X-100 and protease inhibitors). The lysates were centrifuged at 20 000*g* for 10 min at 4°C. The supernatant was subjected to immunoprecipitation. Lysates (1 mg protein in immunoprecipitation buffer) were incubated with 5 μg of antibody for 12 h. Twenty microliters of protein G Sepharose (GE Healthcare) was then added, and incubation was continued for 1 h. For the immunoprecipitation of FLAG-tagged proteins, lysates (1–2 mg protein in immunoprecipitation buffer) were incubated with 30 μl anti-FLAG M2 affinity gel (Sigma) for 2 h. After the beads were washed three times with immunoprecipitation buffer, proteins were eluted with SDS sample buffer (10 mM Tris, pH 7.8, 3% SDS, 5% glycerol and 0.02% bromophenol blue). In some experiments, proteins were eluted with SDS sample buffer containing 2% 2-mercaptoethanol. For the immunoprecipitation of endogenous UCH-L1 (Fig. 5D), 100 μg anti-UCH-L1 antibody (53) or 100 μg normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was immobilized to 100 μl of protein G beads using a Seize X Protein G Immnoprecipitation Kit (Pierce, Rockford, IL, USA). Cell lysates (1 mg protein in 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.25% Triton X-100 and protease inhibitors) were incubated with 25 μl of beads for 12 h. Protein G beads without antibody and protein G beads cross-linked with normal rabbit IgG were used as controls.

Mass spectrometry analysis

Protein bands were sliced from the gel and subjected to in-gel trypsin digestion, and LC-MS/MS analysis was performed at APRO Life Science Institute, Inc. (Naruto, Japan) as a custom service.

Circular dichroism

CD measurements of 0.1 mg/ml (4 μM) of recombinant human UCH-L1 without a tag (Boston Biochem, Cambridge, MA, USA) in 20 mM sodium phosphate buffer (pH 8.0) were performed as described previously (9,10). Since two cysteine residues in UCH-L1, Cys-90 and Cys-152, are major targets of HNE modification (Fig. 4), 4 μM UCH-L1 was reacted with 8 μM HNE. Far UV CD spectra (190–250 nm) were recorded in a 1 mm quartz cuvette on a Jasco J-820 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a temperature controller by scanning at a rate of 50 nm/min at 25°C. For all spectra, 12 scans were averaged. All CD spectra were corrected by background subtraction of the spectrum obtained with buffer alone and smoothed. Spectra were analyzed for the percentage of secondary structural elements by a computer program, based on an algorithm that compares experimental spectra with those of known proteins (54).

Preparation of recombinant proteins

6HN-tagged human UCH-L1 proteins were prepared as described previously (9). For purification of UCH-L1 without a tag, the pGEX UCH-L1 vectors were transformed into *Escherichia coli* BL21. Production of fusion proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM. After a 4 h induction at 37°C, the cells were harvested and lysed by sonication in PBS containing 1% Triton X-100 and protease inhibitors. Puri-

fication of GST-tagged UCH-L1 was performed using glutathione Sepharose 4B (GE Healthcare), and UCH-L1 was released from GST by digestion using PreScission Protease (GE Healthcare). Purified proteins were resolved by SDS–PAGE under reducing conditions and visualized by Coomassie brilliant blue R-250 to confirm purity (Supplementary Material, Fig. S6).

Pull-down assay

TALON resin (Clontech, Palo Alto, CA, USA) was blocked with 3% BSA for 1 h in order to prevent non-specific binding of tubulin (data not shown) and washed three times with PBS containing 0.05% Triton X-100. Five micrograms of recombinant UCH-L1 with an HN tag and 5 μg of purified tubulin (>99% pure tubulin, Cytoskeleton, Denver, CO, USA) were mixed and incubated for 4 h in PBS containing 0.05% Triton X-100. As a control, vehicle was mixed instead of UCH-L1. Twenty microliters of TALON resin blocked with BSA was then added, and incubation was continued for 1 h. After beads were washed three times with PBS containing 0.05% Triton X-100, proteins were eluted with SDS sample buffer.

Tubulin polymerization assay

An *in vitro* tubulin polymerization assay was performed using a tubulin polymerization assay kit, OD based, >99% pure tubulin (Cytoskeleton), according to the manufacturer's protocol. Briefly, recombinant UCH-L1 without a tag and tubulin were mixed to give a final concentration of 0.05 mg/ml UCH-L1 and 3 mg/ml tubulin in tubulin polymerization buffer (80 mM PIPES, pH 6.9, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM GTP, 5% glycerol) and subjected to a tubulin polymerization assay. As a control, vehicle was mixed instead of UCH-L1. Since two cysteine residues in UCH-L1 are major targets of HNE modification (Fig. 4), 40 μM UCH-L1 was reacted with 80 μM HNE to prepare the HNE-modified UCH-L1. To analyze the interaction between UCH-L1 and polymerized tubulin, the polymerized tubulin was pelleted by centrifugation after a tubulin polymerization assay. The supernatant (100 μl) was mixed with 50 μl of 3× SDS sample buffer (30 mM Tris, pH 7.8, 9% SDS, 15% glycerol, 0.06% bromophenol blue). The pellet was washed twice with tubulin polymerization buffer and then dissolved in 150 μl of SDS sample buffer.

Preparation of cell extracts containing soluble and polymeric tubulin

Preparation of soluble and polymeric fractions of tubulin was performed as described (55) with slight modification. Briefly, cells were washed very gently with a microtubule stabilizing buffer (0.1 M *N*-morpholinoethanesulfonic acid, pH 6.75, 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM EDTA, 4 M glycerol). Soluble proteins were extracted at 37 °C for 5 min in microtubule stabilizing buffer containing 0.04% saponin. The remaining cytoskeletal fraction in the culture dish was washed with microtubule stabilizing buffer containing 0.4% saponin and dissolved in SDS sample buffer.

Quantitative assessment of cell death

Neuro2a cells were transfected with plasmids. Four hours after transfection, neuronal cell differentiation was induced by addition of 5 mM dibutyryl cAMP as described in the literature (40), and cells were incubated for 24 h. Cells were then incubated with or without 5 μM paclitaxel for another 24 h. Cell death was assessed by a lactate dehydrogenase release assay, as described previously (17).

Statistical analysis

For comparison of two groups, the statistical difference was determined by Student's *t*-test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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Aberrant Interaction between Parkinson Disease-associated Mutant UCH-L1 and the Lysosomal Receptor for Chaperone-mediated Autophagy*[S]

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Parkinson disease (PD) is the most common neurodegenerative movement disorder. An increase in the amount of α-synuclein protein could constitute a cause of PD. α-Synuclein is degraded at least partly by chaperone-mediated autophagy (CMA). The I93M mutation in ubiquitin C-terminal hydrolase L1 (UCH-L1) is associated with familial PD. However, the relationship between α-synuclein and UCH-L1 in the pathogenesis of PD has remained largely unclear. In this study, we found that UCH-L1 physically interacts with LAMP-2A, the lysosomal receptor for CMA, and Hsc70 and Hsp90, which can function as components of the CMA pathway. These interactions were abnormally enhanced by the I93M mutation and were independent of the monoubiquitin binding of UCH-L1. In a cell-free system, UCH-L1 directly interacted with the cytosolic region of LAMP-2A. Expression of I93M UCH-L1 in cells induced the CMA inhibition-associated increase in the amount of α-synuclein. Our findings may provide novel insights into the molecular links between α-synuclein and UCH-L1 and suggest that aberrant interaction of mutant UCH-L1 with CMA machinery, at least partly, underlies the pathogenesis of PD associated with I93M UCH-L1.

