

FIGURE 2. Localization of leucine-rich repeat kinase 2 (LRRK2) in brains from Alzheimer disease cases. **(A, B)** The immunoreactivity of LRRK2 in the hippocampal CA region stained with antibody JH5514 **(A)** or NB300-267 **(B)**. **(C–E)** Double immunofluorescent labeling for LRRK2 **(C, D, JH5514; E, JH5517)** and paired helical filament (PHF)-tau shows that LRRK2 either does not colocalize (arrowheads) or only partially colocalizes (arrows) with PHF-tau-positive neurofibrillary tangles. Scale bar = 20 μ m.

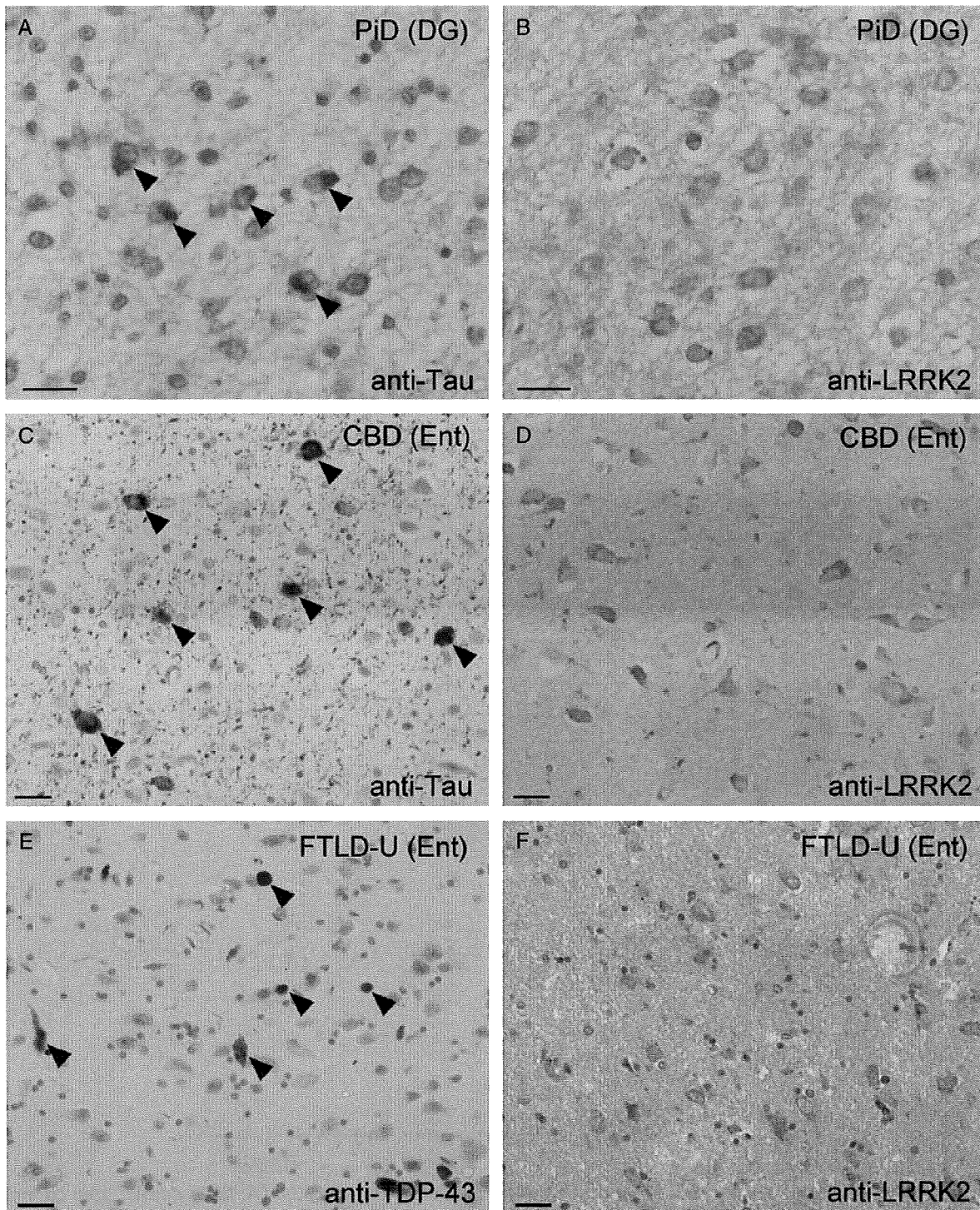


FIGURE 3. Localization of leucine-rich repeat kinase 2 (LRRK2) in various neurodegenerative diseases. **(A, B)** Consecutive paired sections from the dentate gyrus (DG) of a case of Pick disease (PiD) stained with anti-paired helical filament (PHF)-tau **(A)** or anti-LRRK2, NB300-267 antibody **(B)**. Arrowheads in **(A)** indicate tau-positive Pick bodies. **(C, D)** Consecutive sections from the entorhinal cortex (Ent) of a case of corticobasal degeneration (CBD) stained with anti-PHF-tau **(C)** or anti-LRRK2, NB300-267 antibody **(D)**. Arrowheads in **(C)** highlight tau-positive pretangles. **(E, F)** Consecutive sections from the Ent of a case of frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) stained with anti-TAR-DNA binding protein 43 (TDP-43) **(E)** or anti-LRRK2, NB300-267 antibody **(F)**. Arrowheads in **(E)** indicate TDP-43-positive dystrophic neurites. **(A)** and **(B)**, **(C)** and **(D)**, and **(E)** and **(F)** are paired consecutive sections obtained from the same affected brain region of a single case for each disorder. Scale bar= 20 μ m.

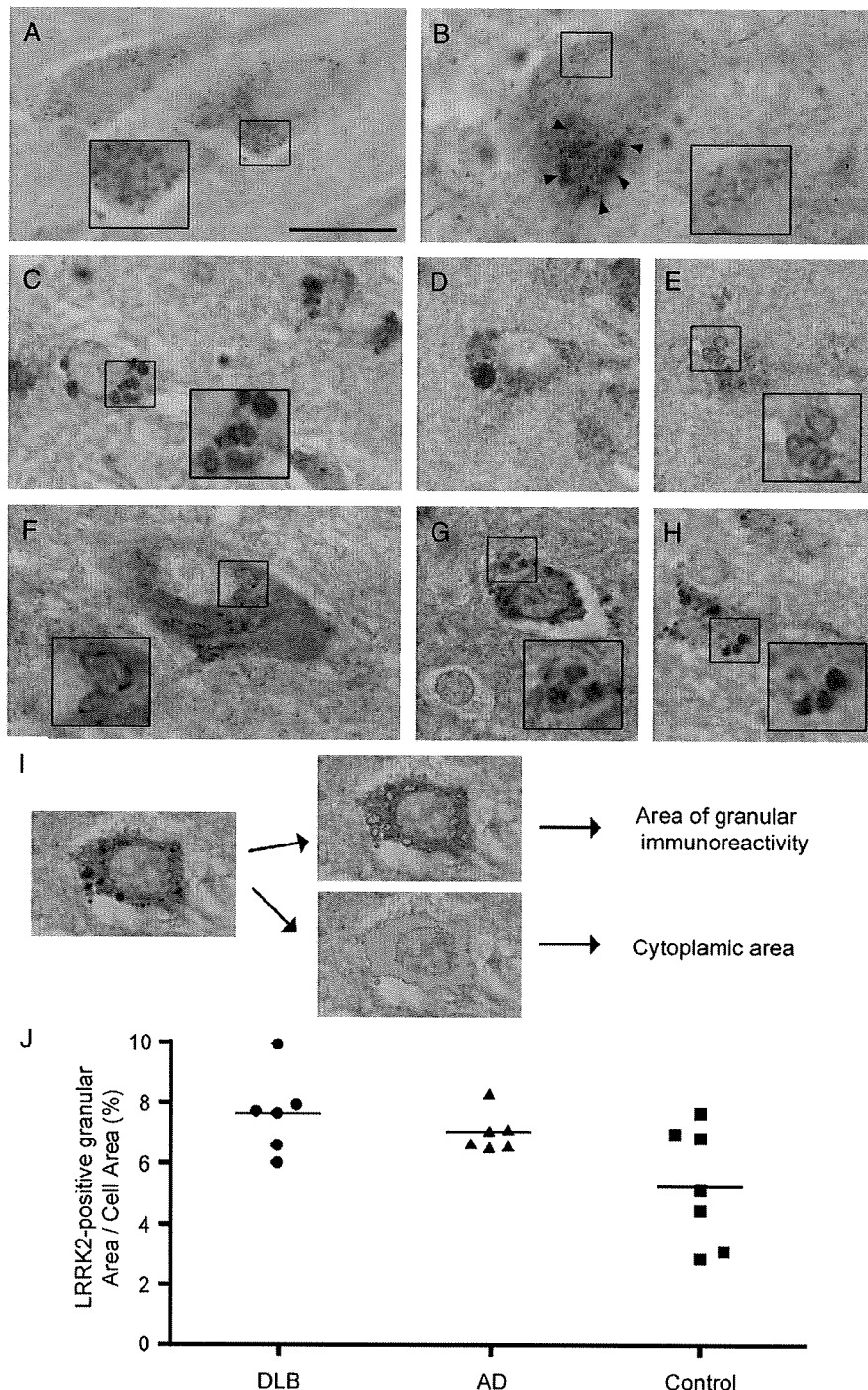


FIGURE 4. Leucine-rich repeat kinase 2 (LRRK2)-positive enlarged granules and vacuoles. **(A, B)** In normal aged controls, LRRK2 immunoreactivity has a small punctate pattern throughout the neuronal soma and processes in the entorhinal cortex **(A)** or neuromelanin-containing dopaminergic neurons (arrowheads) of the substantia nigra pars compacta **(B)**. **(C–E)** In the entorhinal cortex of dementia with Lewy bodies (DLB) cases, LRRK2 immunoreactivity in neurons is mostly in abnormal enlarged **(C)** or giant **(D)** granules or clustered vacuoles **(E)**. **(F, G)** The LRRK2-positive vacuoles or granules in neurons of the substantia nigra pars compacta **(F)** and entorhinal cortex **(G)** from Parkinson disease cases. **(H)** The LRRK2-positive enlarged granules in neurons of the entorhinal cortex from an Alzheimer disease (AD) case. Scale bars **(A–H)** 20 μ m. **(I)** Computerized image analysis for estimation of the volume of enlarged granules immunostained with anti-LRRK2 antibody (JH5514) or total cytoplasmic area outlined by brown (diaminobenzidine) immunoreactivity, as described in the Materials and Methods section. **(J)** Percentage of total cell area occupied by LRRK2-positive granules in neurons of the entorhinal cortex of DLB brains (n = 6), AD brains (n = 6), and aged control brains (n = 7). Values are the mean percentage calculated from 25 neurons per brain.

1000

© 2009 American Association of Neuropathologists, Inc.

By contrast, LRRK2 immunoreactivity in the hippocampus, entorhinal cortex and amygdala from DLB cases did not appreciably colocalize with phosphorylated α -synuclein-positive cortical-type LBs (data not shown). Instead, LRRK2-positive enlarged granular structures were frequently observed in the cytoplasm of neurons in these brain regions (Fig. 1J). Furthermore, LRRK2-positive dystrophic neurites (DNs) were occasionally observed in PD or DLB brain sections immunostained with the LRRK2 antibody NB300-267 but to a lesser extent compared with phosphorylated α -synuclein-positive DNs (data not shown). The LRRK2-positive DNs were not usually observed in brain sections immunostained with the anti-LRRK2 antibodies JH5517 and JH5514.

