Deubiquitinating enzymes (DUBs) are negative regulators of protein ubiquitination and play an important role in ubiquitin-dependent processes. Recent studies have found that diverse cellular mechanisms are employed to control the activity of DUBs. Ubiquitin C-terminal hydrolase-L1 (UCH-L1) is a highly expressed neuronal DUB linked to Parkinson disease; however, little is known about its specific functions or modes of regulation. Here, we demonstrate that UCH-L1 is post-translationally modified by monoubiquitin in cells, at lysine residues near the active site. This modification restricts enzyme activity by preventing binding to ubiquitinated targets, and permanent monoubiquitination, as mimicked by a ubiquitin-UCH-L1 fusion, inhibits UCH-L1 in its capacity to increase free ubiquitin levels in cells. Interestingly, UCH-L1 catalyzes its own deubiquitination in an intramolecular manner, thereby regulating the lifetime of this modification. Our results illustrate monoubiquitination as a reversible regulatory mechanism for DUB activity involving auto-deubiquitination.

Protein ubiquitination is a central regulator in numerous cellular processes, including protein degradation, cell cycle progression, and transcriptional regulation (1). The enzymatic system for conjugating ubiquitin to protein substrates is well characterized and results in conjugation of ubiquitin to the ε-amino group of lysine residues or in some cases to the N-terminal amino group of target proteins (2, 3). Proteins can be modified by monomeric ubiquitin (termed monoubiquitination) or by ubiquitin chains formed by conjugation of additional ubiquitin molecules to various lysines within ubiquitin (referred to as polyubiquitination). The number and conformation of appended ubiquitin molecules determine the fate of the target protein. While Lys-48-linked polyubiquitin chains typically target proteins for proteasomal degradation, monoubiquitination at one or more lysines triggers a variety of effects depending on the substrate protein, including endocytosis, gene silencing, and DNA repair (4, 5). Our understanding of the many functions associated with ubiquitination continues to grow with the identification of additional protein substrates targeted by this versatile modification.

Like protein phosphorylation, ubiquitination is reversible, and deubiquitination plays an important role in regulating ubiquitin-dependent pathways. Removal of ubiquitin is accomplished by DUBs, a diverse class of nearly 80 enzymes in humans, which cleave ubiquitin from proteins, peptides, or small molecules (6, 7). Although specific substrates have been identified for relatively few DUBs, the importance of this enzyme class is highlighted by recent studies which reveal tight regulation of their activity. Transcriptional regulation, protein-protein interactions, and post-translational modifications are known to regulate DUB activity (7, 8). For example, the DUB USP1 (ubiquitin-specific protease-1) is inactivated by a UV irradiation-induced auto-cleavage event, allowing for accumulation of monoubiquitinated PCNA (proliferating cell nuclear antigen) and activation of DNA repair (9). In addition, transient phosphorylation inactivates the DUB CYLD (cylindromatosis gene product), which functions as a tumor suppressor by deubiquitinating key signaling molecules such as TRAF2 (tumor necrosis factor receptor-associated factor 2) (10). These examples demonstrate the importance of post-translational modifications in the regulation of DUB activity.

UCH-L1 is a 223-amino acid, primarily neuronal-specific member of the ubiquitin C-terminal hydrolase (UCH) family of DUBs, and is involved in several neurodegenerative diseases and cancer. UCH-L1 is present in neuronal inclusions associated with Parkinson disease (PD), Alzheimer disease (AD), and Lewy body disease (11, 12); and a common S18Y polymorphism in UCH-L1 is associated with reduced risks of PD and, in females, AD (13–15). In addition, UCH-L1 overexpression occurs in many cancers, including lung and colorectal carcinoma, and may be related to tumor progression (16, 17). Despite its importance in disease, little is known about the normal function of UCH-L1 or its mode of regulation. Physiological substrates are unknown, but in vitro studies suggest that UCHs are involved in the cleavage of ε-amino linked peptides or small molecules from the C terminus of ubiquitin,

---

*This work was supported by National Institutes of Health Grant R21NS047420 and United States Army Department of Defense Grant W81XWH-04-1-0441/04033002. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

* This article was selected as a Paper of the Week.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–6.

‡ To whom correspondence should be addressed: Center for Neurologic Diseases, 65 Landsdowne St., Fourth Floor, Cambridge, MA 02139. Tel.: 617-768-8610; Fax: 617-768-8606; E-mail: plansbury@rics.bwh.harvard.edu.

---

2 The abbreviations used are: DUB, deubiquitinating enzyme; AD, Alzheimer disease; Ni-NTA, nickel-nitrilotriacetic acid; PBS, phosphate-buffered saline; PD, Parkinson disease; UBD, ubiquitin binding domain; UCH, ubiquitin C-terminal hydrolase; UCH-L1, ubiquitin C-terminal hydrolase-L1; WT, wild-type; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; UBB, ubiquitin binding buffer; PCNA, proliferating cell nuclear antigen.
and in the co-translational processing of pro-ubiquitin and ribosomal proteins translated as ubiquitin fusions (18). In vivo studies suggest that UCH-L1 is involved in regulating ubiquitin homeostasis, apoptosis, and learning and memory; however, the precise role of UCH-L1 in these processes is not clear (19–22). As one of the most abundant neuronal proteins, estimated to comprise 1–2% of soluble brain protein (23), it is likely that UCH-L1 activity is regulated at the post-translational level.