Parkinson disease (PD)[4] is the most common neurodegenerative movement disorder characterized by progressive degeneration confined mostly to dopaminergic neurons in the substantia nigra pars compacta. Although the majority of PD cases occur sporadically, nine genes have been reported to be associated with familial forms of PD. Several missense mutations in the α-synuclein gene are linked to dominantly inherited PD (1–3). Duplication and triplication of the α-synuclein gene were also shown to cause familial PD or parkinsonism (4–6), indicating that increases in the levels of α-synuclein could constitute a cause of PD. α-Synuclein is a major component of cytoplasmic inclusions called Lewy bodies in the brains of patients with sporadic PD (7, 8). These findings raised the idea that α-synuclein plays a central role in the pathogenesis of PD. Therefore, elucidating the molecular relationships between α-synuclein and other familial PD-associated proteins is important for understanding the mechanisms that underlie the pathology of PD.

A missense mutation in the ubiquitin C-terminal hydrolase L1 (UCH-L1) gene, leading to an I93M substitution at the protein level, has been reported in two affected siblings of a German family with dominantly inherited PD (9). In this family, four of seven family members were affected with PD. However, the family members, except the two siblings, were not genotyped. There was an unaffected presumed carrier of the I93M mutation in the family. Therefore, the link between the I93M mutation and the development of PD has been questioned (10, 11). To clarify the link between the mutation and PD, we have generated UCH-L1^{I93M} transgenic mice and reported that these mice exhibit progressive dopaminergic cell loss (12). In addition, we have shown that, compared with UCH-L1^{WT}, UCH-L1^{I93M} exhibits increased insolubility and levels of interactions with other proteins in mammalian cells, features that are characteristic of several neurodegenerative disease-linked mutants (13). These findings suggest that the I93M mutation in UCH-L1 contributes to the pathogenesis of PD. UCH-L1 has also been identified as a component of several inclusion bodies characteristic of neurodegenerative diseases including Lewy bodies (14). A polymorphism in the UCH-L1 gene, resulting in an S18Y substitution at the amino acid residue level, has been reported to be associated with decreased risk of PD in certain populations but not in other populations (15, 16). We have also reported that UCH-L1^{I93M} and carbonyl-modified UCH-L1, which is associated with sporadic PD (17), display shared aberrant properties (13), suggesting that carbonyl-modified UCH-L1 constitutes one of the causes of sporadic PD.

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[4] The abbreviations used are: PD, Parkinson disease; UCH-L1, ubiquitin C-terminal hydrolase L1; WT, wild-type; CMA, chaperone-mediated autophagy; LAMP-2, lysosome-associated membrane protein type 2; Hsc70, heat shock cognate protein 70; Hsp90, heat shock protein 90; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

UCH-L1 is one of the most abundant proteins in the brain (1–5% of total soluble protein) (18) and is thought to hydrolyze ubiquitin conjugates into monoubiquitin (19). UCH-L1 was also reported to function as a ubiquitin ligase for monoubiquitinated α-synuclein in a cell-free system (20). Other than these enzymatic activities, we have reported that UCH-L1 stabilizes monoubiquitin by binding to monoubiquitin in neurons (21). Although the hydrolase activity of UCH-L1^{I93M} and the binding of UCH-L1^{I93M} to monoubiquitin are decreased compared with those of UCH-L1^{WT} (9, 13, 22), we have shown that mice deficient in UCH-L1 do not display obvious dopaminergic cell loss (21, 23). These observations indicate that the main cause of UCH-L1^{I93M}-associated PD may not be a loss of UCH-L1 function but an acquired toxicity of UCH-L1^{I93M}. Our previous studies also suggest that aberrantly enhanced physical interactions between UCH-L1^{I93M} and multiple proteins, including tubulin, underlie the toxic functions of UCH-L1^{I93M} (13).

However, the molecular relationship between α-synuclein and UCH-L1 in the pathogenesis of PD has remained largely unclear. α-Synuclein is known to be degraded at least partly by chaperone-mediated autophagy (CMA) (24), in which substrate proteins are selectively transported to and degraded in lysosomes (25). In this study, we sought to identify novel UCH-L1-interacting proteins. We found that UCH-L1 physically interacts with lysosome-associated membrane protein type 2A (LAMP-2A), heat shock cognate protein 70 (Hsc70), and heat shock protein 90 (Hsp90), all of which are components of the CMA pathway (26). These interactions were enhanced by the I93M mutation in UCH-L1 and were independent of the interaction between monoubiquitin and UCH-L1. We also provide the data suggesting that the aberrant interaction of UCH-L1 with CMA machinery results in the accumulation of α-synuclein.

EXPERIMENTAL PROCEDURES

Plasmids—pCI-neo-hUCH-L1 plasmids containing human WT UCH-L1 and UCH-L1 variants with or without a FLAG tag were prepared as described previously (13). The regulatory expression plasmids pTRE-Tight-hUCH-L1 containing WT and I93M UCH-L1 with a FLAG tag at the C terminus of UCH-L1 were constructed by ligating the cDNA encoding UCH-L1 into the pTRE-Tight (Clontech) vector. The expression plasmid pCI-neo-hα-synuclein was constructed using the pCI-neo mammalian expression vector (Promega), and the expression plasmid pCI-neo-ΔDQ α-synuclein was generated using a QuikChange site-directed mutagenesis kit (Stratagene).

Cell Culture and Transfection—COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS). IMR-90 cells, which have been used to study CMA (27), were cultured as described in the literature (27). NIH-3T3 cells stably expressing human UCH-L1 with a FLAG-hemagglutinin double tag at the N terminus were cultured as described previously (13). Transient transfection of COS-7 and IMR-90 cells with each vector was performed using Lipofectamine reagent (Invitrogen) and Lipofectamine LTX reagent (Invitrogen), respectively. There was no notable difference in the transfection efficiency among the culture dishes (wells) in our experimental conditions (data not shown).