With antibody NB300-267, there was robust LRRK2 immunoreactivity that was frequently observed in obvious tangle-bearing neurons of the hippocampus and entorhinal cortex in a subset of AD cases (Fig. 2B); however, LRRK2 immunoreactivity reminiscent of tau-positive NFTs or DNs was not generally observed in these AD cases using antibodies JH5517 and JH5514 (Fig. 2A). Double immunofluorescent labeling using anti-PHF-tau and anti-LRRK2 antibodies (JH5517 and JH5514) revealed that LRRK2 immunoreactivity either does not (Fig. 2D) or only partially (Figs. 2C–E) colocalizes with PHF tau-positive NFTs. We also did not observe LRRK2-positive inclusions in consecutive sections from PiD and corticobasal degeneration brains immunostained for LRRK2 and tau (Figs. 3A–D).

Because tau-negative, α -synuclein-negative, ubiquitin-positive inclusions were reported in brain tissue from a patient with an *LRRK2* mutation (17), we investigated whether LRRK2 localized to TDP-43-positive inclusions in FTLD-U cases. Neither LRRK2-positive inclusions nor dystrophic neurites were observed in consecutive paired sections of brains of FTLD-U cases immunostained for LRRK2 and TDP-43 (Figs. 3E, F).

In summary, these data indicate that LRRK2 is localized to a small subset of brainstem-type LBs but does not localize to other proteinaceous pathological inclusions in the brains of patients with diverse neurodegenerative disorders.

Abnormal Localization of LRRK2 to Enlarged Granules or Vacuoles in Neurons of PD, DLB, and AD Brains

The immunoreactivity of LRRK2 was localized to the neuronal soma and processes in the brains of aged control cases and appeared in a punctate pattern ($\leq 1 \mu\text{m}$ in diameter) even in regions vulnerable to LB diseases, such as the entorhinal cortex (Fig. 4A) and the substantia nigra pars compacta (Fig. 4B). LRRK2 immunoreactivity frequently localized to numerous dense enlarged granules within neurons of affected regions of DLB brains, such as the entorhinal cortex, amygdala, and to a lesser extent, inferior temporal cortex, using the 3 anti-LRRK2 antibodies (Fig. 1J). These enlarged granules were typically greater than or equal to $1.5 \mu\text{m}$ in diameter (Fig. 4C) and occasionally greater than $4 \mu\text{m}$ in diameter (Fig. 4D); they were mostly observed in the neuronal soma but occasionally in neuronal processes. In addition to the enlarged granules, LRRK2 immunoreactivity

was often localized to clustered vacuoles in neurons of these limbic regions (Fig. 4E). In PD and DLB brains, the LRRK2-positive enlarged structures are also observed in surviving dopaminergic neurons of the substantia nigra pars compacta (Fig. 4F), but they were not observed in nigral dopaminergic neurons in aged control brains (Fig. 4B). In addition, LRRK2-positive enlarged structures in neurons of the entorhinal cortex were also observed in PD (Fig. 4G) and AD brains (Fig. 4H), albeit to a lesser extent compared with DLB brains. Thus, abnormal neuronal LRRK2 immunoreactivity has similar morphological characteristics in the limbic regions of DLB brains and the substantia nigra pars compacta

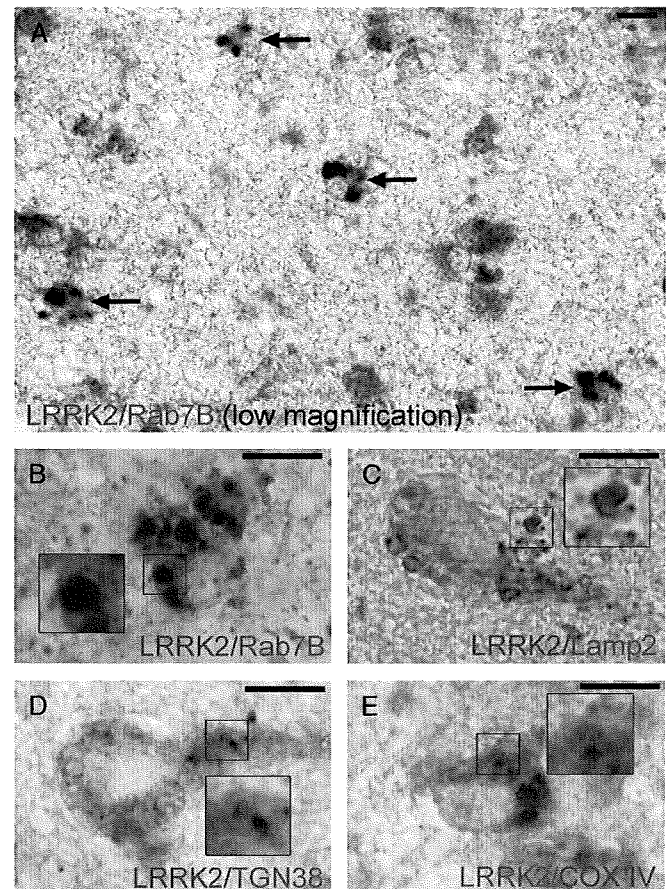


FIGURE 5. Double colorimetric immunolabeling for leucine-rich repeat kinase 2 (LRRK2) and organelle markers in neurons of the entorhinal cortex from cases of dementia with Lewy bodies. **(A, B)** Late-endosome (Rab7B); **(C)** lysosome (lysosomal-associated membrane protein 2 [LAMP-2]); **(D)** trans-Golgi network (TGN38); **(E)** mitochondria (cytochrome oxidase c subunit IV [COX IV]). Each color (brown or blue) corresponding to LRRK2 immunoreactivity or organelle marker immunoreactivity is indicated in each image. Insets in **(B–E)** are enlarged regions as indicated. Arrows in **(A)** highlight neurons with double-positive enlarged granules for LRRK2 and Rab7B (dark brown). The LRRK2-positive granules mainly colocalize with Rab7B **(A, B)** and to a lesser extent with LAMP-2 **(C)** but show minimal overlap with TGN38 **(D)** and COX IV **(E)**. Scale bar = $10 \mu\text{m}$.

of PD and DLB brains. Therefore, we speculate that these LRRK2-positive enlarged structures may be pathological features common to LB diseases and possibly also AD.

To evaluate the difference in volume of these abnormal granular structures between DLB and AD, we used compu-

terized morphometry to determine the percentages of total neuronal area occupied by LRRK2-positive granular structures in the entorhinal cortex of brains from DLB, AD, and aged control cases. This morphometric analysis enabled a precise estimate of the cell area occupied by granular

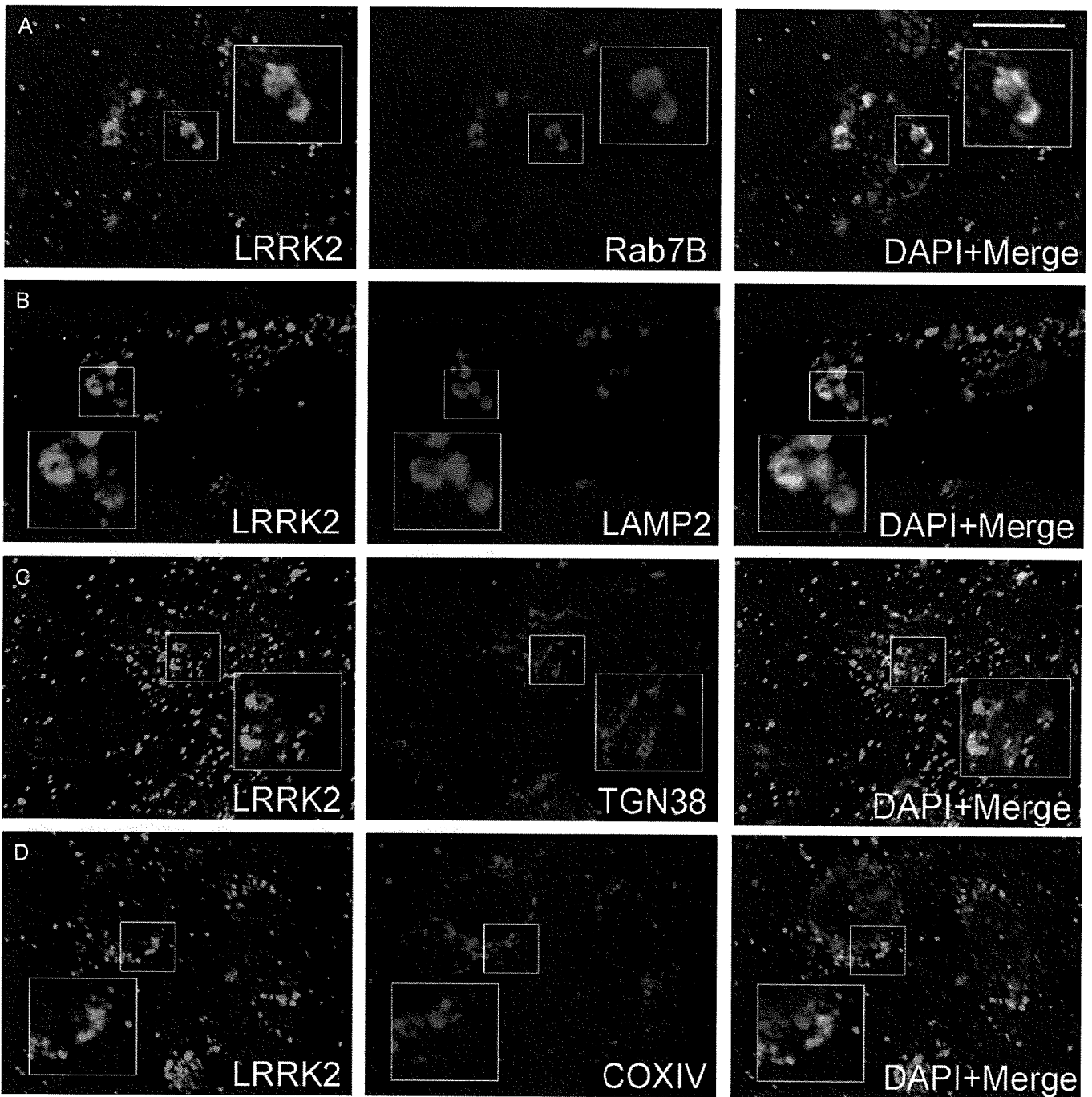


FIGURE 6. Confocal double immunofluorescent images for leucine-rich repeat kinase 2 (LRRK2) and organelle markers in neurons of the entorhinal cortex from cases of dementia with Lewy bodies. **(A)** Late-endosome (Rab7B); **(B)** lysosome (lysosomal-associated membrane protein 2 [LAMP-2]); **(C)** trans-Golgi network (TGN38); **(D)** mitochondria (cytochrome oxidase c subunit IV [COX IV]). Insets in **(A–D)** represent enlarged regions as indicated. The LRRK2-positive granules mainly colocalize with Rab7B **(A)** and to a lesser extent with LAMP-2 **(B)** but show minimal overlap with TGN38 **(C)** and COX IV **(D)**. Scale bar = **(A–D)** 10 μ m. DAPI, 4',6-diamidino-2-phenylindole.