In our efforts to understand the regulation of UCH-L1 activity, we have identified and characterized a monoubiquitinated species of UCH-L1, which to our knowledge represents the first example of a monoubiquitinated UCH enzyme. We show that monoubiquitination inhibits ubiquitin binding in vitro and prevents UCH-L1 from regulating free ubiquitin levels in cells. Monoubiquitination of the enzyme is dependent on its ubiquitin binding site, which directly interacts with the conjugated ubiquitin. Furthermore, the stability of this modification is governed by an auto-deubiquitination reaction, in which ubiquitin is cleaved from UCH-L1 in an intramolecular manner. We discuss the implications of these findings in the context of UCH-L1 structure and function and the emerging role of monoubiquitination in regulating the machinery of the ubiquitin pathway.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—MG-132 and lactacystin were obtained from Calbiochem. Ni-NTA-agarose beads and plasmid purification kits were purchased from Qiagen. Electrophoresis and transfer reagents were purchased from Invitrogen. Other chemicals and reagents were purchased from Sigma.

**Plasmid Construction**—All plasmids were constructed using pCDNA3.1zeo (+) (Invitrogen) as vector. PCR mutagenesis and standard molecular biology techniques were used to generate the following constructs: N-terminally His6-tagged UCH-L1 (epitope = MGHHHHHHHGS), N-terminally FLAG-tagged ubiquitin (epitope = MDYKDDDK), and ubiquitin-UCH-L1 fusions. Single and double amino acid changes were generated by standard PCR mutagenesis as described previously (24) using PfuTurbo DNA polymerase (Stratagene). Multiple amino acid mutations were generated using the QuikChange multisite-directed mutagenesis kit (Stratagene). Templates for PCR and subcloning were human UCH-L1 and human ubiquitin. Restriction enzymes, DNA ligase, and dNTP mix were purchased from New England Biolabs. XL-10 Gold Ultracompetent cells were purchased from Stratagene.

**Cell Culture and Transfection**—COS-7 cells were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, l-glutamine, and penicillin-streptomycin. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Tissue culture media and reagents were purchased from Invitrogen and ATCC.

**Pulldown Assays**—COS-7 cells were transfected with His-tagged UCH-L1 constructs in 12-well plates. 24–48 h post-transfection, cells were washed with PBS, resuspended in denaturing lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 1% Nonidet P-40, 8 mM urea, pH 8), sonicated briefly on ice using a Fisher Scientific sonic dismembrator model 500 with microtip attachment, and incubated with Ni-NTA beads for 1–2 h at 4 °C. Beads were washed with His tag wash buffer (50 mM NaH2PO4, 500 mM NaCl, 20 mM imidazole, 0.1% Nonidet P-40, pH 8), and protein was eluted using SDS-PAGE sample buffer and loaded onto gels or stored at −20 °C until analysis by Western blot.

**Western Blots and Immunostaining**—Cell lysates or protein samples were separated by SDS-PAGE using NuPAGE 4–12% Bis-Tris gels, transferred to polyvinylidene difluoride membranes, subjected to immunoblotting, and detected by enhanced chemiluminescence (Amersham Biosciences). The following primary antibodies were used: UCH-L1 (AB1761) and actin (MAB1501R) from Chemicon; ubiquitin (MMS-257P) from Covance, and parkin (2132) from Cell Signaling. Anti-rabbit (W401B) and anti-mouse (W402B) secondary antibodies conjugated to horseradish peroxidase were purchased from Promega. Densitometric analysis was performed using the public domain NIH Image program (rsb.info.nih.gov/nih-image/). For fluorescence immunostaining, cells were grown on #1.5 glass coverslips (Warner Instruments) and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) overnight at 4 °C or in 70% ethanol at −20 °C for at least 20 min. After washing in PBS, cells were immunostained with anti-His (sc-803, Santa Cruz Biotechnology), anti-ubiquitin (MMS-257P, Covance), and anti-UCH-L1 (AB1761, Chemicon) antibodies in PBS, 1% bovine serum albumin, 0.3% Triton-X100. Rhodamine red anti-rabbit (111-295-144) and Cy-2 anti-mouse (115-225-146) from Jackson ImmunoResearch were used as secondary antibodies. Coverslips were mounted on slides with Prolong gold anti-fade reagent with DAPI (Molecular Probes), prior to analysis using a Nikon Eclipse E600 microscope equipped with Y-FL epifluorescence and differential interference contrast attachments and SPOT RT-Slider digital camera and software.

**Purification of Ubiquitinated UCH-L1**—COS-7 cells were co-transfected with plasmids encoding His-tagged UCH-L1 and FLAG-tagged ubiquitin. 24–48 h post-transfection, cells were rinsed with PBS and resuspended in native lysis buffer (50 mM NaH2PO4, 1% Tween 20, 500 mM NaCl, pH 7.4), sonicated at 15% amplitude for 3 × 15 s, followed by centrifugation for 10 min at 10,000 × g. Supernatants were incubated overnight with EZview Red anti-FLAG M2 affinity gel beads (Sigma). Beads were washed with lysis buffer, and protein was eluted by incubation with 200 μg/ml 3× FLAG peptide (Sigma) in His tag wash buffer for 2–4 h. Eluate was incubated with Ni-NTA beads for 1–2 h, and beads were washed with His tag wash buffer and eluted in appropriate buffer for analysis by either mass spectrometry or in vitro deubiquitination assay. For mass spectrometric analysis, one to three 15-cm plates of cells were used per sample, and protein was eluted from Ni-NTA beads using SDS-PAGE sample buffer. Samples were run on SDS-PAGE and stained with Colloidal Blue staining kit (Invitrogen) prior to excision of bands for analysis by microcapillary liquid chromatography-tandem mass spectrometry at the Taplin Biological Mass Spectrometry Facility at Harvard Medical School. For in vitro deubiquitination assays, one 10-cm plate of cells was used per sample, lysis buffer was supplemented with 0.5 mM ubiquitin (Sigma U6253) to displace non-covalently associated FLAG-ubiquitin from UCH-L1, and protein was eluted...
from Ni-NTA beads using UCH enzyme assay buffer supplemented with imidazole (40 mM Tris-Cl, 250 mM imidazole, 0.5 mM EDTA, 5 mM dithiothreitol, pH 8). All lysis and purification steps were performed on ice or at 4°C for both procedures.