Immunoblotting and Immunoprecipitation—Preparation of the detergent (1% Triton X-100)-soluble fraction was performed as described previously (28). The cytosolic fraction that does not contain LAMP-2, a marker of lysosomes, and the crude lysosomal fraction containing LAMP-2 (supplemental Fig. S1*A*) were prepared according to the method described by Pertoft *et al.* (29). SDS-PAGE was performed under reducing conditions. Immunoblotting was performed according to standard procedures as described previously (30). For some experiments, Can Get Signal Immunoreaction Enhancer Solution (Toyobo, Osaka, Japan) was used. The signal intensity was quantified by densitometry using FluorChem software (Alpha Innotech, San Leandro, CA). Immunoprecipitation was performed using anti-FLAG M2 affinity gel (Sigma) or 10 μg/ml antibodies (unless otherwise mentioned) with protein G-Sepharose (GE Healthcare), as described previously (13). The antibodies used were as follows. Antibodies against UCH-L1, Cu,Zn-superoxide dismutase 1, Hsc70, and Hsp90 were purchased from Ultra-Clone, Stressgen Bioreagents (Victoria, Canada), Affinity BioReagents (Golden, CO), and BD Transduction Laboratories (Franklin Lakes, NJ), respectively. Anti-β-actin, Mcl-1, and FLAG antibodies were from Sigma. Antibodies against α-synuclein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Chemicon (Temecula, CA). Anti-p53 and Bcl-xL antibodies were from Cell Signaling. Anti-Bcl-2, Ubc9, NF-κB p65, and LAMP-2 antibodies were from Santa Cruz Biotechnology. The rabbit polyclonal anti-LAMP-2A antibody was raised in rabbit against a synthetic peptide (CYFIGLKHHHAGYEQF) containing an amino acid sequence corresponding to the cytosolic region of human LAMP-2A. The specificity of the anti-LAMP-2A antibody was confirmed as shown in supplemental Fig. S1, *B* and *C*.

Pulldown Assay—Recombinant human UCH-L1 proteins without a tag were prepared as described previously (13). A pulldown assay was performed as described previously (13) with slight modifications. Streptavidin-Sepharose (GE Healthcare) was blocked with 3% bovine serum albumin for 15 h to prevent nonspecific binding of UCH-L1 to the beads and washed three times with phosphate-buffered saline containing 0.05% Triton X-100. Ten μg of UCH-L1 (wild-type or I93M) and 2 nmol of synthetic peptides conjugated to biotin (control or LAMP-2A peptide, Invitrogen) were mixed and incubated for 15 h in phosphate-buffered saline containing 0.05% Triton X-100. Twenty μl of streptavidin beads blocked with bovine serum albumin was then added, and incubation was continued for 1 h. After beads were washed three times with phosphate-buffered saline containing 0.05% Triton X-100, proteins were eluted with SDS sample buffer and subjected to SDS-PAGE.

UCH-L1 Degradation Assay—COS-7 cells were cotransfected with pTet-Off and pTRE-Tight-hUCH-L1. Twenty-four h after transfection, transcription of UCH-L1-FLAG gene was suppressed by adding 100 ng/ml doxycycline and incubating for 4 h. Then, cells were harvested at the 0-, 24-, and 48-h time

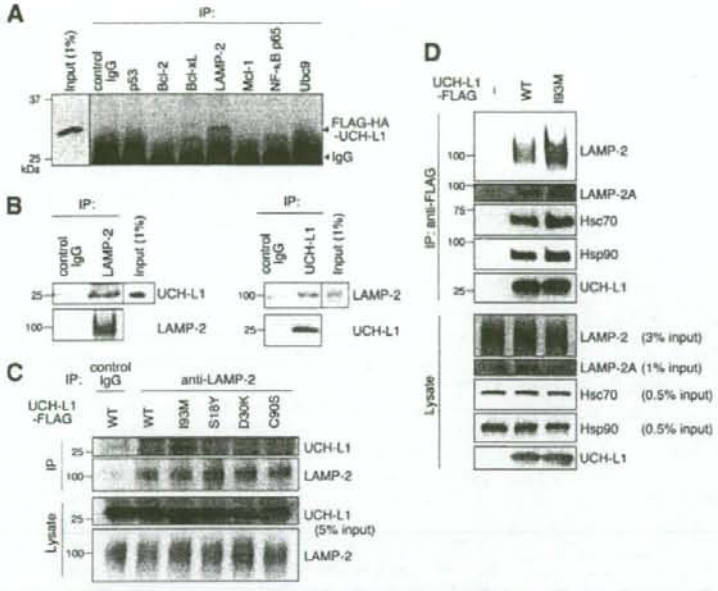

FIGURE 1. **Physical interactions of UCH-L1 with LAMP-2A, Hsc70, and Hsp90.** *A*, lysates of NIH-3T3 cells stably expressing FLAG-hemagglutinin (*HA*)-tagged UCH-L1 were immunoprecipitated (*IP*) with antibodies against cell death- or protein degradation-related proteins and analyzed by immunoblotting using anti-UCH-L1 antibody. A representative blot including immunoprecipitant with anti-LAMP-2 antibody is shown. *B*, mouse (C57BL/6J) whole brain lysates were immunoprecipitated with control IgG, anti-LAMP-2, or anti-UCH-L1 antibody and immunoblotted with anti-UCH-L1 and LAMP-2 antibodies. *C*, lysates of COS-7 cells transfected with the indicated constructs were immunoprecipitated with 5 μg/ml control IgG or anti-LAMP-2 antibody and analyzed by immunoblotting using anti-UCH-L1 antibody. *D*, lysates of COS-7 cells transfected with the indicated constructs (−, empty vector) were immunoprecipitated with anti-FLAG beads and immunoblotted using anti-LAMP2, LAMP-2A, Hsc70, Hsp90, and UCH-L1 antibodies.

points after the suppression of the gene and analyzed by immunoblotting. Pulse-chase analyses were performed as described previously (21) with some modifications. COS-7 cells transfected with pCI-neo-hUCH-L1-FLAG were washed and incubated with methionine-, cysteine-, and cystine-free medium for 1 h. The cells were pulsed with 0.1 mCi/ml [^{35}S]Met and [^{35}S]Cys (Expre^{35}S^{35}S protein labeling mixture, PerkinElmer Life Sciences) for 1 h and then washed and chased with 3 mM methionine and cysteine for 48 h. At the 0-, 24-, and 48-h time points, the cells were harvested for immunoprecipitation with anti-FLAG M2 affinity gel. Following SDS-PAGE on a 15% gel, radioactive bands were detected and analyzed by using a BAS-5000 imaging analyzer (Fujifilm, Tokyo, Japan).

Statistical Analysis—For comparison of two groups, the statistical significance of differences was determined by the Student's *t* test.

RESULTS

UCH-L1 Interacts with LAMP-2A, Hsc70, and Hsp90—We have previously shown that soluble UCH-L1 interacts with multiple proteins in mammalian cells and that one of the UCH-L1-interacting proteins is α/β-tubulin (13). In this study, we further screened for UCH-L1-interacting proteins using a coimmunoprecipitation assay (Fig. 1*A*). We identified LAMP-2 as a novel UCH-L1-interacting protein (Fig. 1*A*). To confirm this interaction *in vivo*, a coimmunoprecipitation assay was performed using mouse whole brain lysate. Interaction between endogenous UCH-L1 and endogenous LAMP-2 was observed (Fig. 1*B*). LAMP-2 exists in three different isoforms, LAMP-2A, LAMP-2B, and LAMP-2C, which are produced by the alternative splicing of the LAMP-2 pre-mRNA (31). LAMP-2A forms a complex with chaperones such as Hsc70 and Hsp90 and functions as a receptor for CMA at the lysosomal membrane (26). Because α-synuclein has been reported to interact with LAMP-2A (24), we tested for interactions between UCH-L1 and LAMP-2A, Hsc70, and Hsp90. The UCH-L1 immunoprecipitant included LAMP-2A as well as Hsc70 and Hsp90 (Fig. 1*D*). These results indicate that UCH-L1 interacts with LAMP-2A, Hsc70, and Hsp90 in mammalian cells.