LRRK2 immunoreactivity (Fig. 4I). The mean percentage of cell area occupied by granular LRRK2 immunoreactivity was significantly increased in DLB brains compared with control brains (DLB, $7.62\% \pm 0.55\%$ vs controls, $5.27\% \pm 0.73\%$ [mean \pm SEM], $p = 0.026$) (Fig. 4J). In contrast, there was no significant difference between AD and control brains, despite a strong trend toward increased LRRK2-immunoreactive volumes in the AD brains compared with the control brains (AD, $7.04\% \pm 0.26\%$, $p = 0.057$ vs controls) (Fig. 4J). We also analyzed the correlation between cell area occupied by LRRK2-positive granular structures and the stage of neurofibrillary and LB pathology using the Spearman rank correlation coefficient, but the differences were not significant ($p > 0.05$). The LRRK2-positive enlarged structures were only sparsely observed in brains from PiD, progressive supranuclear palsy, corticobasal degeneration, and FTLN-U cases.

LRRK2-Positive Enlarged Granules or Vacuoles Colocalize With Late-Endosomal and Lysosomal Markers, Rab7B and LAMP-2

We next examined the subcellular localization of LRRK2-positive enlarged granules or vacuoles in limbic brain regions from DLB subjects using antibodies to proteins that localize to distinct cytoplasmic organelles. We selected these markers based on the reported localizations of LRRK2 to the Golgi apparatus (35, 40), mitochondria (35, 36, 40), endosomes (35, 41), and lysosomes (35, 42). The LRRK2-positive enlarged granules or vacuoles mostly colocalized with the late endosomal marker, Rab7B (Figs. 5A, 5B, 6A). The proportions of neurons with double-positive granules (LRRK2/Rab7B) in DLB brains varied from case to case: from approximately 5% to 50% (mean \pm SD, $24.6\% \pm 20.9\%$). The 2 DLB brains with the most severe LB pathology (Stage IV, Table 2) displayed the highest frequency of neurons bearing double-positive granules ($49.4\% \pm 5.9\%$). Some LRRK2-positive enlarged granules or vacuoles also colocalized with LAMP-2-positive lysosomal structures (Figs. 5C, 6B), albeit in approximately less than 5% of the neurons. In contrast, LRRK2-positive enlarged structures only rarely colocalize with the trans-Golgi marker TGN38 or the mitochondrial marker COX IV (Figs. 5D, E, 6C, D) in neurons of these limbic brain regions from DLB subjects. This indicates that LRRK2 localizes to the endosomal-lysosomal compartment under pathological conditions in LB disease and AD.

DISCUSSION

In the present study, we demonstrate that LRRK2 immunoreactivity colocalizes to a subset of α -synuclein-positive brainstem-type LBs, especially to the inner core and its outer rim of LBs, in PD and DLB brains, whereas α -synuclein immunoreactivity localizes predominantly to the LB halos. In contrast, LRRK2 immunoreactivity does not appreciably overlap with α -synuclein-positive cortical-type LBs and DNPs in the cerebral cortex or amygdala of PD and DLB brains. The immunostaining pattern of LRRK2 in LB diseases is clearly distinct from that of α -synuclein and

tends to resemble that of synphilin 1 (43). In contrast to the LB halo, which is composed of Lewy filaments, the core of LBs is mainly composed of densely packed vesicular structures (44). Therefore, we speculate that LRRK2 might localize to vesicular structures of unknown origin rather than to filamentous aggregates of α -synuclein within LBs, as has also been speculated for synphilin 1 localization (43).

We also found that LRRK2 immunoreactivity did not colocalize with tau-positive NFTs, other tau inclusions, or with TDP-43-positive inclusions in brains of AD, various tauopathies or TDP-43 proteinopathy cases. In AD brains, LRRK2 immunoreactivity did not localize to NFTs when the anti-LRRK2 antibodies JH5517 and JH5514 were used, whereas it localized with a subset of NFTs when the commercial anti-LRRK2 antibody NB300-267 was used. The 3 distinct rabbit polyclonal antibodies to LRRK2 used here were rigorously evaluated in our previous study, in which we show that each antibody can detect endogenous human LRRK2 protein (38). The inconsistent findings between the JH5514 and JH5517 antibodies and the NB300-267 antibody may be caused by additional non-specific cross reactivity of NB300-267 because the JH5514 antibody has been validated using LRRK2-knockout mice and detects endogenous LRRK2 as the major protein species in brain tissue in wild-type control mice (38). We conclude, therefore, that LRRK2 is not a component common to different protein aggregates of these neurodegenerative disorders.

We next examined whether the subcellular localization or expression of LRRK2 is altered under pathological conditions in neurodegenerative disease. We previously reported that LRRK2 localizes exclusively to neuronal membrane-bound and vesicular structures including endosomes, lysosomes, multivesicular bodies, various transport vesicles, and mitochondria in vivo (35). In addition, LRRK2 immunoreactivity is morphologically observed as a distinct punctate staining pattern in human and rodent brain tissues and mouse primary cortical neurons (19, 35), consistent with the present data in aged human control brains. In the present study, morphological alterations of LRRK2 immunoreactivity were frequently observed as enlarged granules or clustered vacuoles in neurons within the affected limbic areas of DLB brains. Morphometric analysis reveals that the volume of cell area occupied by these LRRK2-positive structures in neurons was elevated in brain regions with LB pathology in DLB compared with similar regions of aged control brains. Recent studies have shown that certain disease-linked mutations in *LRRK2* produce a hyperactive kinase and induce toxicity in primary neurons (36, 40, 45, 46). In addition, autosomal-dominant inheritance of *LRRK2* mutations in families with PD is most likely consistent with a gain-of-function mechanism for the pathogenic properties of *LRRK2* mutations (47). Therefore, it is most likely that morphological alterations and increased volume of LRRK2 immunoreactivity may reflect an elevated expression level and thus greater overall kinase or enzymatic activity of LRRK2 per neuron in brain regions with Lewy pathology. At this juncture, however, it is not possible to rule out improved accessibility of LRRK2 epitopes for binding to each antibody

that would also result in increased LRRK2 immunoreactivity in brain tissue. This seems unlikely because the 3 antibodies we used detect distinct amino- or carboxyl-terminal epitopes. Because it has been exceedingly difficult to detect full-length LRRK2 in postmortem human samples by Western analysis with numerous LRRK2 antibodies, it is not currently possible to determine whether there is an increase in LRRK2 protein expression in these DLB brain regions. This issue will require further analysis as the tools for detecting LRRK2 improve.

The LRRK2-positive enlarged granules or vacuoles frequently colocalized with Rab7B-positive late endosomes and occasionally with LAMP-2-positive lysosomes, suggesting that LRRK2 may be associated with the endosomal-lysosomal system in these pathological brains. Growing evidence indicates that the autophagy or lysosomal systems are associated with the pathogenesis of PD, including accumulation of autophagic vacuoles in PD brains (48), clearance of misfolded α -synuclein by autophagy (49–51), and mutations in the lysosomal ATPase *ATP13A2* leading to a parkinsonian phenotype (7). In addition, there is recent evidence of an important role for LRRK2 in the endosomal-lysosomal system. For example, increased autophagic or lysosomal compartments were observed in primary cortical neurons (52) and in SH-SY5Y cells expressing the *LRRK2* G2019S variant (42). Moreover, LRRK2 is reported to interact with Rab5B, a component of the endosomal pathway that regulates synaptic vesicle endocytosis (41). Shin et al (41) recently demonstrated that LRRK2 overexpression impaired synaptic vesicle endocytosis in rat primary hippocampal neurons, indicating that LRRK2 may influence endosomal pathway function (39). These observations suggest that PD-linked mutations in *LRRK2* may lead to neuronal dysfunction and parkinsonism through perturbations in the endosomal-lysosomal trafficking pathway. Given that LRRK2 is localized to the core of LBs that are typically enriched in vesicular structures as previously described, localization of LRRK2 to the endosomal-lysosomal subcellular compartments within neurons suggests that LRRK2 may function as part of the endocytic machinery that normally regulates vesicular trafficking.

Intriguingly, LRRK2-positive enlarged structures are also observed in neurons of the limbic area of AD brains. The volume of cell area occupied by LRRK2-positive structures in neurons of AD brains showed a strong tendency to increase compared with that in aged control brains; however, this was not statistically significant. Involvement of the endosomal-lysosomal system in the pathogenesis of AD is well documented, and expression of various endosomal or lysosomal proteins are reported to be increased in neurons in regions affected in AD (53, 54). Late endosome/multi-vesicular bodies contain amyloid- β (A β) peptide (55), and intracellular A β peptide may be generated in the endosomal compartment by cleavage of amyloid precursor protein (56). In addition, with reference to abnormalities of the endosomal-lysosomal system in several neurodegenerative disorders (57), recent studies suggest that impaired autophagy through genetic disruption of *ATG5* or *ATG7* in neurons causes frank neuronal degeneration characterized by ubiquitin-positive inclusions, a pathological feature common to many neuro-

degenerative disorders (58, 59). Thus, disruption of the endosomal-lysosomal system is considered to be a pathogenic event common not only to LB diseases such as PD and DLB, but also to other neurodegenerative disorders including AD, as suggested by our finding of abnormal LRRK2-positive enlarged late endosomal compartments in neurons of the limbic area of AD brains and in DLB brains. Because *LRRK2* mutations usually cause a PD phenotype, however, the involvement of LRRK2 in the pathogenesis of AD may be indirect. It is of great interest to determine whether the function of LRRK2 in the endosomal machinery is associated with the pathogenesis of AD or with the diverse pathology observed in brains with *LRRK2* mutations.

In conclusion, the present observation that LRRK2 is localized only to the core of α -synuclein-positive brainstem-type LBs but not to α -synuclein-positive cortical-type LBs and DNs, tau-positive NFTs and other inclusions, or TDP-43-positive inclusions suggests that LRRK2 is not a common cardinal protein component of filamentous proteinaceous aggregates in various neurodegenerative disorders. In addition, morphological alterations and increased volume of LRRK2 immunoreactivity in DLB brains may indicate perturbed expression or enzymatic activity of LRRK2 associated with Lewy pathology and could be associated with a principal pathogenic event that triggers the development of the disease. The present observation that LRRK2-positive granules or vacuoles colocalize with the endosomal-lysosomal compartment may be associated with the pathogenic role of LRRK2 leading to neuronal degeneration. These findings may provide important insight into the contribution of LRRK2 dysfunction to the pathogenesis of LB diseases.

ACKNOWLEDGMENTS

The authors thank the patients and their families who made this research possible. Ted M. Dawson is the Leonard and Madlyn Abramson Professor in Neurodegenerative Diseases at Johns Hopkins.