Ubiquitin Binding Assays—COS-7 cells were transiently transfected with UCH-L1 expression plasmids and were harvested 24–48 h post-transfection. For ubiquitin binding assays using purified His-UCH-L1, cells were resuspended in native lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 1% Nonidet P-40, pH 8) and sonicated briefly on ice. Following incubation of lysates with Ni-NTA beads at 4°C for 1–2 h, beads were washed, and proteins were eluted using ubiquitin binding buffer (UBB) (50 mM NaH₂PO₄, 1 mM β-mercaptoethanol, 0.05% Tween, pH 8) supplemented with 250 mM imidazole. Eluates were diluted in UBB and incubated with ubiquitin-agarose beads for 2 h at 4°C. Following incubation, unbound fraction was set aside, beads were washed, and bound proteins were eluted in SDS-PAGE sample buffer. Unbound fraction was diluted in UBB supplemented with 300 mM NaCl and incubated with Ni-NTA beads for 1–2 h. Ni-NTA beads were washed, and bound proteins were eluted using SDS-PAGE sample buffer, prior to analysis by Western blotting. For ubiquitin binding assays performed directly with cell lysates, cells were lysed in UBB, sonicated briefly on ice, and incubated with ubiquitin-agarose beads. Beads were washed, and protein was eluted in SDS-PAGE sample buffer.

Statistical Analysis—Statistical analysis was performed using MINITAB statistical software.

RESULTS

UCH-L1 Is Monoubiquitinated in Cell Culture—To identify post-translational modifications of UCH-L1, we undertook studies of UCH-L1 transiently transfected in COS-7 monkey kidney cells, which express only very low levels of UCH-L1 endogenously. In Western blots of transfected cell lysates, the majority of UCH-L1 migrated at the expected mobility of 25 kDa; however, long exposures revealed an additional 33-kDa species (Fig. 1A). This modified form of UCH-L1 was stable to SDS, reducing agents, and boiling (data not shown), suggesting a covalent linkage, and the shift in mobility implied addition of a protein modifier such as ubiquitin. To determine whether the modified species was monoubiquitinated UCH-L1, we performed nickel-affinity pulldown assays using Ni-NTA beads, from cells expressing His-tagged wild-type UCH-L1 (His-UCH-L1⁵WT). A catalytically inactive mutant of UCH-L1, in which the active site cysteine is mutated to serine (His-UCH-L1C⁹⁰S), was also analyzed because it exhibited higher levels of inhibition, transfected cells were treated with lactacystin or MG-132, and pulldown assays were performed. Although high molecular weight ubiquitinated proteins accumulated in total cell lysates of lactacystin- and MG-132-treated cells, UCH-L1 did not accumulate, and no high molecular weight forms of UCH-L1 were detected (Fig. 1C). These results suggest that monoubiquitination of UCH-L1 is unrelated to the formation of polyubiquitin chains for proteasomal degradation and may serve as a novel regulatory mechanism for UCH-L1.

Monoubiquitination Inhibits Ubiquitin Binding by UCH-L1—We next wished to determine the effect of monoubiquitination on UCH-L1 activity. Substrate recognition by UCHs is mediated by a ubiquitin binding site on the enzyme (25, 26), and UCH-L1 binds free ubiquitin in brain and cell lysates (19). To determine whether monoubiquitination affects the ability of the enzyme to bind ubiquitin, we performed ubiquitin binding assays with unmodified and monoubiquitinated His-UCH-L1⁵WT and His-UCH-L1C⁹⁰S purified from COS-7 cells. In contrast to unmodified UCH-L1, monoubiquitinated UCH-L1 did not appreciably bind ubiquitin-agarose beads (Fig. 2A). We hypothesized that inhibition of ubiquitin binding may result from the conjugated ubiquitin occupying the ubiquitin binding site of UCH-L1. If so, disrupting the interaction between conjugated ubiquitin and the ubiquitin binding site should restore binding between free ubiquitin and the enzyme. To test this hypothesis, we first generated a monoubiquitin-UCH-L1 translational fusion protein, Ub⁶⁰⁷—UCH-L1, in which
the C terminus of ubiquitin was linked to Lys-4 of UCH-L1, and the last two amino acids of ubiquitin were mutated from glycine to valine (G75,76V) to prevent cleavage by endogenous DUBs (27) (Fig. 2B). To determine whether UbVV-UCH-L1 effectively mimics post-translationally ubiquitinated UCH-L1, we confirmed that UbVV-UCH-L1 was deficient in ubiquitin binding (Fig. 2C). To address the mechanism of this inhibition, and to concurrently control for the possibility that inhibition of ubiquitin binding by UbVV-UCH-L1 was due to improper folding, we generated a second fusion protein, UbVV/L8A-UCH-L1, which contains an L8A mutation in the ubiquitin moiety of this fusion protein. The L8A mutation, which disrupts binding of ubiquitin to UCH-L1 in vitro (28), was introduced to prevent interaction of the translationally fused ubiquitin with the ubiquitin binding site of UCH-L1. In contrast to UbVV-UCH-L1, UbVV/L8A-UCH-L1 efficiently bound ubiquitin-agarose beads (Fig. 2C), suggesting that the fusion proteins are properly folded and, furthermore, that monoubiquitinated UCH-L1 is unable to interact with ubiquitinated targets or free ubiquitin because conjugated ubiquitin occupies the ubiquitin binding site of the enzyme.