UCH-L1 Can Be Degraded by Macroautophagy—Although UCH-L1 physically interacts with LAMP-2A, UCH-L1 is not a presumable substrate for CMA because UCH-L1 does not contain a KFERQ-like motif, which is required for substrate proteins to be degraded by CMA (32). Therefore, we speculated that UCH-L1 is degraded by other degradation pathways in mammalian cells. We used a regulatory protein expression system to switch off the expression of UCH-L1 by adding doxycycline, to follow UCH-L1 degradation. Degradation of UCH-L1 was observed 24 or 48 h after expression was switched off, compared with the time point at which expression was switched off (Fig. 2*A*). The half-life of UCH-L1 was >48 h (Fig. 2*A*). Long-lived proteins are known to be mainly degraded by macroautophagy (33). We therefore investigated whether UCH-L1 was degraded by macroautophagy using 3-MA, an inhibitor of macroautophagy (24, 28, 34). The 3-MA treatment significantly inhibited the degradation of UCH-L1 (Fig. 2*A*). Similar results were obtained when we used UCH-L1^{I93M} (Fig. 2*B*). Pulse-chase experiments also showed that the degradations of UCH-L1^{WT} and UCH-L1^{I93M} were significantly inhibited by 3-MA treatment (Fig. 2, *C* and *D*). These results suggest that macroautophagy is one of the major pathways that degrade UCH-L1 in our cell model.

The Interactions of UCH-L1 with LAMP-2A, Hsc70, and Hsp90 Are Enhanced by the I93M Mutation in UCH-L1 and Are Independent of the Interaction between Monoubiquitin and

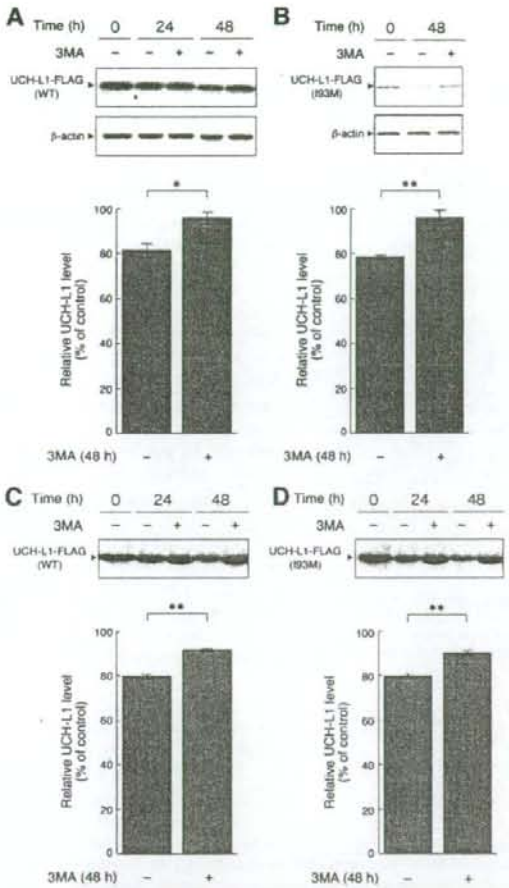

FIGURE 2. **Degradation of UCH-L1 by macroautophagy.** *A* and *B*, COS-7 cells were transfected with pTet-Off and pTRE-Tight-hUCH-L1^{WT} (*A*) or pTRE-Tight-hUCH-L1^{I93M} (*B*). Twenty-four h after transfection, transcription of UCH-L1-FLAG gene was suppressed by adding 100 ng/ml doxycycline and incubating for 4 h. Then, 3-MA (+) or vehicle (−) was added, and cells were harvested at the indicated times after the suppression of the gene and analyzed by immunoblotting (*upper panels*). The relative levels of UCH-L1-FLAG at 48 h after the suppression (% of 0-h control) were quantified by densitometry. Mean values are shown with S.E. (*A*, $n = 4$; *B*, $n = 3$). *, $p < 0.05$; **, $p < 0.01$. *C* and *D*, COS-7 cells were transfected with pCI-neo-hUCH-L1^{WT}-FLAG (*C*) or pCI-neo-hUCH-L1^{I93M}-FLAG (*D*). Twenty-four h after transfection, cells were labeled with [^{35}S]Met and [^{35}S]Cys. Autoradiograms of anti-FLAG immunoprecipitates pulse-chased at the indicated times in the absence or presence of 3-MA are shown (*upper panels*). Relative band intensities at 48 h (% of 0-h control) are quantified. Mean values are shown with S.E. ($n = 3$). **, $p < 0.01$.

UCH-L1—We have previously shown that the amount of each protein interacting with UCH-L1^{I93M} is mostly higher than the amount interacting with UCH-L1^{WT} (13). Consistent with this observation, we found that the amount of LAMP-2A, Hsc70, and Hsp90 interacting with UCH-L1^{I93M} is higher than the amount interacting with UCH-L1^{WT} (~1.8-, 1.3-, and 1.3-fold increases, respectively) (Fig. 1, *C* and *D*, and supplemental Fig. S2*A*). The interactions of LAMP-2, Hsc70, or Hsp90 with UCH-L1^{S18Y}, UCH-L1^{D30K}, which lacks hydrolase activity and binding affinity for ubiquitin (21), and UCH-L1^{C90S}, which lacks hydrolase activity but maintains binding affinity for ubiquitin (21), were not notably changed compared with those of UCH-L1^{WT} (Fig. 1*C*, supplemental Fig. S2*A*, and data not shown). These results suggest that the interaction between UCH-L1 and CMA machinery is independent of both UCH-L1-binding affinity for ubiquitin and the hydrolase activity of UCH-L1. To further show that these interactions are independent of monoubiquitin binding to UCH-L1, and to elucidate the amino acid residues of UCH-L1 involved in the interaction with LAMP-2A, Hsc70, and Hsp90, we performed coimmunoprecipitation assays using a series of alanine substitutions (13) of basic and acidic residues located on the surface of UCH-L1 (Fig. 3*A*). The R63A mutant displayed increased levels of interactions with LAMP-2, Hsc70, and Hsp90, whereas other mutations had no notable effect on the interactions (Fig. 3*A*). We further performed alanine-scanning mutagenesis experiments and found that E174A, D176A, and H185A mutants also displayed increased levels of interactions with LAMP-2, Hsc70, and Hsp90 (Fig. 3*B* and data not shown). Glu^{174}, Asp^{176}, and His^{185} are located near Arg^{63} (Fig. 3*C*). The surface region containing Arg^{63} and His^{185} possesses features that are characteristic of a protein-protein interacting site (35). These observations suggest that this surface region, which is distinct from the ubiquitin-binding region (13, 35), is involved in the interactions with LAMP-2, Hsc70, and Hsp90. The R63A, E174A, D176A, or H185A mutation possibly causes partial misfolding, resulting in increased interactions.