REFERENCES

1. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 1997;276:2045–47
2. Kitada T, Asakawa S, Hattori N, et al. Mutations in the *parkin* gene cause autosomal recessive juvenile parkinsonism. *Nature* 1998;392:605–8
3. Valente EM, Abou-Sleiman PM, Caputo V, et al. Hereditary early-onset Parkinson's disease caused by mutations in *PINK1*. *Science* 2004;304:1158–60
4. Bonifati V, Rizzu P, van Baren MJ, et al. Mutations in the *DJ-1* gene associated with autosomal recessive early-onset parkinsonism. *Science* 2003;299:256–59
5. Zimprich A, Biskup S, Leitner P, et al. Mutations in *LRRK2* cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron* 2004;44:601–7
6. Paisan-Ruiz C, Jain S, Evans EW, et al. Cloning of the gene containing mutations that cause *PARK8*-linked Parkinson's disease. *Neuron* 2004;44:595–600
7. Ramirez A, Heimbach A, Grundemann J, et al. Hereditary parkinsonism with dementia is caused by mutations in *ATP13A2*, encoding a lysosomal type 5 P-type ATPase. *Nat Genet* 2006;38:1184–91
8. Gilks WP, Abou-Sleiman PM, Gandhi S et al. A common *LRRK2* mutation in idiopathic Parkinson's disease. *Lancet* 2005;365:415–16

9. Kachergus J, Mata IF, Hulihan M, et al. Identification of a novel LRRK2 mutation linked to autosomal dominant parkinsonism: Evidence of a common founder across European populations. *Am J Hum Genet* 2005; 76:672–80
10. Healy DG, Falchi M, O'Sullivan SS, et al. Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: A case-control study. *Lancet Neurol* 2008;7:583–90
11. Hulihan MM, Ishihara-Paul L, Kachergus J, et al. LRRK2 Gly2019Ser penetrance in Arab-Berber patients from Tunisia: A case-control genetic study. *Lancet Neurol* 2008;7:591–94
12. Giasson BI, Covy JP, Bonini NM, et al. Biochemical and pathological characterization of Lrrk2. *Ann Neurol* 2006;59:315–22
13. Giordana MT, D'Agostino C, Albani G, et al. Neuropathology of Parkinson's disease associated with the LRRK2 Ile1371Val mutation. *Mov Disord* 2007;22:275–78
14. Ross OA, Toft M, Whittle AJ, et al. Lrrk2 and Lewy body disease. *Ann Neurol* 2006;59:388–93
15. Wszolek ZK, Pfeiffer RF, Tsuboi Y, et al. Autosomal dominant parkinsonism associated with variable synuclein and tau pathology. *Neurology* 2004;62:1619–22
16. Rajput A, Dickson DW, Robinson CA, et al. Parkinsonism, Lrrk2 G2019S, and tau neuropathology. *Neurology* 2006;67:1506–8
17. Dachsel JC, Ross OA, Mata IF, et al. Lrrk2 G2019S substitution in frontotemporal lobar degeneration with ubiquitin-immunoreactive neuronal inclusions. *Acta Neuropathol* 2007;113:601–6
18. Hasegawa K, Kowa H. Autosomal dominant familial Parkinson disease: Older onset of age, and good response to levodopa therapy. *Eur Neurol* 1997;38(suppl 1):39–43
19. Higashi S, Biskup S, West AB, et al. Localization of Parkinson's disease-associated LRRK2 in normal and pathological human brain. *Brain Res* 2007;1155:208–19
20. Higashi S, Moore DJ, Colebrooke RE, et al. Expression and localization of Parkinson's disease-associated leucine-rich repeat kinase 2 in the mouse brain. *J Neurochem* 2007;100:368–81
21. Galter D, Westerlund M, Carmine A, et al. LRRK2 expression linked to dopamine-innervated areas. *Ann Neurol* 2006;59:714–19
22. Taymans JM, Van den Haute C, Baekelandt V. Distribution of PINK1 and LRRK2 in rat and mouse brain. *J Neurochem* 2006;98:951–61
23. Melrose H, Lincoln S, Tyndall G, et al. Anatomical localization of leucine-rich repeat kinase 2 in mouse brain. *Neuroscience* 2006;139:791–94
24. Melrose HL, Kent CB, Taylor JP. A comparative analysis of leucine-rich repeat kinase 2 (Lrrk2) expression in mouse brain and Lewy body disease. *Neuroscience* 2007;147:1047–58
25. Perry G, Zhu X, Babar AK, et al. Leucine-rich repeat kinase 2 colocalizes with alpha-synuclein in Parkinson's disease, but not tau-containing deposits in tauopathies. *Neurodegener Dis* 2008;5:222–24
26. Zhu X, Babar A, Siedlak SL, et al. LRRK2 in Parkinson's disease and dementia with Lewy bodies. *Mol Neurodegener* 2006;1:17
27. Zhu X, Siedlak SL, Smith MA, et al. LRRK2 protein is a component of Lewy bodies. *Ann Neurol* 2006;60:617–8; [author reply 618–19]
28. Mikosy J, Arai T, Guo JP, et al. LRRK2 expression in normal and pathologic human brain and in human cell lines. *J Neuropathol Exp Neurol* 2006;65:953–63
29. Gelb DJ, Oliver E, Gilman S. Diagnostic criteria for Parkinson disease. *Arch Neurol* 1999;56:33–39
30. McKeith IG, Dickson DW, Lowe J, et al. Diagnosis and management of dementia with Lewy bodies: Third report of the DLB Consortium. *Neurology* 2005;65:1863–72
31. Consensus recommendations for the postmortem diagnosis of Alzheimer's disease. The National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease. *Neurobiol Aging* 1997;18:S1–S2
32. Higashi S, Iseki E, Yamamoto R, et al. Concurrence of TDP-43, tau and alpha-synuclein pathology in brains of Alzheimer's disease and dementia with Lewy bodies. *Brain Res* 2007;1184:284–94
33. Braak H, Alafuzoff I, Arzberger T, et al. Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. *Acta Neuropathol (Berl)* 2006;112:389–404
34. Marui W, Iseki E, Kato M, et al. Pathological entity of dementia with Lewy bodies and its differentiation from Alzheimer's disease. *Acta Neuropathol (Berl)* 2004;108:121–28
35. Biskup S, Moore DJ, Celsi F, et al. Localization of LRRK2 to membranous and vesicular structures in mammalian brain. *Ann Neurol* 2006; 60:557–69
36. West AB, Moore DJ, Biskup S, et al. Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. *Proc Natl Acad Sci U S A* 2005;102:16842–47
37. Greggio E, Jain S, Kingsbury A, et al. Kinase activity is required for the toxic effects of mutant LRRK2/dardarin. *Neurobiol Dis* 2006;23:329–41
38. Biskup S, Moore DJ, Rea A, et al. Dynamic and redundant regulation of LRRK2 and LRRK1 expression. *BMC Neurosci* 2007;8:102
39. Saito Y, Kawashima A, Ruberu NN, et al. Accumulation of phosphorylated alpha-synuclein in aging human brain. *J Neuropathol Exp Neurol* 2003;62:644–54
40. Gloeckner CJ, Kinkl N, Schumacher A, et al. The Parkinson disease causing LRRK2 mutation I2020T is associated with increased kinase activity. *Hum Mol Genet* 2006;15:223–32
41. Shin N, Jeong H, Kwon J, et al. LRRK2 regulates synaptic vesicle endocytosis. *Exp Cell Res* 2008;314:2055–65
42. Plowey ED, Cherra SJ 3rd, Liu YJ, et al. Role of autophagy in G2019S-LRRK2-associated neurite shortening in differentiated SH-SY5Y cells. *J Neurochem* 2008;105:1048–56
43. Wakabayashi K, Engelender S, Yoshimoto M, et al. Synphilin-1 is present in Lewy bodies in Parkinson's disease. *Ann Neurol* 2000;47: 521–23
44. Takahashi H, Wakabayashi K. The cellular pathology of Parkinson's disease. *Neuropathology* 2001;21:315–22
45. Smith WW, Pei Z, Jiang H, et al. Kinase activity of mutant LRRK2 mediates neuronal toxicity. *Nat Neurosci* 2006;9:1231–33
46. West AB, Moore DJ, Choi C, et al. Parkinson's disease-associated mutations in LRRK2 link enhanced GTP-binding and kinase activities to neuronal toxicity. *Hum Mol Genet* 2007;16:223–32
47. Moore DJ. The biology and pathobiology of LRRK2: Implications for Parkinson's disease. *Parkinsonism Relat Disord* 2008;14(suppl 2): S92–98
48. Anglade P, Vyas S, Javoy-Agid F, et al. Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. *Histol Histopathol* 1997;12:25–31
49. Cuervo AM, Stefanis L, Fredenburg R, et al. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. *Science* 2004;305:1292–95
50. Lee HJ, Khoshaghideh F, Patel S, et al. Clearance of alpha-synuclein oligomeric intermediates via the lysosomal degradation pathway. *J Neurosci* 2004;24:1888–96
51. Webb JL, Ravikumar B, Atkins J, et al. Alpha-synuclein is degraded by both autophagy and the proteasome. *J Biol Chem* 2003;278:25009–13
52. MacLeod D, Dowman J, Hammond R, et al. The familial parkinsonism gene LRRK2 regulates neurite process morphology. *Neuron* 2006;52: 587–93
53. Cataldo AM, Barnett JL, Pieroni C, et al. Increased neuronal endocytosis and protease delivery to early endosomes in sporadic Alzheimer's disease: Neuropathologic evidence for a mechanism of increased beta-amyloidogenesis. *J Neurosci* 1997;17:6142–51
54. Nixon RA, Wegiel J, Kumar A, et al. Extensive involvement of autophagy in Alzheimer disease: An immuno-electron microscopy study. *J Neuropathol Exp Neurol* 2005;64:113–22
55. Takahashi RH, Milner TA, Li F, et al. Intraneuronal Alzheimer abeta42 accumulates in multivesicular bodies and is associated with synaptic pathology. *Am J Pathol* 2002;161:1869–79
56. Small SA, Gandy S. Sorting through the cell biology of Alzheimer's disease: Intracellular pathways to pathogenesis. *Neuron* 2006;52:15–31
57. Martinez-Vicente M, Cuervo AM. Autophagy and neurodegeneration: When the cleaning crew goes on strike. *Lancet Neurol* 2007;6:352–61
58. Hara T, Nakamura K, Matsui M, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 2006;441: 885–89
59. Komatsu M, Waguri S, Chiba T, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 2006;441: 880–84



Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Familial amyotrophic lateral sclerosis-linked mutant SOD1 aberrantly interacts with tubulin

Tomohiro Kabuta^{a,*}, Aiko Kinugawa^a, Yukihiro Tsuchiya^a, Chihana Kabuta^a, Rieko Setsuie^a, Minako Tateno^b, Toshiyuki Araki^b, Keiji Wada^{a,*}

^a Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan

^b Department of Peripheral Nervous System Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan

ARTICLE INFO

Article history:

Received 19 June 2009

Available online 1 July 2009

Keywords:

SOD1

ALS

Tubulin

Interaction

ABSTRACT

Mutations in the Cu,Zn-superoxide dismutase (SOD1) gene cause 20–25% of familial amyotrophic lateral sclerosis (ALS). Mutant SOD1 causes motor neuron degeneration through toxic gain-of-function(s). However, the direct molecular targets of mutant SOD1, underlying its toxicity, are not fully understood. In this study, we found that α/β -tubulin is one of the major mutant SOD1-interacting proteins, but that wild-type SOD1 does not interact with it. The interaction between tubulin and mutant SOD1 was detected in the spinal cords of mutant G93A SOD1 transgenic mice before the onset of symptoms. Tubulin interacted with amino acid residues 1–23 and 116–153 of SOD1. Overexpression of mutant SOD1 resulted in the accumulation of tubulin in detergent-insoluble fractions. In a cell-free system, mutant SOD1 modulated tubulin polymerization, while wild-type SOD1 did not. Since tightly regulated microtubule dynamics is essential for neurons to remain viable, α/β -tubulin could be an important direct target of mutant SOD1.

© 2009 Elsevier Inc. All rights reserved.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease caused by selective loss of motor neurons. Five to 10 percent of ALS cases are known to occur in familial forms. Dominant missense mutations in the gene that encodes the Cu/Zn-superoxide dismutase, SOD1, are associated with 20–25% of familial ALS cases (fALS). Mice overexpressing mutant SOD1 develop an ALS-like phenotype and have been used as a mouse model of ALS, whereas mice lacking SOD1 do not [1,2]. These studies indicate that mutant SOD1 causes motor neuron degeneration not by loss of function of SOD1, but by toxic gain-of-function(s) of mutant SOD1.

The toxic properties of mutant SOD1 may be exerted at least partly through the physical interactions of mutant SOD1 with other functional proteins. Recent studies suggest that the physical interactions of mutant SOD1 with other cellular proteins, such as Derlin-1, mediate the toxicity of mutant SOD1 [3]. However, the direct molecular targets of mutant SOD1, underlying its toxicity, are not fully understood. Therefore, elucidating novel mutant SOD1-interacting proteins is important for understanding the mechanisms that underlie the pathology of ALS, and for developing effective therapies for ALS.