We next wished to determine whether the lack of ubiquitin binding exhibited in vitro by monoubiquitinated UCH-L1 would affect UCH-L1 function in cells. It has been shown that UCH-L1 variants capable of binding ubiquitin increase monoubiquitin levels in cultured cells and neurons in vivo (19). We tested this effect in COS-7 cells and found that expression of His-UCH-L1WT or His-UCH-L1C90S (19) represents positive and negative controls, respectively, for ubiquitin binding. Bands marked by an asterisk represent endogenous UCH-L1, and bands marked by an open arrowhead may represent monoubiquitinated fusion protein.

### FIGURE 2. Monoubiquitination prevents UCH-L1 from binding ubiquitin. A, His-UCH-L1WT and His-UCH-L1C90S were expressed in COS-7 cells and isolated by nickel-affinity purification under native conditions. The purified proteins, which contained unmodified and monoubiquitinated UCH-L1, were incubated with ubiquitin-agarose beads to assess ubiquitin binding. Bound and unbound proteins were analyzed by Western blot (WB) using antibodies for UCH-L1 and ubiquitin. Input lanes represent a portion of purified His-UCH-L1 proteins prior to incubation with ubiquitin-agarose beads. B, schematic representation of His-UCH-L1 and ubiquitin-UCH-L1 fusion constructs for ubiquitin binding studies. C, lysates from COS-7 cells expressing the indicated plasmids were incubated with ubiquitin-agarose beads to compare ubiquitin binding by UCH-L1 variants. Ubiquitin-agarose pulldowns (top panel) and total cell lysates (bottom panel) were analyzed by Western blot for UCH-L1. His-UCH-L1WT and His-UCH-L1L8A (19) represent positive and negative controls, respectively, for ubiquitin binding. Bands marked by an asterisk represent endogenous UCH-L1, and bands marked by an open arrowhead may represent monoubiquitinated fusion protein.

### FIGURE 3. Monoubiquitinated UCH-L1 fails to increase monoubiquitin level in COS-7 cells. Lysates of COS-7 cells expressing the indicated plasmids were analyzed by Western blot (WB) using antibodies for ubiquitin, UCH-L1, and actin. Densitometric analysis was performed to quantify band intensities, and monoubiquitin level normalized to actin was calculated for each variant and expressed relative to vector control. The asterisk indicates p < 0.05 against vector control as determined by single factor ANOVA with post hoc Dunnett’s test. Data represent mean ± S.E. Solid line through immunoblot demarcates non-adjacent lanes from the same Western blot. The minor band in the UCH-L1 blot may represent a degradation product of transfected UCH-L1.

UbVV/L8A-UCH-L1 efficiently bound ubiquitin-agarose beads (Fig. 2C), suggesting that the fusion proteins are properly folded and, furthermore, that monoubiquitinated UCH-L1 is unable to interact with ubiquitinated targets or free ubiquitin because conjugated ubiquitin occupies the ubiquitin binding site of the enzyme.
capable of impairing functions of UCH-L1 that are dependent on ubiquitin binding, both in vitro and in a cellular system.

Auto-deubiquitination Regulates the Monoubiquitination of UCH-L1—Although permanent monoubiquitination rendered the enzyme deficient in a cellular assay of UCH-L1 function, the stability of this modification on UCH-L1 was not clear. Because the C90S mutation increased cellular levels of monoubiquitinated UCH-L1, we hypothesized that UCH-L1 may deubiquitinate itself. To examine this idea, we first tested whether a separate crippling active site mutation, H161D (29), would also increase levels of monoubiquitinated UCH-L1. Pulldown assays from COS-7 cells revealed that whereas an estimated 5–10% of total His-UCH-L1WT was monoubiquitinated, 30% of both His-UCH-L1H161D and His-UCH-L1C90S were monoubiquitinated (Fig. 4A), thus confirming that inhibition of catalytic activity is associated with higher levels of monoubiquitinated UCH-L1 in cells. To determine whether this could be explained by auto-deubiquitination of the active enzyme, an in vitro deubiquitination assay was performed using FLAG-monoubiquitinated His-UCH-L1 (Ub-UCH-L1) purified from COS-7 cells. While Ub-UCH-L1WT was hydrolyzed almost completely to the unmodified form, no hydrolysis of Ub-UCH-L1C90S was observed (Fig. 4B, left and center panels), indicating that enzymatically active UCH-L1 is required for the reaction and that hydrolysis was not caused by contaminating DUBs or nonspecific degradation. Furthermore, addition of iodoacetamide, a cysteine protease inhibitor, to the reaction prevented deubiquitination (supplementalFig. 1). We next wished to determine whether deubiquitination occurs intramolecularly, within a single molecule of ubiquitinated UCH-L1, or intermolecularly, whereby one molecule of ubiquitinated UCH-L1 is hydrolyzed by a second molecule of UCH-L1. No hydrolysis was observed when the in vitro deubiquitination assay was repeated with Ub-UCH-L1C90S in the presence of recombinant UCH-L1WT (Fig. 4B, rightmost panel), suggesting that UCH-L1 cannot hydrolyze monoubiquitinated UCH-L1 in an intermolecular manner.