UCH-L1 Directly Interacts with the Cytoplasmic Region of LAMP-2A—LAMP-2 is a type 1 membrane protein, consisting of a short cytoplasmic tail (12 amino acids), one transmembrane domain, and a glycosylated luminal domain (31). To test whether UCH-L1 directly interacts with the cytosolic region of LAMP-2A, we prepared purified recombinant wild-type and I93M UCH-L1 proteins, a peptide containing an amino acid sequence corresponding to the C-terminal cytoplasmic tail of LAMP-2A, and a control peptide (Fig. 4*A*). Purified UCH-L1 proteins and the peptides were mixed, and pulldown assays were performed. A direct interaction between wild-type UCH-L1 and the cytosolic region of LAMP-2A was observed (Fig. 4, *B* and *C*). Consistent with the results of the coimmunoprecipitation assay, UCH-L1^{I93M} exhibited an abnormally increased level of interaction with the cytosolic region of LAMP-2A compared with wild-type UCH-L1 (Fig. 4*D*). Because chaperones, including Hsc70, are considered to be required for the interaction of the CMA substrates with LAMP-2A (36), our results may indicate that UCH-L1 interacts with LAMP-2A in a manner different from the interaction between CMA substrates and LAMP-2A.

UCH-L1^{I93M} Causes Accumulation of α-Synuclein—It has been reported that α-synuclein^{WT} is a CMA substrate, but pathogenic mutants A30P and A53T α-synuclein inhibit CMA by tight binding to LAMP-2A (24). Thus, UCH-L1^{I93M}, which exhibits elevated interactions with LAMP-2A, Hsc70, and Hsp90, may also inhibit CMA. To examine this possibility in mammalian cells, we assessed the effects of UCH-L1^{I93M} on the

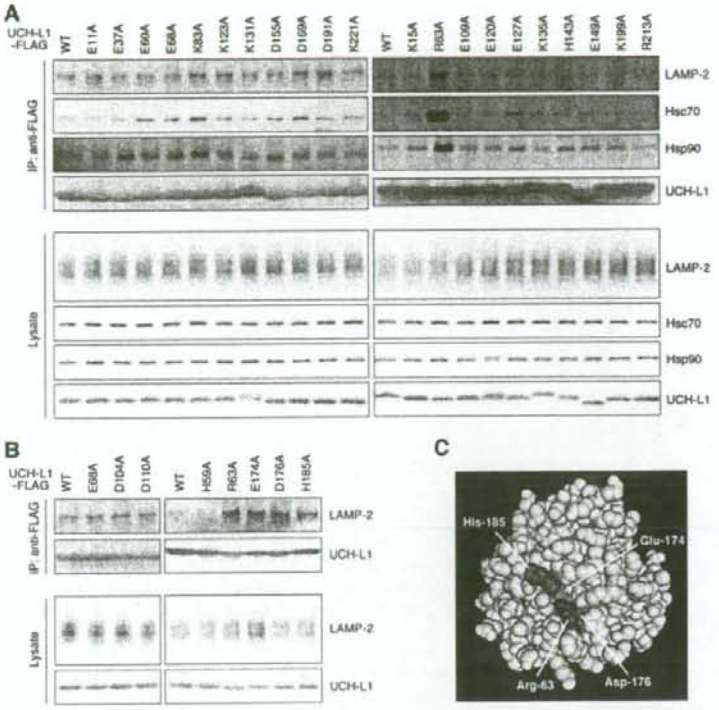

FIGURE 3. **Alanine-scanning mutagenesis of UCH-L1.** *A* and *B*, lysates of COS-7 cells transfected with the indicated constructs were immunoprecipitated (*IP*) with anti-FLAG antibody and analyzed by immunoblotting. *C*, a structural model for human UCH-L1 is shown. Arg^{63}, Glu^{174}, Asp^{176}, and His^{185} are shown in *blue*, *green*, *magenta*, and *red*, respectively, using Cn3D software (version 4.1) and NCBI structural model mmdbId:38174 (35).

protein level of GAPDH, an established substrate of CMA (24), in the lysosomal fraction and whole-cell lysate. The GAPDH level in whole-cell lysate was increased in cells expressing UCH-L1^{I93M} compared with that in cells expressing UCH-L1^{WT} (an ~1.5-fold increase) (Fig. 5*A*), whereas the GAPDH level in the lysosomal fraction was decreased in cells expressing UCH-L1^{I93M} (an ~2.1-fold decrease) (Fig. 5*B*), supporting the idea that the aberrant interaction of UCH-L1^{I93M} with CMA machinery inhibits CMA. The inhibition of CMA also results in the accumulation of other CMA substrates, including α-synuclein (24). We found that the amount of α-synuclein^{WT} was increased in cells expressing UCH-L1^{I93M} compared with cells expressing UCH-L1^{WT} (~1.7 and 1.4-fold increases, respectively) (Fig. 5, *C* and *D*) or control mock cells (data not shown). The physical interaction between UCH-L1 and α-synuclein was not detected under these experimental conditions (data not shown). These results suggest that the accumulation of α-synuclein in cells expressing UCH-L1^{I93M} is due to the inhibition of CMA-dependent degradation of α-synuclein. α-Synuclein contains a CMA recognition motif, ^{95}VKKDQ^{99}, and mutant α-synuclein$^{\Delta DQ}$, in which ^{98}DQ^{99} is replaced by Ala-Ala, is not degraded by CMA (24). To confirm that the accumulation of α-synuclein in cells expressing UCH-L1^{I93M} is associated with CMA-dependent degradation of α-synuclein, we used mutant α-synuclein$^{\Delta DQ}$ and found that the I93M mutation does not affect the α-synuclein$^{\Delta DQ}$ level (~1.0 and 1.0-fold increases, respectively) (Fig. 5, *E* and *F*).

G93A Cu,Zn-superoxide dismutase 1 and WT Cu,Zn-superoxide dismutase 1 are not presumable substrates for CMA because Cu,Zn-superoxide dismutase 1 does not contain a KFERQ-like motif, but they can be degraded by the proteasome and macroautophagy (28). Protein levels of G93A Cu,Zn-superoxide dismutase 1 and WT Cu,Zn-superoxide dismutase 1 in cells transfected with UCH-L1^{I93M} were not increased compared with those in cells expressing UCH-L1^{WT} (an ~1.0-fold increase) (Fig. 5*G* and data not shown), suggesting that the I93M mutation does not considerably affect the degradation of proteins by macroautophagy and the proteasome under these experimental conditions.

Contrary to UCH-L1^{I93M}, UCH-L1^{D30K} and UCH-L1^{C90S} did not increase the amount of α-synuclein in cells (supplemental Fig. S2*B*), indicating that the accumulation of α-synuclein in cells expressing UCH-L1^{I93M} is independent of the hydrolase activity of UCH-L1 and the interaction between monoubiquitin and UCH-L1. These observations are consistent with the results showing that the interaction between UCH-L1 and LAMP-2A, Hsc70, or Hsp90 is independent of the enzymatic activity of UCH-L1 and the interaction between monoubiquitin and UCH-L1 (Figs. 1*C* and 3) and also with the idea that the main cause of UCH-L1^{I93M}-associated PD is not a loss of UCH-L1 function but an acquired toxicity of UCH-L1^{I93M}.