In this study, we sought to identify novel mutant SOD1-interacting proteins using coimmunoprecipitation assays and mass spectrometry analyses. We found that α/β -tubulin is one of the major mutant SOD1-interacting proteins.

Materials and methods

Plasmid constructs and antibodies. These reagents are described in the Supplemental materials and methods.

Cell culture and transfection. HeLa, COS-7 and Neuro2a cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS). Transient expression of each vector in cells was performed using Lipofectamine LTX (Invitrogen) or the FuGENE 6 transfection reagent (Roche, Indianapolis, IN). Differentiation of Neuro2a cells was induced by adding 5 mM dibutyryl cAMP, 4 h after transfection, as described previously [4].

Preparation of recombinant proteins. Recombinant SOD1 proteins were prepared according to methods described previously [4]. Detailed procedures are described in the Supplemental materials and methods.

Immunoprecipitation. Immunoprecipitation was performed as previously described [5]. Detailed procedures are described in the Supplemental materials and methods.

* Corresponding authors. Fax: +81 42 346 1745.

E-mail addresses: kabuta@ncnp.go.jp (T. Kabuta), wada@ncnp.go.jp (K. Wada).

Mutant G93A SOD1 transgenic mice. Transgenic mice expressing G93A hSOD1 (G1L line of B6SJL-TgN(SOD1-G93A)1Gur^{dl}) were obtained from the Jackson Laboratory (Bar Harbor, ME), and backcrossed to C57BL/6J for more than 14 generations. These mice were propagated at the National Institute of Neuroscience, National Center of Neurology and Psychiatry. We observed that the G93A SOD1-transgenic mice we used in this study experienced disease onset around the age of 8 months (246.5 ± 5.8 days) and died around 9.5 months (288.5 ± 7.1 days, mean \pm SEM, $n = 14$). For coimmunoprecipitation assays, brains and spinal cords from male mice at 65 days of age were homogenized in immunoprecipitation buffer. After the lysates were centrifuged at 20,000 g for 10 min at 4 °C, 1 ml of the supernatants (~ 1.5 mg/ml protein in immunoprecipitation buffer) were incubated with 15 μ g of antibody for 15 h, and immunoprecipitation was performed using protein G Sepharose. All experiments using mice were approved by the institution's Animal Investigation Committee.

Immunoblotting analysis. SDS-PAGE was performed under reducing conditions. Immunoblotting was performed according to standard procedures as described previously [6]. The prepara-

tion of detergent (1% Triton X-100)-soluble and -insoluble fractions was performed as described previously [6].

Mass spectrometry analysis. Protein bands (55-kDa proteins in Fig. 1A) were sliced from gel and subjected to in-gel trypsin digestion, and liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis was performed at Shimadzu (Kyoto, Japan) as a custom service.

Pull-down assay. A pull-down assay was performed as described previously [4]. Detailed procedures are described in the Supplemental materials and methods.

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. Recombinant SOD1 without a tag and tubulin were mixed to give a final concentration of ~ 0.04 mg/ml SOD1 and 3 mg/ml tubulin in tubulin polymerization buffer (80 mM PIPES, pH 6.9, 2 mM MgCl₂, 0.5 mM EGTA, 1 mM GTP, 5% glycerol), and subjected to a tubulin polymerization assay. As a control, vehicle was added instead of SOD1.

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

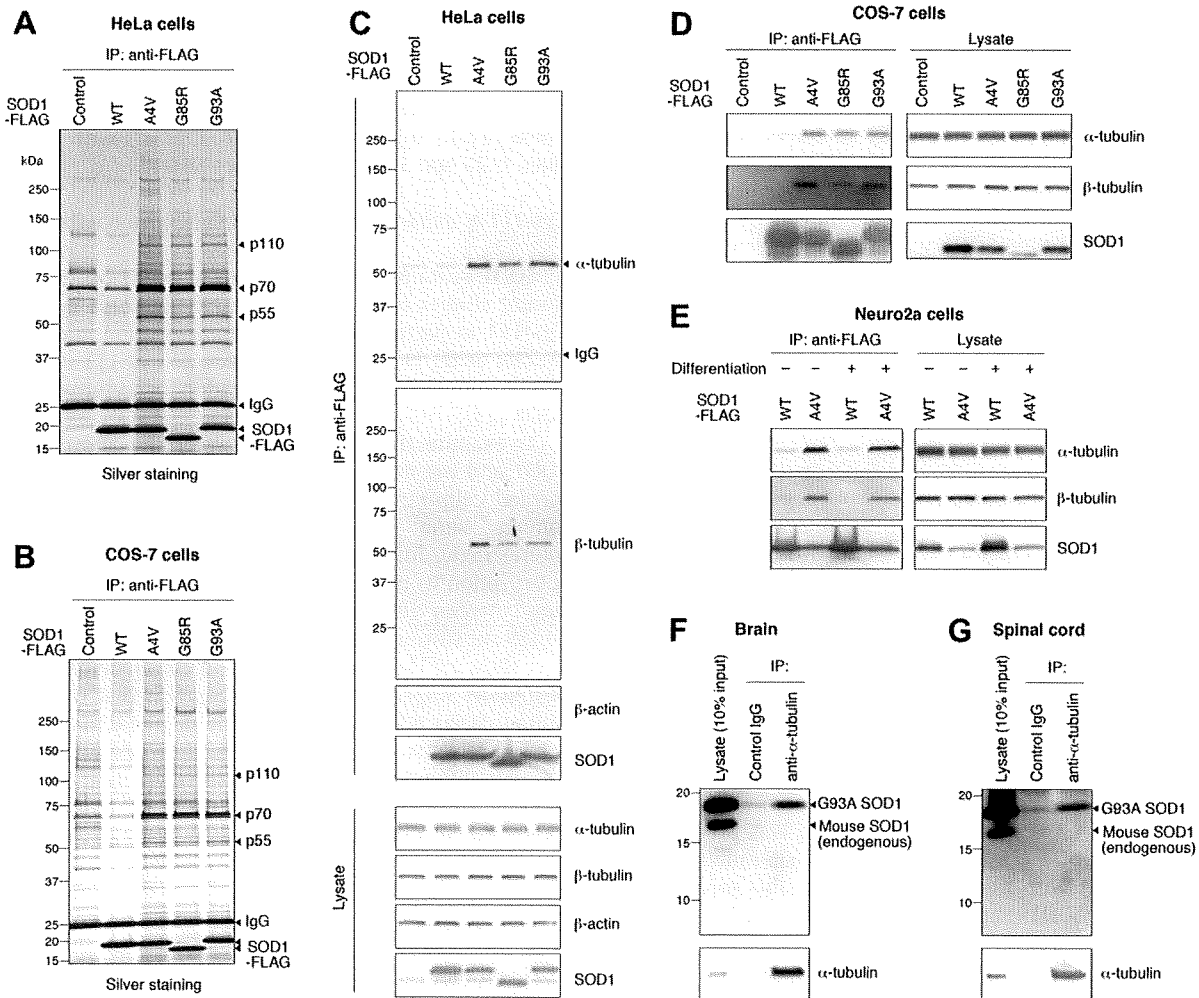


Fig. 1. Interaction between mutant SOD1 and tubulin. (A,B) Lysates of HeLa cells (A) or COS-7 cells (B) transfected with the indicated constructs (Control: empty vector) were immunoprecipitated with anti-FLAG antibody. The immunoprecipitants were analyzed by SDS-PAGE followed by silver staining. In the presence of FLAG-tagged mutant SOD1, mutant SOD1-interacting proteins were coimmunoprecipitated with mutant SOD1, while in the absence of mutant SOD1, proteins were nonspecifically precipitated with anti-FLAG beads. (C–E) HeLa (C), COS-7 (D) or Neuro2a (E) cells were transfected with the indicated constructs. Differentiation of Neuro2a cells was induced by adding 5 mM dibutyryl cAMP. Immunoprecipitation was performed using anti-FLAG antibody. The lysates and immunoprecipitants were analyzed by immunoblotting. (F,G) Lysates were prepared from the brains (F) and spinal cords (G) of mutant G93A transgenic mouse, and immunoprecipitated with anti- α -tubulin antibody or control IgG. The lysates and immunoprecipitants were analyzed by immunoblotting. Mutant SOD1 was clearly precipitated with α -tubulin. The coimmunoprecipitation of mutant SOD1 was also observed when immunoprecipitation was performed using anti- β -tubulin antibody (data not shown).

of more than two groups, Dunnett's multiple comparison test was used.

Results

Mutant SOD1 interacts with α/β -tubulin

To understand the molecular mechanism underlying the gain-of-toxicity of mutant SOD1, we attempted to identify proteins that interact with mutant SOD1 using a coimmunoprecipitation assay. We observed that the SOD1 mutants specifically interacted with multiple proteins, although wild-type SOD1 did not (Fig. 1A). Among these interacting proteins, the band intensities of the 110-kDa, 70-kDa and 55-kDa proteins were considerably higher than those of other interacting proteins (Fig. 1A). The interactions of these proteins with mutant SOD1 were also detected when we used COS-7 or Neuro2a cells (Fig. 1B and 3C).

The 110-kDa protein interacting with mutant SOD1 was previously reported to be heat-shock protein 105 [7]; mutant SOD1 was also previously reported to interact with the 70 kDa heat shock protein 70 and heat shock cognate protein 70 [8]. Using LC-MS/MS analysis, we confirmed that the 70-kDa band contained heat shock cognate protein 70 (data not shown). Using LC-MS/MS analysis, we found that the 55-kDa proteins interacting with mutant SOD1 in Fig. 1A are human α -tubulin and β -tubulin (Supplemental Fig. 1A and Supplemental files 1 and 2), indicating that mutant SOD1 physically interacts with endogenous α -tubulin and β -tubulin. The interactions of mutant SOD1 with α -tubulin and β -tubulin were also detected by immunoblotting (Fig. 1C). Consistent with the silver staining analysis (Fig. 1A), an interaction of wild-type SOD1 with tubulin was not detected (Fig. 1C). The interaction between mutant SOD1 and endogenous tubulin was also observed in COS-7 cells, and in both undifferentiated and differentiated Neuro2a cells (Fig. 1D and E). In contrast to tubulin, an interaction of β -actin with mutant SOD1 was not observed (Fig. 1C), indicating that the interaction between mutant SOD1 and tubulin is not a non-specific interaction of abundant proteins.

To confirm the interaction of mutant SOD1 with tubulin *in vivo*, we performed coimmunoprecipitation assays using the spinal cords and brains of G93A SOD1 transgenic mice before the onset of the symptoms. The interaction of tubulin with G93A SOD1, but not with mouse wild-type SOD1, was clearly detected in the spinal cords and brains (Fig. 1F and G). Since the interaction between mutant SOD1 and tubulin was observed before the onset of symptoms, it is possible that this interaction partly plays a causative role in the pathogenesis of ALS.

To test whether mutant SOD1 directly interacts with tubulin, we purified recombinant mutant SOD1 proteins with a His tag (Supplemental Fig. 1B). Purified α/β -tubulin from bovine brain (Supplemental Fig. 1B) and His-tagged SOD1 proteins were mixed and pull-down assays were performed. Tubulin was precipitated with apo mutant SOD1 (Fig. 2A), indicating that mutant SOD1 can directly interact with tubulin. Tubulin was also precipitated with apo wild-type SOD1 (Supplemental Fig. 2).