To further test this idea, we examined whether additional UCH-L1WT would affect monoubiquitinated UCH-L1 levels in a cellular system. Reflecting the in vitro results, co-expression of untagged UCH-L1WT had no effect on levels of monoubiquitinated His-UCH-L1WT or His-UCH-L1C90S in COS-7 cells (Fig. 4C).

Monoubiquitination Targets Residues Near the UCH-L1 Active Site—To determine how intramolecular deubiquitination would be feasible on a structural level, we analyzed the ubiquitination sites of UCH-L1. Monoubiquitinated His-UCH-L1WT and His-UCH-L1C90S were purified from COS-7 cells and analyzed for post-translational modifications by microcapillary liquid chromatography-tandem mass spectrometry. Ubiquitination site analysis was conducted using mass spectrometry, which allowed for the identification of modified lysines in both proteins: Lys-4, Lys-65, Lys-71, and Lys-157 (supplemental Fig. 1A).
Reversible Monoubiquitination Regulates UCH-L1 Activity

FIGURE 5. UCH-L1 is monoubiquitinated at lysines near the active site. A, location of ubiquitination sites, as detected by mass spectrometric analysis, and their surrounding regions of secondary structure are shown in a model of UCH-L1 with ubiquitin bound to the active site, generated from the co-crystal structure of UCH-L3 with ubiquitin-vinyl methyl ester (26). UCH-L1 is shown in gray and ubiquitin in brown. Lys-4 and the N-terminal loop are shown in blue; Lys-65 and Lys-71 and surrounding helical region are shown in magenta; and Lys-157 and the active site crossover loop are shown in gold. The catalytic cysteine (Cys-90) is indicated in green space-filling format. This image was generated using PyMOL 8. B, Lys → Arg mutants of His-UCH-L1WT (left panel) and His-UCH-L1C90S (right panel) were expressed in COS-7 cells, and cell lysates were subjected to pulldown assays followed by Western blotting for UCH-L1. Single Lys → Arg mutants are shown in lanes 2–5 and 11–14, while multiple Lys → Arg mutants are shown in lanes 6–9 and 15–18. SKR = K4/64/65/71/157R; 8KR = SKR + K83/131/135R; for Cys-90, 15KR = all Lys → Arg except Lys-221; for C90S, 15KR = all Lys → Arg except Lys-199; ΔK = all Lys → Arg.

FIGURE 6. Ubiquitin binding is required for monoubiquitination of UCH-L1. A and B, the indicated UCH-L1 variants were expressed in COS-7 cells and analyzed for monoubiquitination level by pulldown assay and Western blotting (WB) for UCH-L1. Densitometric analysis of monoubiquitinated and unmodified protein bands was used to estimate the amount of monoubiquitinated UCH-L1. Data represent mean ± S.D.

Figs. 2 and 3). As shown in Fig. 5A, Lys-4 is located in a flexible N-terminal loop which passes over the active site of UCH-L1; Lys-65 and Lys-71 are positioned in a region containing an α-helix spanning residues 57–69, immediately adjacent to the active site cleft; and Lys-157 is located in a flexible loop (residues 148–159) which crosses directly over the active site of UCH-L1. Lys-4, Lys-65, Lys-71, and Lys-157 are distanced 17, 23, 24, and 14 Å, respectively, from the active site thiol in the unliganded structure of UCH-L1 (30). To determine the relative importance of each ubiquitination site and whether additional sites exist, a panel of single and multiple lysine to arginine mutants of UCH-L1 were generated and analyzed for ubiquitination level in COS-7 cells. Because mutation of ubiquitination sites may affect both the rates of ubiquitin conjugation (by a ubiquitin ligase) and deconjugation (by UCH-L1), we analyzed the effect of mutations in both catalytically active and inactive (C90S) UCH-L1, the latter of which should only reflect changes in the rate of ubiquitin conjugation. We found that the only single lysine mutant to noticeably reduce monoubiquitination level was C90S/K157R (Fig. 5B, lane 14), suggesting that Lys-157 may be the preferred ubiquitination site. Mutation of all four detected sites, plus an additional adjacent residue, Lys-64, (SKR, Fig. 5B, lanes 6 and 15), failed to eliminate ubiquitination of UCH-L1, indicating that alternate sites are possible, but their relevance under physiological conditions is unclear. Mutation of all 16 lysines in UCH-L1 to arginine (ΔK, Fig. 5B, lanes 9 and 18) greatly reduced, but did not completely eliminate, monoubiquitination, suggesting that N-terminal ubiquitination of UCH-L1 may occur. It should be noted that binding of ΔLys mutants to ubiquitin was preserved (supplemental Fig. 4), suggesting that these mutants are properly folded. Furthermore, ΔLys was comparable with WT in the monoubiquitination level assay (Fig. 3). Overall, these results suggest that the major ubiquitination site(s) of UCH-L1 are located near the active site and are thus consistent with an intramolecular auto-deubiquitination reaction.