DISCUSSION

An increase in the amount of α-synuclein protein could constitute a pathogenic factor underlying sporadic PD because the heterozygous duplication of the α-synuclein gene causes familial PD (4, 5), and the deposition of α-synuclein protein is associated with sporadic PD (7, 8, 37). α-Synuclein^{WT} is a CMA substrate, but mutant A30P and A53T α-synuclein inhibit CMA by aberrant tight binding to LAMP-2A (24). Thus, inhibition of CMA by mutant α-synuclein might result in an increase in the amount of α-synuclein protein, leading to the neurodegeneration in familial PD associated with mutant α-synuclein. To date, the relationships between α-synuclein and other familial PD-

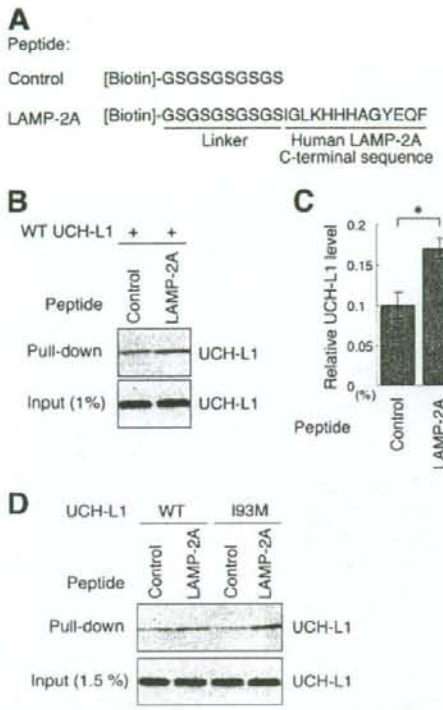

FIGURE 4. **Direct interaction between UCH-L1 and the cytosolic region of LAMP-2A.** *A*, an amino acid sequence of biotin-conjugated peptides is shown. *B* and *C*, 10 μg of recombinant UCH-L1 and 2 nmol of peptides (control or LAMP-2A peptide) were mixed, and a pulldown assay was performed using streptavidin beads. Precipitants were analyzed by immunoblotting (*B*). The levels of UCH-L1 relative to input were quantified by densitometry. Mean values are shown with S.E. ($n = 3$). *, $p < 0.05$. *D*, 10 μg of UCH-L1 (wild-type or I93M) and 2 nmol of peptides (control or LAMP-2A peptide) were mixed, and a pulldown assay was performed.

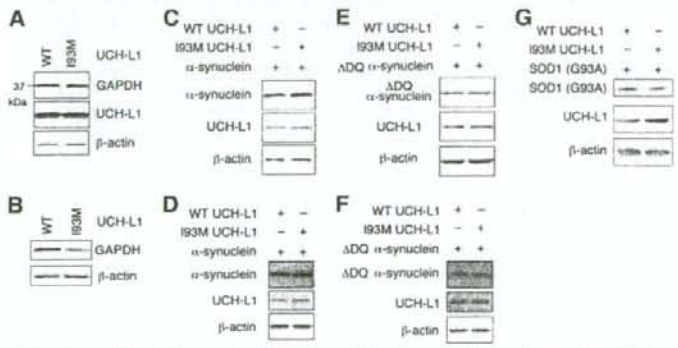

FIGURE 5. **Effects of the I93M mutation of UCH-L1 on CMA and α-synuclein levels.** *A* and *B*, COS-7 cells were transfected with the indicated constructs. Forty-eight h after transfection, whole-cell lysates (*A*) and a lysosomal fraction (*B*) were prepared and analyzed by immunoblotting. *C–G*, COS-7 cells (*C*, *E*, and *G*) or IMR-90 cells (*D* and *F*) were transfected with the indicated constructs. Cell lysates were prepared and analyzed by immunoblotting. Accumulation of α-synuclein^{WT} in cells transfected with UCH-L1^{I93M} was observed in COS-7 (*C*), IMR-90 (*D*), and SH-SY5Y cells (data not shown). The assays were performed at least three times; representative results are shown. *SOD1*, Cu,Zn-superoxide dismutase 1.

associated mutant proteins in the pathogenesis of PD have remained largely unclear. Although it was reported that UCH-L1 polyubiquitinates monoubiquitinated α-synuclein in a cell-free system (20), the relationship between UCH-L1 and non-ubiquitinated α-synuclein in the pathogenesis of PD has also remained unknown.

In the present study, we have shown that familial PD-associated UCH-L1^{I93M} abnormally interacts with LAMP-2A, Hsc70, and Hsp90 and causes an increase in the amounts of α-synuclein and GAPDH, which are CMA substrates, in cultured cells. The increase can be explained by an inhibition of CMA via an aberrant interaction between UCH-L1^{I93M} and CMA machinery because the GAPDH level in the lysosomal fraction was decreased in cells expressing UCH-L1^{I93M} (Fig. 5*B*), and the I93M mutation in UCH-L1 does not affect the levels of α-synuclein$^{\Delta DQ}$, which is not degraded by CMA (Fig. 5, *E* and *F*). These findings suggest that an increase in the amount of α-synuclein protein by inhibition of CMA via the interaction between UCH-L1^{I93M} and CMA machinery underlies one of the causes of familial PD associated with mutant UCH-L1. It is also possible that increases in the amount of other CMA substrates, such as GAPDH, are involved in the pathogenesis of PD. Taken together with a report that pathogenic mutant A30P and A53T α-synuclein exhibit an enhanced interaction with LAMP-2A compared with α-synuclein^{WT} (24), our results indicate that UCH-L1^{I93M} and mutant A30P and A53T α-synuclein share aberrant biochemical properties with respect to their interactions with LAMP-2A. These observations further support the idea that the I93M mutation in UCH-L1 contributes to the pathogenesis of PD.

We revealed that the R63A, E174A, D176A, or H185A substitution in UCH-L1 increases the levels of interactions of UCH-L1 with LAMP-2, Hsc70, and Hsp90 (Fig. 3), suggesting that the surface region containing Arg^{63} and His^{185} in UCH-L1 (35) is involved in its interaction with LAMP-2, Hsc70, and Hsp90. We have previously reported that the R63A or H185A substitution in UCH-L1 enhances the interaction of UCH-L1 with tubulin (13). These results suggest that tubulin, LAMP-2A, Hsc70, and Hsp90 interact with the same region in UCH-L1. Arg^{63} and His^{185} are distinct from Asp^{30}, which is one of the ubiquitin-binding sites (21, 38), and from Cys^{90} (13), which is a catalytic center cysteine residue. We have shown that D30K or C90S mutation in UCH-L1 does not alter its interactions with tubulin (13) and LAMP-2 (Fig. 1*C*). Thus, the interactions of UCH-L1 with tubulin and LAMP-2 are independent of the monoubiquitin-binding and hydrolase activity of UCH-L1.

It is known that the majority of PD cases occur sporadically and that oxidative/carbonyl stresses are

elevated in PD brains (17, 39). In the brains of sporadic PD patients, UCH-L1 is a major target of carbonyl formation (17). We previously reported that carbonyl-modified UCH-L1 and UCH-L1^{I93M} share biochemical properties: both of these UCH-L1 variants display increased insolubility, elevated interactions with multiple proteins including tubulin, and decreased interaction with monoubiquitin compared with UCH-L1^{WT} (13). We have also shown that both carbonyl-modified UCH-L1 and UCH-L1^{I93M} abnormally promote tubulin polymerization (13). Our previous studies using circular dichroism suggest that both of these UCH-L1 variants display decreased α-helix and increased β-sheet content (13, 22, 40). Thus, both carbonyl modification and the I93M mutation in UCH-L1 may alter its conformation, resulting in changes in the biochemical and functional properties of UCH-L1. It is an interesting issue whether carbonyl-modified UCH-L1 can also inhibit CMA. Other than tubulin, LAMP-2A, Hsc70, and Hsp90, UCH-L1 interacts with multiple proteins (13). These other interactors may also be involved in the mechanism of UCH-L1-mediated PD and are currently under investigation. It is also possible that the interaction of Hsc70 or Hsp90 with UCH-L1 plays roles other than in the CMA pathway.