The mutant SOD1-binding site within tubulin partly overlaps with the binding site for nocodazole

To determine the mutant SOD1-binding region within tubulin, we performed pull-down assays using the tubulin-binding agents paclitaxel, noscapine and nocodazole. We found that nocodazole inhibited the interaction between mutant SOD1 and tubulin, while paclitaxel and noscapine did not (Fig. 2A–C). The nocodazole binding site overlaps with that for colchicine [9]. Colchicine also inhibited

the interaction between mutant SOD1 and tubulin (data not shown). The paclitaxel binding site within tubulin is distinct from those for noscapine and colchicine [10,11]. Thus the mutant SOD1-binding region of tubulin in part overlaps with the region(s) bound by nocodazole and colchicine, but does not overlap with the regions bound by paclitaxel and noscapine.

Amino acid residues 1–23 and 116–153 of SOD1 interact with tubulin

We next assessed whether tubulin interacts with the C-terminal deletion mutant T116X SOD1, which has been reported to cause ALS-like phenotype in mice [12]. Coimmunoprecipitation assays using FLAG-tagged T116X SOD1 (Fig. 2D) revealed that mutant T116X SOD1 interacts with tubulin (Fig. 2E), indicating that tubulin interacts with amino acid residues 1–115 of mutant SOD1. Silver staining analysis also showed that, similarly to A4V SOD1, mutant T116X SOD1 interacts with multiple proteins (Fig. 2F). This indicates that interacting with various proteins is a common abnormal property shared by ALS-associated mutant SOD1 proteins. We observed that tubulin also interacts with amino acid residues 116–153 of SOD1 (Fig. 2G and H). To determine the tubulin-binding region within residues 1–115 of SOD1, we further performed coimmunoprecipitation assays using deletion mutants of SOD1 (Fig. 2I). We found that amino acid residues 11–115 and 1–85 of SOD1 interact with tubulin, while amino acid residues 24–115 and 38–115 do not (Fig. 2J). Moreover, amino acid residues 1–10 and 1–23 of SOD1 both interacted with tubulin (Fig. 2K and L). These results suggest that tubulin interacts with amino acid residues 1–23 and 116–153 of mutant SOD1.

Cys-111 of mutant SOD1 affects the interaction with tubulin

SOD1 contains four cysteine residues, Cys-6, 57, 111 and 146. Recent studies have shown that, among these cysteine residues, Cys-111 in particular affects the aggregation and cytotoxicity of mutant SOD1 [13]. The substitution of Cys-111 to serine (C111S) significantly inhibits the aggregation or cytotoxicity of mutant SOD1 [13].

To examine the correlation between the cytotoxicity of mutant SOD1 and the interaction of mutant SOD1 with tubulin, we performed coimmunoprecipitation assays using mutant SOD1 containing serine substitutions at each cysteine residue. Interestingly, immunoblotting analyses showed that the C111S substitution in mutant SOD1 significantly decreased the interaction between tubulin and mutant SOD1, although C6S, C57S and C146S substitutions did not (Fig. 3A and B). Together with the report showing the role of Cys-111 in cytotoxicity and aggregation of mutant SOD1 [13], our results suggest that the cytotoxicity of mutant SOD1 is correlated with the degree of its interaction with tubulin. Silver staining analyses also showed that the C111S substitution in mutant SOD1 decreased the interaction between multiple proteins and mutant SOD1 (~50% decrease), while C6S, C57S and C146S substitutions did not (Fig. 3C).

Overexpression of mutant SOD1 causes accumulation of tubulin in insoluble fractions

Given that mutant SOD1 physically interacts with tubulin, and that mutant SOD1 forms insoluble aggregates when overexpressed in cells, we hypothesized that mutant SOD1 causes aggregation of tubulin. To examine the effects of mutant SOD1 on the aggregation of tubulin, we prepared 1% Triton X-100-soluble and insoluble fractions from cells overexpressing wild-type or mutant SOD1, and the amount of tubulin in each fraction was analyzed. An increased level of SOD1 was detected in the insoluble fraction of cells overexpressing mutant SOD1 compared with that of cells

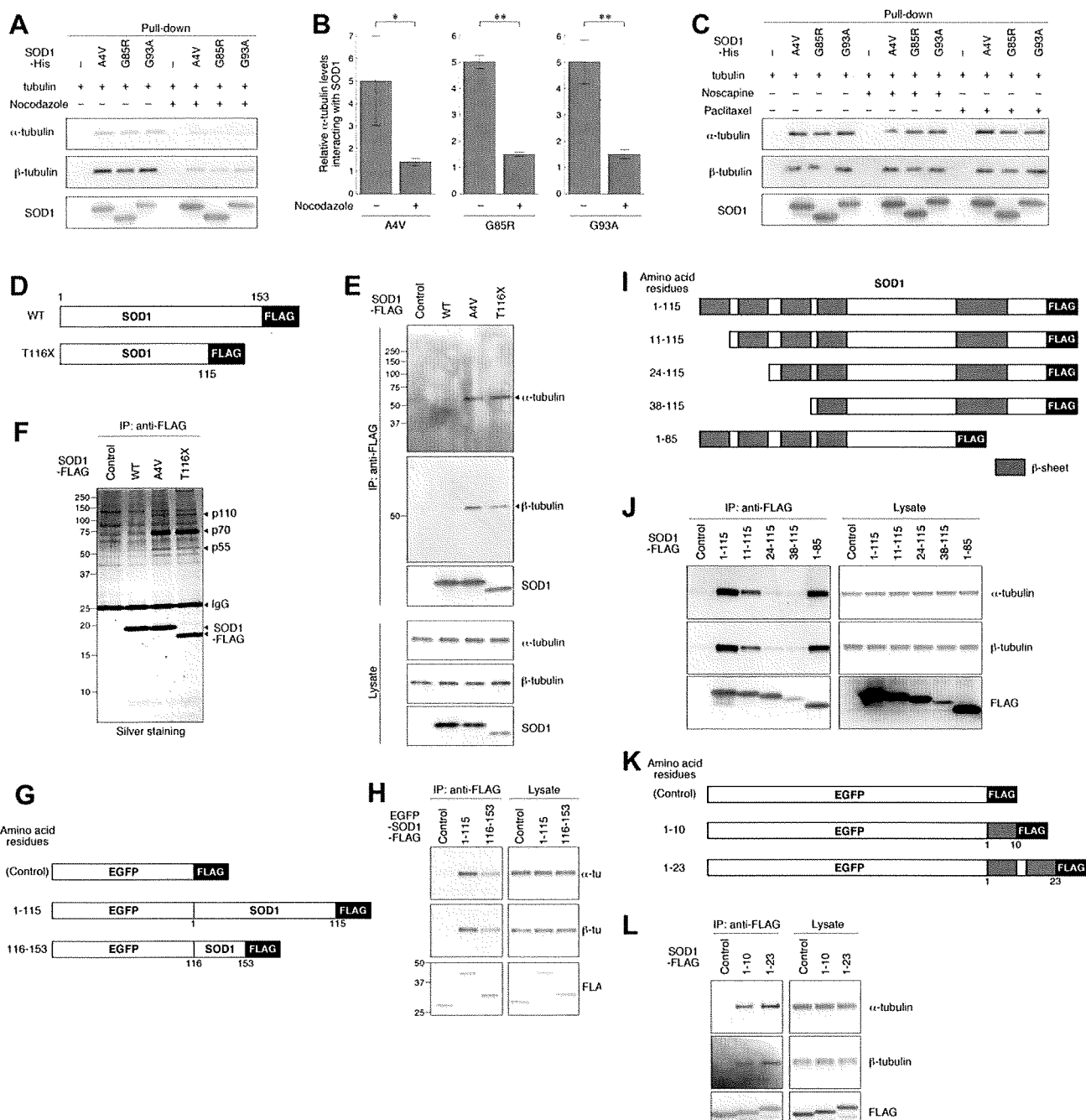


Fig. 2. Binding site within tubulin and mutant SOD1. (A–C) Forty micromolars of nocodazole (A), paclitaxel (C), noscapine (C) or vehicle was mixed with the indicated purified proteins and pull-down assays were performed. Precipitants were analyzed by immunoblotting. The relative levels of α -tubulin interacting with mutant SOD1 were quantified by densitometry. Mean values are shown with SD ($n = 3$). * $p < 0.05$; ** $p < 0.01$ (B). (D, G, I, and K) Schematic representation of deletion mutants of SOD1. (E, F, H, J, and L) Lysates of COS-7 cells transfected with the indicated constructs were immunoprecipitated with an anti-FLAG antibody and analyzed by immunoblotting (E, H, J, and L) or silver staining (F).

overexpressing wild-type SOD1 (Fig. 3D) We found that overexpression of mutant SOD1 resulted in increased levels of α - and β -tubulin in the insoluble fraction, but had no effect on the protein levels in the soluble fraction (Fig. 3D and E). By contrast, overexpression of wild-type SOD1 did not alter the levels of α - and β -tubulin in either fraction (Fig. 3D and E). In both fractions, the level of β -actin, which does not interact with mutant SOD1 (Fig. 1C), was not affected by the overexpression of wild-type or mutant SOD1 (Fig. 3D). These results indicate that the accumulation of tubulin in insoluble fractions is mediated by the interaction between mutant SOD1 and tubulin. Since the insoluble aggregates associated

with neurodegenerative diseases exhibit protease resistance [14], it is possible that aggregated tubulin exhibits resistance to constitutive degradation, resulting in the accumulation of insoluble tubulin in cells overexpressing mutant SOD1.

Mutant SOD1 directly modulates tubulin polymerization

To elucidate the direct effect of mutant SOD1 on microtubule dynamics, we used a cell-free tubulin polymerization assay. We confirmed that paclitaxel, a microtubule-stabilizing agent, promotes tubulin polymerization, and that nocodazole, a microtu-