Ubiquitin Binding Is Required for Monoubiquitination of UCH-L1—Finally, we wished to examine how properties of UCH-L1, other than auto-deubiquitination, might affect its monoubiquitination level in cells. Two mutations which impair ubiquitin binding, D30K and D176N (29), virtually eliminated the monoubiquitination of UCH-L1, even in the C90S background (Fig. 6A). The protective S18Y polymorphism, which has no effect on hydrolytic activity (31), did not affect cellular levels of monoubiquitinated UCH-L1. In contrast, an I93M mutation, which reduces catalytic activity by 50% and was identified in a kindred with familial PD (31, 32), approximately doubled the monoubiquitination level of UCH-L1 (Fig. 6B). These findings indicate that a functional ubiquitin binding site is necessary to initiate ubiquitination of UCH-L1 and suggest that catalytic activity level of the enzyme is a major determinant of the stability of this modification and of total cellular levels of monoubiquitinated UCH-L1.

Subcellular Localization of Monoubiquitinated UCH-L1—In some cases, monoubiquitination alters subcellular localization of target proteins (4, 33). However, no effects of this modifica-
Reversible Monoubiquitination Regulates UCH-L1 Activity

The ubiquitin hydrolase UCH-L1 is one of the most abundant brain proteins, and is associated with several neurodegenerative diseases and cancer, yet its specific functions in vivo are poorly understood. In an effort to identify regulatory post-translational modifications, which may shed light on UCH-L1 function, we discovered a reversible modification, monoubiquitination, which has the capacity to negatively regulate UCH-L1 by preventing binding to ubiquinated targets or free ubiquitin.

The discovery that UCH-L1 is monoubiquitinated adds to the rapidly growing number of proteins known to be targeted by this modification and provides the first example of a UCH enzyme that is monoubiquitinated. The best characterized class of monoubiquitinated proteins is a subset of modular ubiquitin-binding proteins that undergo monoubiquitination in a manner dependent on their ubiquitin binding domains (UBDs) (34, 35). The similarities between monoubiquitination of UBD-containing proteins and that of UCH-L1 are striking: only a small fraction of the total expressed protein is ubiquitinated; ubiquitin binding is required for their monoubiquitination; and the conjugated ubiquitin interacts with the ubiquitin binding site of the protein in an intramolecular manner. These parallels may suggest that monoubiquitination of UCH-L1 and UBD-containing proteins occur through similar mechanisms.

To our knowledge, UCH-L1 is the first example of a DUB that is monoubiquitinated in a manner dependent on its interaction with ubiquitin. At least one other DUB, TRE17, is monoubiquitinated, and its ubiquitination occurs in a calcium-dependent manner and is dependent upon its interaction with calmodulin (36). In addition to DUBs and ubiquitin-binding proteins, ubiquitin-conjugating enzymes are targets of monoubiquitination as well. It was recently reported that UBE2T, an E2 ligase in the Fanconi anemia pathway, is inactivated by auto-monoubiquitination (37). It will be important to determine whether monoubiquitination is a common regulatory mechanism for DUBs and other machinery of the ubiquitin pathway and how this modification is related to the function of these proteins.

Our studies indicate that the lifetime of ubiquitin modification on UCH-L1 is regulated by auto-deubiquitination. Experimental evidence supports an intramolecular reaction, which in addition to being entropically favorable to an intermolecular reaction, is reasonable based on UCH structure and substrate specificity. While the distances of the scissile bonds from the active site thiol imply that significant structural flexibility is needed to support intramolecular deubiquitination, protein motions of this magnitude are not uncommon (38–40). Furthermore, Lys-157 and Lys-4 of UCH-L1 are located in flexible regions that undergo significant conformational change upon ligand binding by UCHs (25, 26). Deubiquitination from Lys-65 and Lys-71, located in a helical region adjacent to the active site, could be facilitated by hinge-type movement of this helix toward the active site. In addition, intramolecular deubiquitination appears favorable to an intermolecular reaction due to the lack of a leaving group. In vitro studies suggest that UCHs prefer small or unstructured leaving groups C-terminal to ubiquitin (18), and an active site crossover loop and narrow substrate binding pocket appear to exclude larger, tightly folded proteins as substrates (25, 26, 30). These structural constraints would have little impact on an intramolecular reaction, which would not require the enzyme active site to accommodate an additional molecule of UCH-L1.

Cellular levels of monoubiquitinated UCH-L1 are likely determined by the balance of its ubiquitination and deubiquitination, as is the case with other proteins such as p53 and PCNA (9, 33). Although the proportion of monoubiquitinated UCH-L1 is small in our experimental system, it is possible that UCH-L1 monoubiquitination is increased under certain conditions in vivo. For example, auto-deubiquitination could be inhibited by endogenous inhibitors of UCH-L1. UCH-L1 is a target of oxidative modifications in AD and PD brain (41), and its activity is inhibited in vitro by 4-hydroxynonenal, an endogenous mediator of oxidative damage and cell death (42). Therefore, it is possible that oxidative stress in vivo could lead to increased stability and abundance of monoubiquitinated UCH-L1. In addition to regulation by auto-deubiquitination, it will be interesting to understand what directly promotes the monoubiquitination of UCH-L1. While UCH-L1 has been shown to exhibit ubiquitin ligase activity in vitro (31), the fact that catalytically inactive UCH-L1 is ubiquitinated precludes the possibility of UCH-L1 ubiquitinating itself. Rather, it is likely that ubiquitin bound to an E2 or E3 ligase is recruited to the ubiquitin binding site of UCH-L1. Such a mechanism has been proposed for UBD-containing proteins (43) and is consistent with the necessity of a functional ubiquitin binding surface for monoubiquitination. Ubiquitin ligase interacting partners have not yet been detected for UCH-L1 by yeast two-hybrid screens (44), and we found that co-expression of the PD-linked E3 ligase parkin had no effect on monoubiquitination level of UCH-L1 in COS-7 cells (supplemental Fig. 6). It will be interesting to determine which ubiquitin-conjugating enzyme is responsible for the ubiquitination of UCH-L1 and whether physiological or pathological triggers of UCH-L1 ubiquitination exist.