α-Synuclein and UCH-L1 have been reported to be expressed abundantly in dopaminergic neurons in the human brain (41). Thus, UCH-L1^{I93M} is possibly overproduced in dopaminergic neurons in familial PD, leading to an accumulation of α-synuclein and the selective loss of dopaminergic neurons. In conclusion, familial PD-associated mutant UCH-L1^{I93M} physically interacts with LAMP-2A, Hsc70, and Hsp90 and causes an increase in the amount of α-synuclein in cells. We propose that aberrant interaction of mutant UCH-L1 with CMA machinery, at least in part, underlies the pathogenesis of familial PD associated with UCH-L1^{I93M}.

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Article Addendum

Insights into links between familial and sporadic Parkinson's disease

Physical relationship between UCH-L1 variants and chaperone-mediated autophagy

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Abbreviations: UCH-L1, ubiquitin C-terminal hydrolase L1; PD, Parkinson's disease; CMA, chaperone-mediated autophagy; WT, wild-type; LAMP-2, lysosome-associated membrane protein type 2; Hsc70, heat shock cognate protein 70; Hsp90, heat shock protein 90; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAE, 4-hydroxy-2-alkenals; HNE, 4-hydroxy-2-nonenal

Key words: ubiquitin C-terminal hydrolase L1 (UCH-L1), Parkinson's disease, LAMP-2, chaperone-mediated autophagy, α-synuclein

Ubiquitin C-terminal hydrolase L1 (UCH-L1) is expressed abundantly in neurons and has been reported to be a major target of oxidative/carbonyl damage associated with sporadic Parkinson's disease (PD). The I93M mutation in UCH-L1 is also associated with familial PD. We recently reported that UCH-L1 physically interacts with LAMP-2A, the lysosomal receptor for chaperone-mediated autophagy (CMA), and Hsc70 and Hsp90, both of which can function as components of the CMA pathway. We found that the levels of these interactions were aberrantly increased by the I93M mutation, and that expression of I93M UCH-L1 in cells induced the CMA inhibition-associated increase in the amount of α-synuclein, a risk factor for PD. The interactions of UCH-L1 with LAMP-2A, Hsc70 and Hsp90 were also abnormally enhanced by carbonyl modification of UCH-L1. We propose that aberrant interactions of UCH-L1 variants with CMA machinery, at least partly, underlie the pathogenesis of I93M UCH-L1-associated PD, and possibly of sporadic PD. Our findings may provide novel insights into the links between familial and sporadic PD.

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. It is characterized by progressive cell loss of dopaminergic neurons in the substantia nigra pars compacta. A missense mutation in the ubiquitin C-terminal hydrolase L1 (UCH-L1) gene, leading to an I93M substitution at the amino acid residue level, has been reported in a German family with dominantly inherited PD.[1] We have previously shown that I93M UCH-L1-transgenic mice exhibit progressive cell loss of dopaminergic neurons.[2] Compared with wild-type (WT) UCH-L1, I93M UCH-L1 displays increased insolubility and levels of interactions with other proteins in mammalian cells, features that are characteristic of several neurodegenerative disease-linked mutants.[3] These findings suggest that the I93M mutation in UCH-L1 is a causative mutation for PD. Although the binding of I93M UCH-L1 to monoubiquitin as well as the hydrolase activity of I93M UCH-L1 are decreased compared with those of WT UCH-L1,[1,3,4] mice deficient in UCH-L1 do not display obvious dopaminergic cell loss.[5,6] Thus, the main cause of I93M UCH-L1-associated PD may not be a loss of UCH-L1 function but an acquired toxicity of I93M UCH-L1. Our previous studies suggest that aberrantly enhanced physical interactions between I93M UCH-L1 and multiple proteins, including tubulin, underlie the toxic functions of I93M UCH-L1 (Fig. 1).[3]

Several missense mutations in the α-synuclein gene are also linked to dominant-inherited PD.[7-9] α-Synuclein is thought to be a major component of cytoplasmic inclusions called Lewy bodies in the brains of patients with sporadic PD.[10,11] Increases in the levels of α-synuclein could constitute a cause of PD, since duplication and triplication of the α-synuclein gene cause familial PD or parkinsonism.[12-14] α-Synuclein is degraded at least partly by chaperone-mediated autophagy (CMA).[15]

To elucidate the molecular relationship between α-synuclein and UCH-L1 in the pathogenesis of PD, we sought to identify novel UCH-L1-interacting proteins. We found that UCH-L1 interacts with lysosome-associated membrane protein type 2A (LAMP-2A), heat shock cognate protein 70 (Hsc70) and heat shock protein 90 (Hsp90),[16] all of which are components of the CMA pathway.[17] These interactions were enhanced by the I93M mutation in UCH-L1.[16] Expression of I93M UCH-L1 in cells induced the increase in the amount of α-synuclein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH),[16] both of which are substrates of CMA,[15] but had almost no effects on the amount of ΔDQ α-synuclein,[16] which lacks the CMA recognition motif.[15] Based on these results, we propose that the aberrant interaction of I93M UCH-L1 with CMA machinery causes the accumulation of α-synuclein and GAPDH by inhibiting CMA. Besides its role in glycolysis, GAPDH is known to initiate a cell-death cascade.[18] Thus, it is possible that the increases in

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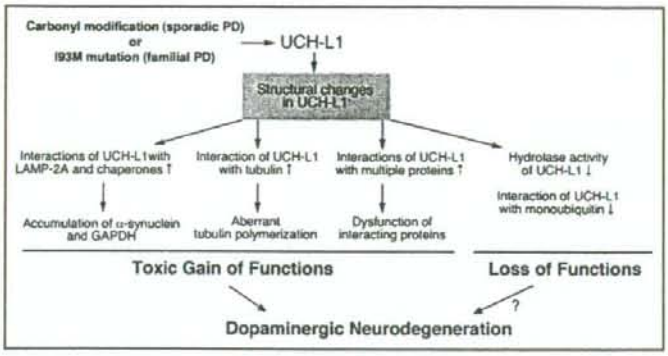

Figure 1. Possible role of UCH-L1 in PD. The I93M mutation (as occurs in familial PD associated with I93M UCH-L1) and carbonyl-modification (as occurs in sporadic PD) cause structural changes in UCH-L1. The hydrolase activity and binding affinity to monoubiquitin of these UCH-L1 proteins are decreased. The involvement of loss of UCH-L1 functions in the pathogenesis of PD is currently unclear. Abnormal UCH-L1 interacts tightly with LAMP-2A, Hsc70 and Hsp90. These abnormal UCH-L1 may inhibit CMA-dependent degradation, and cause CMA substrates including α-synuclein and GAPDH to accumulate. The increased amount of α-synuclein or GAPDH proteins possibly contributes to the neurodegeneration of dopaminergic neurons. The aberrant interactions of UCH-L1 with other proteins, including tubulin, may also contribute to neurodegeneration. ↑: increase compared with wild-type UCH-L1, ↓: decrease compared with wild-type UCH-L1.

the amount of α-synuclein and GAPDH are involved in the pathogenesis of PD (Fig. 1).