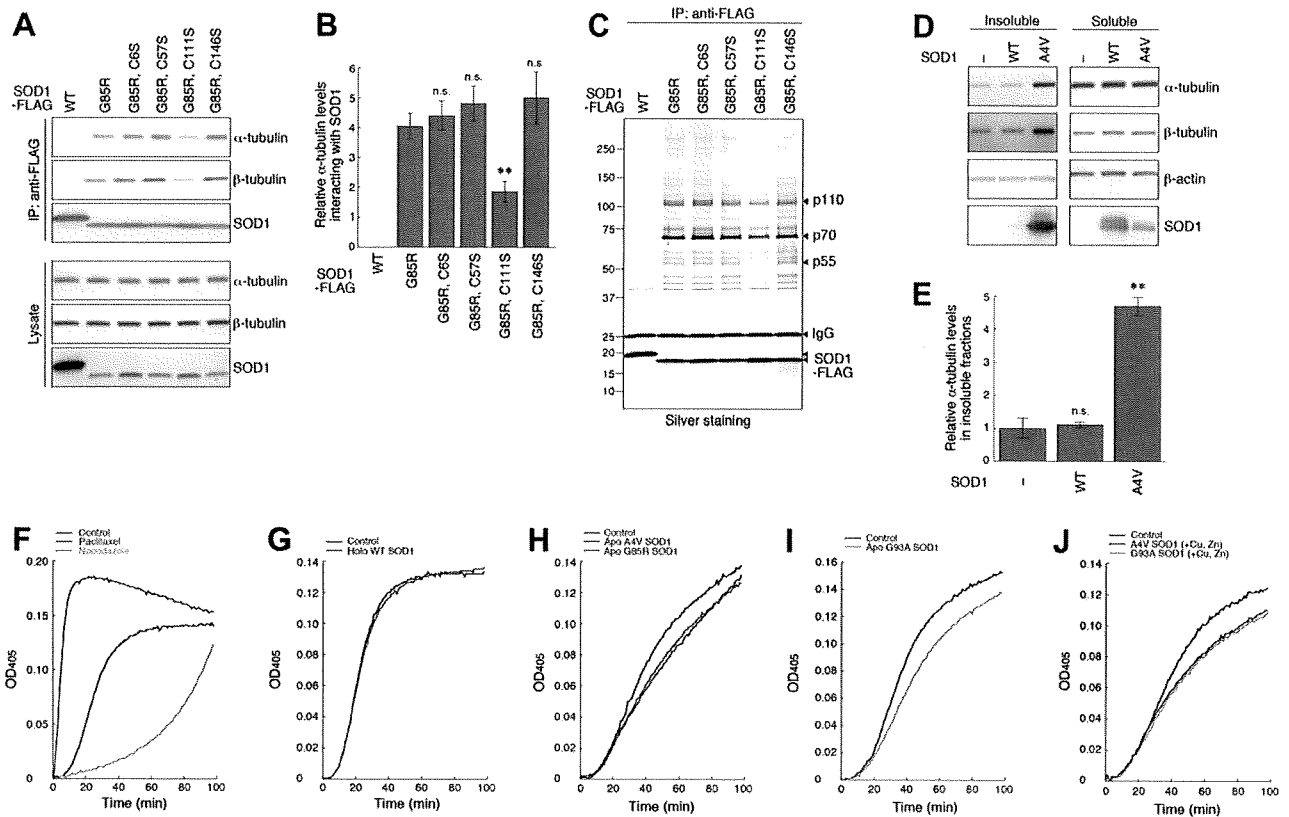


Fig. 3. Effects of mutant SOD1 on tubulin. (A–C) Lysates of Neuro2a cells transfected with the indicated constructs were immunoprecipitated with anti-FLAG antibody and analyzed by immunoblotting (A) or silver staining (C). The relative levels of α -tubulin interacting with mutant SOD1 were quantified by densitometry (B). Mean values are shown with SD ($n = 3$). n.s., not significant; $**p < 0.01$ (B, Dunnett's multiple comparison test). (D,E) pEF-hSOD1 (WT or A4V) or empty vector was transfected into COS-7 cells. Three days after transfection, soluble and insoluble fractions were prepared and analyzed by immunoblotting (D). The relative levels of α -tubulin in the insoluble fractions were quantified by densitometry. Mean values are shown with SD ($n = 3$). n.s., not significant; $**p < 0.01$ (E, Dunnett's multiple comparison test). (F) A tubulin polymerization assay was performed in the presence of 10 μ M paclitaxel, 10 μ M nocodazole or vehicle (control, DMSO). Light scattering was used to measure the amount of polymerized microtubules. (G–J) Four microliters of 1 μ g/ μ l holo wild-type SOD1 or vehicle (200 μ M CuSO_4 , 200 μ M ZnCl_2 in 20 mM sodium phosphate buffer, pH 8.0) was mixed with 100 μ l of 3 mg/ml tubulin (G). Twenty microliters of 0.2 μ g/ μ l apo mutant SOD1 or vehicle (20 mM sodium phosphate buffer, pH 8.0) was mixed with 80 μ l of 3.75 mg/ml tubulin (H,I). Twenty microliters of 0.2 μ g/ μ l holo mutant SOD1 or vehicle (200 μ M CuSO_4 , 200 μ M ZnCl_2 in 20 mM sodium phosphate buffer, pH 8.0) was mixed with 80 μ l of 3.75 mg/ml tubulin (J). Then tubulin polymerization assays were performed. The assays were performed at least three times; representative results are shown. Wild-type SOD1 did not inhibit tubulin polymerization when we used 20 μ l of 0.2 μ g/ μ l holo wild-type SOD1 (data not shown).

bule-destabilizing agent inhibits tubulin polymerization in a cell-free system (Fig. 3F). We prepared wild-type and mutant SOD1 proteins without a tag (Supplemental Fig. 1C and Supplemental Table 1), and performed tubulin polymerization assays. Because apo wild-type SOD1 interacts with tubulin, we used holo wild-type SOD1 as a control native SOD1. Wild-type SOD1 had almost no effect on tubulin polymerization (Fig. 3G). Interestingly, mutant SOD1 inhibited tubulin polymerization in tubulin polymerization assays (Fig. 3H–J). These results are consistent with the results showing that tubulin interacts with mutant SOD1, but not with wild-type SOD1, and indicate that mutant SOD1 directly affects the stability of microtubules.

Discussion

The microtubule-destabilizing agent colchicine has been reported to induce neuronal cell death in mice [15]. Microtubule-stabilizing agents such as paclitaxel are effective chemotherapeutic agents for the treatment of many cancers. However, neuropathy is a major adverse effect of microtubule-stabilizing agent-based chemotherapy [16]. Paclitaxel is also reported to induce cell death in cortical neurons [17]. Thus tightly regulated microtubule dynamics, namely controlled tubulin polymerization/depolymerization, are required for neurons to remain viable, and our results may indicate that the physical interaction of mutant SOD1 with α/β -tubulin at

least partly underlies the neurodegeneration mediated by mutant SOD1.

A recent report has shown that microtubule dynamics are increased in the motor neurons of mutant G93A SOD1 transgenic mice, and that this increase in microtubule dynamics in motor neurons is associated with altered axonal transport and motor neuron degeneration [18]. We have shown that mutant SOD1 directly interacts with tubulin (Fig. 2A), and that mutant SOD1 has a similar effect on microtubules to a microtubule-destabilizing agent, but to a lesser extent (Fig. 3F–J). Since microtubules in neurons are relatively stable due to their interactions with microtubule-associated proteins, destabilization of microtubules may cause increased microtubule dynamics in neurons. Taken together, our results suggest that mutant SOD1 can directly increase microtubule dynamics through its destabilizing effect on microtubules in neurons.

Using tubulin-binding agents, we have shown that the mutant SOD1-binding site within tubulin in part overlaps with the sites bound by colchicine and nocodazole (Fig. 2A–C). Considering that colchicine destabilizes microtubules through the interaction with tubulin at microtubule ends [19], mutant SOD1 may also modulate microtubule dynamics through the interaction with tubulin at microtubule ends. Structural analysis of the interaction of SOD1 with tubulin, such as X-ray crystallography, is required for further understanding of the mechanism that underlies the modulation of microtubule dynamics by mutant SOD1. Native SOD1 forms a sta-

ble homodimer, while mutant SOD1 partly exists as a monomer [20]. We showed that amino acid residues 1–23 and 116–153 in SOD1 interact with tubulin (Fig. 2D–L). These amino acid residues contain residues 4–8 and 143–151, which are sequestered in the SOD1 dimer interface and are inaccessible in native SOD1 [20]. We also showed that tubulin interacts with apo wild-type SOD1 (Supplemental Fig. 2). Apo SOD1 has been reported to exist substantially as a monomer [8]. Together, our results suggest that tubulin interacts with monomeric SOD1. The inhibitory effects of holo mutant SOD1 on tubulin polymerization (Fig. 3J) may be due to the misfolded or remaining apo SOD1 in the recombinant SOD1 sample, considering that the enzymatic activities of holo mutant SOD1 were reduced compared with that of wild-type SOD1 (Supplemental Table 1).

In addition to tubulin, we observed that SOD1 mutants specifically interact with multiple proteins (Fig. 1A and B and 3C). The C111S substitution in mutant SOD1 decreases the degree of interaction between mutant SOD1 and various proteins (Fig. 3C), suggesting that most mutant SOD1-interacting proteins bind to SOD1 in a similar way. These other interactors may also be involved in the mechanism underlying SOD1-mediated neurodegeneration. We have identified some of these interactors, and these proteins are currently under investigation.

Our present study demonstrates that mutant SOD1 can directly modulates tubulin polymerization by physically interacting with tubulin. The findings in the present study provide novel insights into the mechanisms underlying the pathology of ALS.

Acknowledgments

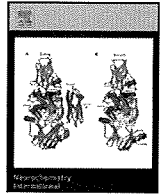
We thank Dr. Ryosuke Takahashi (Kyoto University) and Dr. Makoto Urushitani (Shiga University of Medical Science) for the gift of pcDNA3-hSOD1-FLAG plasmids. We also thank Dr. H. Akiko Popiel (National Institute of Neuroscience) for support with English. This work was supported by Grants-in-Aid for Scientific Research of Japan Society for the Promotion of Science; Research Grant in Priority Area Research of the Ministry of Education, Culture, Sports, Science and Technology, Japan; Grants-in-Aid for Scientific Research of the Ministry of Health, Labour and Welfare, Japan and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.138.