In discerning the function of UCH-L1 monoubiquitination in vivo, a key aspect may be the reversible nature of this modification. Therefore, it is interesting to speculate how transient covalent monoubiquitination of UCH-L1 may affect its cellular functions. Kinetics of ubiquitination can be critical in determining the functional effects of this modification. For example, ubiquitination and deubiquitination of β-arrestin2 mediates its association and dissociation, respectively, with G protein-coupled receptors and facilitates their internalization (45). Different receptor subtypes trigger either transient (<15 min) or stable ubiquitination of β-arrestin2, leading to contrasting outcomes in their endocytic trafficking and in downstream activation of ERK (extracellular signal-regulated kinase) (45, 46). It is possible that transient monoubiquitination of UCH-L1 facilitates (yet unidentified) protein-protein interactions, which would then be subject to regulation by auto-deubiquitination.

3 R. K. Meray and P. T. Lansbury, unpublished observations.
Reversible Monoubiquitination Regulates UCH-L1 Activity

nation. Alternatively, it is possible that transient monoubiquitin-
nation is related to the ubiquitin homeostasis role of UCH-L1. UCH-L1 non-covalently binds ubiquitin in brain and cell culture, and this interaction increases monoubiquitin levels by protecting ubiquitin from degradation (19). This interaction may provide a readily accessible pool of ubiquitin when cellular ubiquitin concentrations drop. It is possible that transiently monoubiquitinated UCH-L1 provides an additional pool of monomeric ubiquitin, which is released at a rate dependent on its auto-deubiquitination reaction, rather than on the timescale of equilibrium binding events, i.e. more slowly and in a manner potentially subject to regulation. Because many ubiquitin-de