Although the majority of PD cases occur sporadically, the molecular mechanisms that underlie the pathology of sporadic PD are poorly understood. It is known that oxidative/carbonyl stresses are elevated in PD brains.[19,20] In the brains of sporadic PD patients, UCH-L1 is a major target of carbonyl formation,[19] which is the most widely used marker for oxidative damage to proteins. Carbonyl groups can be introduced into proteins in vivo mainly by reactions with 2-alkenals, 4-hydroxy-2-alkenals (HAE) or ketoaldehydes,[21,22] which are endogenous aldehyde products formed by lipid peroxidation or glycooxidation. Protein carbonyls can also be produced by metal-catalyzed reactions with H_2O_2 in vitro.[22,23] It has been suggested that 4-hydroxy-2-nonenal (HNE) can accumulate in biological membranes at concentrations of over 10–100 μM in response to oxidative stress.[24,25] In mammalian cells, carbonyl-modified UCH-L1 can be produced by reactions with 10–100 μM HAE or 2-alkenals, but not by 100–500 μM ketoaldehydes or 0.1–1 mM H_2O_2.[3] Furthermore, I93M UCH-L1 and carbonyl-modified UCH-L1 display shared aberrant properties,[3] suggesting that carbonyl-modified UCH-L1 constitutes one of the causes of sporadic PD. Therefore, we tested the effects of carbonyl modification of UCH-L1 on the interaction of UCH-L1 with LAMP-2A and chaperones. We found that HNE modification of UCH-L1 promotes interactions between UCH-L1 and LAMP-2A, Hsc70 or Hsp90 (Fig. 2A). 4-hydroxy-2-hexenal or 2-propenal modification of UCH-L1 have similar effects on the interactions between UCH-L1 and LAMP-2A, Hsc70 or Hsp90 as HNE modification (data not shown). In a pull-down assay, HNE-modified UCH-L1 exhibits an abnormally increased level of interaction with the cytosolic region of LAMP-2A, compared with wild-type UCH-L1 (data not shown). Thus, I93M UCH-L1 and carbonyl-modified UCH-L1 also exhibit common biochemical properties with respect to their interactions with LAMP-2A, Hsc70 and Hsp90. These results support the idea that carbonyl-modified UCH-L1 constitutes one of the causes of sporadic PD (Fig. 1). A coimmunoprecipitation assay using C90S, C132S and C152S UCH-L1 mutants shows less binding of C90S UCH-L1 to LAMP-2A, Hsc70 or Hsp90 than WT UCH-L1, when cells are treated with HNE (Fig. 2B and C). Thus, HNE modification of Cys-90 of UCH-L1 promotes the interactions of UCH-L1 with LAMP-2A, Hsc70 and Hsp90. These results are consistent with our previous data showing that the HAE modification of Cys-90 of UCH-L1 promotes the interactions of UCH-L1 with multiple proteins.[3]

The appearance of HNE-modified proteins in nigral neurons is associated with sporadic PD.[26,27] We have previously observed that cysteine residues in UCH-L1 are main targets for HNE-modification of UCH-L1.[3] UCH-L1 is modified by 10–100 μM HNE, whereas α-synuclein, which contains no cysteine residues, is not modified by 100 μM HNE in mammalian cells,[3] suggesting that, in mammalian cells, HNE reacts with proteins mainly via cysteine residues. It is possible that, in sporadic PD, cysteine residue-reactive carbonyl stresses such as HAE result in the accumulation of α-synuclein, not by direct modification of α-synuclein, but via reaction with UCH-L1.

In conclusion, aberrant interactions of UCH-L1 variants with multiple proteins, including CMA machinery, may underlie the pathogenesis of I93M UCH-L1-associated PD, and possibly of sporadic PD. We propose that carbonyl modification of UCH-L1 can be a therapeutic target for the treatment of sporadic PD.

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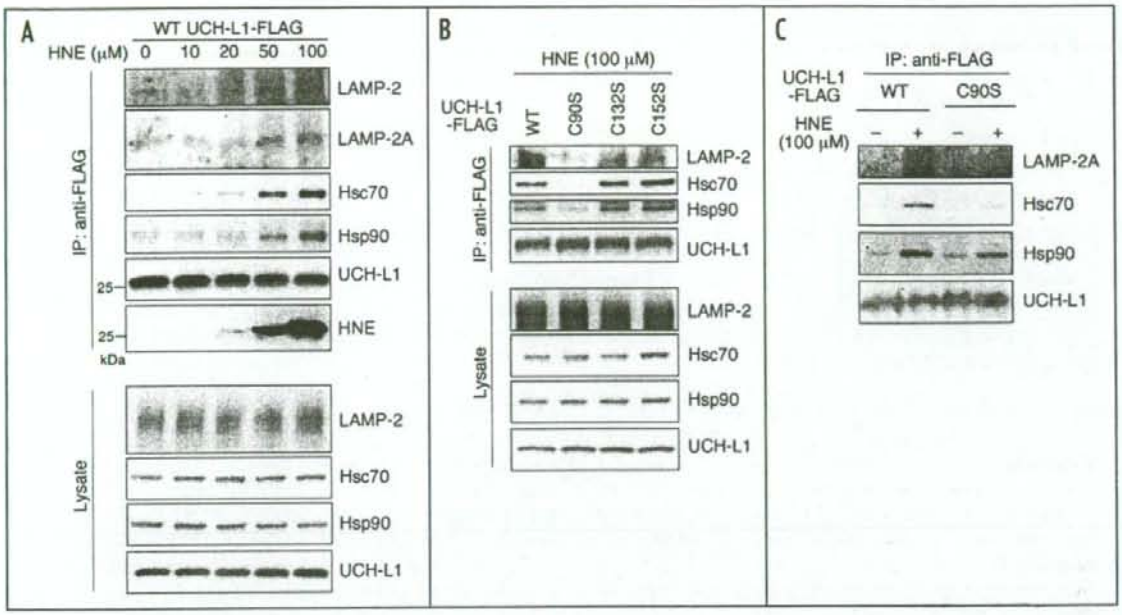

Figure 2. Effects of HNE-modification of UCH-L1 on the interactions of UCH-L1 with LAMP-2A, Hsc70 and Hsp90 (A) COS-7 cells transfected with FLAG-tagged WT UCH-L1 were treated with the indicated concentrations of HNE. Lysates were immunoprecipitated with anti-FLAG antibody, and analyzed by immunoblotting using anti-LAMP-2, LAMP-2A, Hsc70, Hsp90, HNE and FLAG antibodies. (B and C) COS-7 cells transfected with the indicated constructs were treated with or without 100 μM HNE. Lysates were immunoprecipitated using anti-FLAG antibody, and analyzed by immunoblotting.

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