REFERENCES

1. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–467
2. Pickart, C. M., and Eddins, M. J. (2004) Biochim. Biophys. Acta 1695, 55–72
3. Ciechanover, A., and Ben-Saadon, R. (2004) Trends Cell Biol. 14, 103–106
4. Schnell, J. D., and Hicke, L. (2003) J. Biol. Chem. 278, 35857–35860
5. Huang, T. T., and D’Andrea, A. D. (2006) Nat. Rev. Mol. Cell Biol. 7, 323–334
6. Amerik, A. Y., and Hochstrasser, M. (2004) Biochim. Biophys. Acta 1695, 189–207
7. Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., and Bernards, R. (2005) Cell 123, 773–786
8. Wing, S. S. (2003) Int. J. Biochem. Cell Biol. 35, 590–605
9. Huang, T. T., Nijman, S. M., Mirchandani, K. D., Galardy, P. J., Cohn, M. A., Haas, W., Gyggi, S. P., Ploegh, H. L., Bernards, R., and D’Andrea, A. D. (2006) Nat. Cell Biol. 8, 339–347
10. Reiley, W., Zhang, M., Wu, X., Granger, E., and Sun, S. C. (2005) Mol. Cell. Biol. 25, 3886–3895
11. Lowe, J., McDermott, H., Landon, M., Mayer, R. J., and Wilkinson, K. D. (1990) J. Pathol. 161, 153–160
12. Dickson, D. W., Schmidt, M. L., Lee, V. M., Zhao, M. L., Yen, S. H., and Trojanowski, J. Q. (1994) Acta Neuropathol. (Berl.) 87, 269–276
13. Facheris, M., Strain, K. J., Lesnick, T. G., de Andrade, M., Landon, M., Mayer, R. J., and Wilkinson, K. D. (2005) Neurosci. Lett. 381, 131–134
14. Satoh, J., and Kuroda, Y. (2001) J. Neurosci. 18, 113–117
15. Xue, S., and Ji, J. (2006) Brain Res. 1087, 28–32
16. Hibi, K., Westra, W. H., Borges, M., Goodman, S., Sidransky, D., and Jen, J. (1999) Am. J. Pathol. 155, 711–715
17. Yamazaki, T., Hibi, K., Takase, T., Tezel, E., Nakayama, H., Kasai, Y., Ito, K., Akiyama, S., Nagasaka, T., and Nakao, A. (2002) Clin. Cancer Res. 8, 192–195
18. Larsen, C. N., Krantz, B. A., and Wilkinson, K. D. (1998) Biochemistry 37, 3358–3368
19. Osaka, H., Wang, Y. L., Takada, K., Takizawa, S., Setsui, R., Li, H., Sato, Y., Nishikawa, K., Sun, Y. J., Sakurai, M., Harada, T., Hara, Y., Kimura, I., Chiba, S., Namikawa, K., Kiyama, H., Noda, M., Aoki, S., and Wada, K. (2003) Hum. Mol. Genet. 12, 1945–1958
20. Harada, T., Harada, C., Wang, Y. L., Osaka, H., Amanai, K., Tanaka, K., Takizawa, S., Setsui, R., Sakurai, M., Sato, Y., Noda, M., and Wada, K. (2004) Am. J. Pathol. 164, 59–64
21. Kwon, J., Wang, Y. L., Setsui, R., Sekiguchi, S., Sato, Y., Sakurai, M., Noda, M., Aoki, S., Yoshikawa, Y., and Wada, K. (2004) Am. J. Pathol. 165, 1367–1374
22. Gong, B., Cao, Z., Zheng, P., Vitolo, O. V., Liu, S., Staniszewski, A., Moon, D., Zhang, H., Shelanski, M., and Arancio, O. (2006) Cell 126, 775–788
23. Doran, J. F., Jackson, P., Kynoch, P. A., and Thompson, R. J. (1983) J. Neurochem. 40, 1542–1547
24. Papworth, C., Bauer, J. C., Braman, J., and Wright, D. A. (1996) Strategies 9, 3–4
25. Johnston, S. C., Riddle, S. M., Cohen, R. E., and Hill, C. P. (1999) EMBO J. 18, 3877–3887
26. Misaghi, S., Galardy, P. J., Meester, W. J., Ovaa, H., Ploegh, H. L., and Gaudet, R. (2005) J. Biol. Chem. 280, 1512–1520
27. Zhu, Q., Wani, G., Wang, Q. E., El-mahdy, M., Snapka, R. M., and Wani, A. A. (2005) Exp. Cell Res. 307, 436–451
28. Luchsang, S. J., Lansbury, P. T., and Stein, R. L. (2006) Biochemistry 45, 14177–14172
29. Larsen, C. N., Price, J. S., and Wilkinson, K. D. (1996) Biochemistry 35, 6735–6744
30. Das, C., Hoang, Q. Q., Kleirnegg, C. A., Luchsang, S. J., Meray, R. K., Ray, S. S., Lansbury, P. T., Ringe, D., and Petsko, G. A. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 4675–4680
31. Liu, Y., Fallon, L., Lashuel, H. A., Liu, Z., and Lansbury, P. T., Jr. (2002) Cell 111, 209–218
32. Neves, E., Boyer, R., Auburger, G., Leube, R., Ulm, G., Mezey, E., Harta, G., Brownstein, M. J., Jonnalageda, S., Chernova, T., Deheja, A., Lavedan, C., Gasser, T., Steinbach, P. J., Wilkinson, K. D., and Polymereopolous, M. H. (1998) Nature 395, 451–452
33. Brooks, C. L., and Gu, W. (2006) Mol. Cell 21, 307–315
34. Hoeller, D., Crosetto, N., Blagoev, B., Raiborg, C., Tikkannen, R., Wagner, S., Kowannetz, K., Breitling, R., Mann, M., Sternmark, H., and Dikic, I. (2006) Nat. Cell Biol. 8, 163–169
35. Miller, S. L., Malotky, E., and O’Bryan, J. P. (2004) J. Biol. Chem. 279, 35328–35337
36. Shen, C., Ye, Y., Robertson, S. E., Lau, A. W., Mak, D. O., and Chou, M. M. (2005) J. Biol. Chem. 280, 35967–35973
37. Machida, Y., Machida, Y., Chen, Y., Kurtan, A. M., Kupfer, G. M., D’Andrea, A. D., and Dutta, A. (2006) Mol. Cell 23, 589–596
38. Gerstein, M., and Krebs, W. (1998) *Nucleic Acids Res.* **26**, 4280–4290
39. Callaci, S., Heyduk, E., and Heyduk, T. (1999) *Mol. Cell* **3**, 229–238
40. Huse, M., and Kuriyan, J. (2002) *Cell* **109**, 275–282
41. Choi, I., Levey, A. I., Weintraub, S. T., Rees, H. D., Gearing, M., Chin, L. S., and Li, L. (2004) *J. Biol. Chem.* **279**, 13256–13264
42. Nishikawa, K., Li, H., Kawamura, R., Osaka, H., Wang, Y. L., Hara, Y., Hirokawa, T., Manago, Y., Amano, T., Noda, M., Aoki, S., and Wada, K. (2003) *Biochem. Biophys. Res. Commun.* **304**, 176–183
43. Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M. R., Bossi, G., Chen, H., De Camilli, P., and Di Fiore, P. P. (2002) *Nature* **416**, 451–455
44. Caballero, O. L., Resto, V., Patturajan, M., Mez Ramadan, D., Guo, M. Z., Engles, J., Yochem, R., Ratovitski, E., Sidransky, D., and Jen, J. (2002) *Oncogene* **21**, 3003–3010
45. Shenoy, S. K., and Lefkowitz, R. J. (2003) *J. Biol. Chem.* **278**, 14498–14506
46. Shenoy, S. K., and Lefkowitz, R. J. (2005) *J. Biol. Chem.* **280**, 15315–15324
47. Swaminathan, S., Amerik, A. Y., and Hochstrasser, M. (1999) *Mol. Biol. Cell* **10**, 2583–2594
48. Burbea, M., Dreier, L., Dittman, J. S., Grunwald, M. E., and Kaplan, J. M. (2002) *Neuron* **35**, 107–120
49. DiAntonio, A., and Hicke, L. (2004) *Annu. Rev. Neurosci.* **27**, 223–246