References

- [1] M.E. Gurney, H. Pu, A.Y. Chiu, M.C. Dal Canto, C.Y. Polchow, D.D. Alexander, J. Caliendo, A. Hentati, Y.W. Kwon, H.X. Deng, W. Chen, P. Zhai, R.L. Sufit, T. Siddique, Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation, *Science* 264 (1994) 1772–1775.
- [2] A.G. Reaume, J.L. Elliott, E.K. Hoffman, N.W. Kowall, R.J. Ferrante, D.F. Siwek, H.M. Wilcox, D.G. Flood, M.F. Beal, R.H. Brown Jr., R.W. Scott, W.D. Snider, Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury, *Nat. Genet.* 13 (1996) 43–47.
- [3] H. Nishitoh, H. Kadowaki, A. Nagai, T. Maruyama, T. Yokota, H. Fukutomi, T. Noguchi, A. Matsuzawa, K. Takeda, H. Ichijo, ALS-linked mutant SOD1 induces ER stress- and ASK1-dependent motor neuron death by targeting Derlin-1, *Genes Dev.* 22 (2008) 1451–1464.
- [4] T. Kabuta, R. Setsuie, T. Mitsui, A. Kinugawa, M. Sakurai, S. Aoki, K. Uchida, K. Wada, Aberrant molecular properties shared by familial Parkinson's disease-associated mutant UCH-L1 and carbonyl-modified UCH-L1, *Hum. Mol. Genet.* 17 (2008) 1482–1496.
- [5] T. Kabuta, A. Furuta, S. Aoki, K. Furuta, K. Wada, Aberrant interaction between Parkinson disease-associated mutant UCH-L1 and the lysosomal receptor for chaperone-mediated autophagy, *J. Biol. Chem.* 283 (2008) 23731–23738.
- [6] T. Kabuta, Y. Suzuki, K. Wada, Degradation of amyotrophic lateral sclerosis-linked mutant Cu, Zn-superoxide dismutase proteins by macroautophagy and the proteasome, *J. Biol. Chem.* 281 (2006) 30524–30533.
- [7] H. Yamashita, J. Kawamata, K. Okawa, R. Kanki, T. Nakamizo, T. Hatayama, K. Yamanaka, R. Takahashi, S. Shimohama, Heat-shock protein 105 interacts with and suppresses aggregation of mutant Cu/Zn superoxide dismutase: clues to a possible strategy for treating ALS, *J. Neurochem.* 102 (2007) 1497–1505.
- [8] M. Urushitani, J. Kurisu, M. Tateno, S. Hatakeyama, K. Nakayama, S. Kato, R. Takahashi, CHIP promotes proteasomal degradation of familial ALS-linked mutant SOD1 by ubiquitinating Hsp/Hsc70, *J. Neurochem.* 90 (2004) 231–244.
- [9] J. Hoebke, G. Van Nijen, M. De Brabander, Interaction of oncodazole (R 17934), a new antitumoral drug, with rat brain tubulin, *Biochem. Biophys. Res. Commun.* 69 (1976) 319–324.
- [10] J. Zhou, K. Gupta, J. Yao, K. Ye, D. Panda, P. Giannakakou, H.C. Joshi, Paclitaxel-resistant human ovarian cancer cells undergo c-Jun NH₂-terminal kinase-mediated apoptosis in response to noscapine, *J. Biol. Chem.* 277 (2002) 39777–39785.
- [11] R.B. Ravelli, B. Gigant, P.A. Curmi, I. Jourdain, S. Lachkar, A. Sobel, M. Knossow, Insight into tubulin regulation from a complex with colchicine and a stathmin-like domain, *Nature* 428 (2004) 198–202.
- [12] D. Han-Xiang, J. Hujun, F. Ronggen, Z. Hong, S. Yong, L. Erdong, H. Makito, C.D. Mauro, S. Teepu, Molecular dissection of ALS-associated toxicity of SOD1 in transgenic mice using an exon-fusion approach, *Hum. Mol. Genet.* 17 (2008) 2310–2319.
- [13] M. Cozzolino, I. Amori, M.G. Pesaresi, A. Ferri, M. Nencini, M.T. Carri, Cysteine 111 affects aggregation and cytotoxicity of mutant Cu, Zn-superoxide dismutase associated with familial amyotrophic lateral sclerosis, *J. Biol. Chem.* 283 (2008) 866–874.
- [14] R.M. Murphy, Peptide aggregation in neurodegenerative disease, *Annu. Rev. Biomed. Eng.* 4 (2002) 155–174.
- [15] Y. Liang, S. Li, C. Wen, Y. Zhang, Q. Guo, H. Wang, B. Su, Intrastriatal injection of colchicine induces striatonigral degeneration in mice, *J. Neurochem.* 106 (2008) 1815–1827.
- [16] J.J. Lee, S.M. Swain, Peripheral neuropathy induced by microtubule-stabilizing agents, *J. Clin. Oncol.* 24 (2006) 1633–1642.
- [17] X.A. Figueroa-Masot, M. Hetman, M.J. Higgins, N. Kokot, Z. Xia, Taxol induces apoptosis in cortical neurons by a mechanism independent of Bcl-2 phosphorylation, *J. Neurosci.* 21 (2001) 4657–4667.
- [18] P. Fanara, J. Banerjee, R.V. Hueck, M.R. Harper, M. Awada, H. Turner, K.H. Husted, R. Brandt, M.K. Hellerstein, Stabilization of hyperdynamic microtubules is neuroprotective in amyotrophic lateral sclerosis, *J. Biol. Chem.* 282 (2007) 23465–23472.
- [19] R.L. Margolis, L. Wilson, Addition of colchicine-tubulin complex to microtubule ends: the mechanism of substoichiometric colchicine poisoning, *Proc. Natl. Acad. Sci. USA* 74 (1977) 3466–3470.
- [20] R. Rakhit, J. Robertson, C. Vande Velde, P. Horne, D.M. Ruth, J. Griffin, D.W. Cleveland, N.R. Cashman, A. Chakrabarty, An immunological epitope selective for pathological monomer-misfolded SOD1 in ALS, *Nat. Med.* 13 (2007) 754–759.



Ubiquitin dimers control the hydrolase activity of UCH-L3

Rieko Setsuie^a, Mikako Sakurai^{a,1}, Yuriko Sakaguchi^b, Keiji Wada^{a,*}

^a Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan

^b Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

ARTICLE INFO

Article history:

Received 29 September 2008

Received in revised form 3 December 2008

Accepted 15 December 2008

Available online 25 December 2008

Keywords:

Ubiquitin

Ubiquitin dimer

Ubiquitin carboxy terminal hydrolase-L1 (UCH-L1)

Ubiquitin carboxy terminal hydrolase-L3 (UCH-L3)

ABSTRACT

Ubiquitin (Ub) carboxy terminal hydrolase (UCH)-L1 and UCH-L3 are two of the deubiquitinating enzymes expressed in the brain. Both *gad* mice, which lack UCH-L1 expression and *Uchl3* knockout mice exhibit neurodegeneration, although at distinct areas. These phenotypes indicate the importance of UCH-L1 and UCH-L3 in the regulation of the central nervous system. However, molecular substrates and the molecular regulators of UCH-L1 and UCH-L3 remain poorly identified. Here we show that Ub dimers interact non-covalently with UCH-L3 *in vitro* and in cells. These interactions were not observed with UCH-L1 in cells. *In vitro*, K48-linked Ub dimers pronouncedly inhibited the hydrolase activity of UCH-L3, while mono-Ub, a previously identified interacting protein, inhibited the hydrolase activity of UCH-L1. These results indicate that mono-Ub and Ub dimers may regulate the enzymatic functions of UCH-L1 and UCH-L3, respectively, *in vivo*.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

The ubiquitin (Ub) system is involved in the regulation of various physiological phenomena including development, inflammatory response, and intracellular trafficking (Hershko and Ciechanover, 1998; Hicke, 2001; Glickman and Ciechanover, 2002; Mukhopadhyay and Riezman, 2007). Malfunction of the Ub system in the central nervous system (CNS) may lead to neurodegeneration or synaptic dysfunction (Rubinsztein, 2006; Hegde and Upadhyay, 2007). Ub can be covalently conjugated to substrate proteins by the sequential action of E1, E2 and E3 enzymes forming an isopeptide bond between the carboxy terminus of Ub and the lysine residues of the substrates (Hershko and Ciechanover, 1998). The attachment of Ub to the substrates may occur either in the form of a single Ub or a poly-Ub chain, where the carboxy terminus of one Ub moiety is covalently linked to one of the seven Lys residues of an adjacent Ub molecule.

Deubiquitination involves the hydrolysis of the isopeptide bonds between the carboxy terminus glycine of the Ub and the lysine of the substrates or Ub, which requires the activity of deubiquitinating enzymes (DUBs) (Nijman et al., 2005). DUBs can be divided into six distinct families: the Ub-specific processing proteases, the Ub carboxyl-terminal hydrolases (UCHs), the

Ataxin-3/Josephin domains, the ovarian tumor domain-containing proteases, the viral processing proteases and the JAMM proteases.

UCH is a cysteine protease with relatively small molecular weight. There is one UCH in yeast, and four UCH isozymes in mammals: UCH-L1, UCH-L3, UCH37 and BAP1. Of these, UCH-L1 and UCH-L3 are the most closely related family members with about 53% identity. These UCHs have also been shown to hydrolyze Nedd8 (neural precursor cell expressed developmentally down regulated 8), a Ub-like protein with 68% identity to Ub (Wada et al., 1998; Hemelaar et al., 2004). The expression of UCH-L3 is ubiquitous whereas that of UCH-L1 is mainly restricted to the brain and the testis/ovary (Wilkinson et al., 1989; Kurihara et al., 2000; Kurihara et al., 2001).

Previous studies using mutant mice of *Uchl1* and *Uchl3* indicated that these enzymes are involved in the regulation of the brain function (Setsuie and Wada, 2007). The I93M point mutation in UCH-L1 is associated with familial Parkinson's disease (Leroy et al., 1998) and we have previously shown that I93M UCH-L1 expression in the cell or in transgenic mice induces dopaminergic neuron death (Setsuie et al., 2007; Kabuta et al., 2008). We also reported that *gad* (gracile axonal dystrophy) mice, which lack *Uchl1* expression, show axonal degeneration of the gracile tract, which results in sensory ataxia (Saigoh et al., 1999). Our analysis also indicated that UCH-L1 is involved in many biological aspects including neuronal apoptosis (Harada et al., 2004), neurotransmitter receptor activation (Manago et al., 2005), neural progenitor regulation (Sakurai et al., 2006), learning and memory (Sakurai et al., 2008). On the other hand, UCH-L3 knockout mice show photoreceptor cell degeneration (Semenova

* Corresponding author. Tel.: +81 42 346 1715; fax: +81 42 346 1745.

E-mail address: wada@ncnp.go.jp (K. Wada).

¹ Present address: Department of Pathology and Taub Institute, Columbia University, New York, NY 10032, USA.

et al., 2003; Sano et al., 2006) and defects in memory function (Wood et al., 2005). These different phenotypes of mutant mice indicate that although both enzymes share similar properties in terms of biochemical identity (Johnston et al., 1997; Larsen et al., 1998; Misaghi et al., 2005; Das et al., 2006), UCH-L1 and UCH-L3 should be differentially controlled or be regulated by distinct molecules.

To identify the proteins that regulate UCH-L1 and UCH-L3 in a diverse manner, we performed immunoaffinity purification assay using UCH-L1 and UCH-L3 and found that UCH-L3 is inhibited by Ub dimers (di-Ub) while UCH-L1 is inhibited by mono-Ub.

2. Experimental procedures

2.1. Isolation and culture of mouse embryonic fibroblasts (MEFs) and their immortalization

Pregnant female *Uchl3* heterozygote mice (Kurihara et al., 2000) were sacrificed at 14–15 days postcoitus. The head, the viscera and the bones of each embryo were removed under a dissecting microscope and a part of these samples were used for genotyping. The remaining portion of embryo was placed in 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA), cut finely with razor blade, and incubated for 15 min at 37 °C. The cell suspension was passed several times through a 1 ml pipette tip until no clumps were visible. The cell suspension prepared from each individual embryo was washed with medium containing 10% (v/v) fetal bovine serum (FBS). Viable cells were determined by trypan-blue exclusion assay and 4,000,000 viable cells from each embryo were plated in 100 mm culture dishes. MEFs were maintained in Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, MO, USA) supplemented with 10% (v/v) FBS (JRH, Biosciences, Lenexa, KS, USA), 100 U/ml penicillin and streptomycin (Invitrogen). The genotype was determined using the remnants of tissues, and polymerase chain reactions (PCR) were conducted using the primers and the thermal cycling as follows: L3KO-1 primer (5'-ggaactactgagccatagtgc-3') L3KO-2 primer (5'-cggactactccatcacttcac-3') L3KO-3 primer (5'-cttgtgagcgcaagtgc-3'), one cycle of 98 °C for 5 min, 30 cycles at 98 °C for 10 s, 60 °C for 20 s, and 72 °C for 30 s. For the immortalization of MEFs, basic 3T3 protocols were carried out as described (Todaro and Green, 1963).

2.2. Construction of cell lines expressing human wild-type or mutant UCH-L3

Retrovirus vectors encoding human wild-type UCH-L3 were constructed as follows. Full-length human UCH-L3 cDNA was cloned from human liver cDNA library and inserted into the pGEM-T vector (Promega, Madison, WI, USA). For PCR-based amplification of the insert, the *huchl3-retro-s2* primer (5'-GGGGCTCGAGCGCGG-CATGAGGGTCAACGCTGGCT-3') and *huchl3-pci-as1* primer (5'-GGGGCGGGCC-CCTATGCTGCAGAAAGAGC AATCG-3') were used with 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The amplified PCR fragment and pOZ-N retrovirus vector were successively digested with XhoI and NotI. The enzymatically digested PCR fragment and pOZ-N vector were subsequently applied to electrophoresis, gel extraction and ligated using the ligation system ver2 (Takara, Tokyo, Japan). This resulted in the insertion of human UCH-L3 without a starting methionine after the HA and FLAG double-tag sequence between the XhoI and NotI sites at the multicloning site of the pOZ-N double-tag retrovirus vector. To obtain Ub hydrolase-deficient C95S mutant- and Ub affinity deficient D33A mutant-encoding retrovirus vectors, the Cys95 or Asp33 of hUCH-L3 were mutated to Ser or Ala, respectively, using PCR-based *in vitro* mutagenesis (Stratagene, La Jolla, CA, USA). The primers used for mutagenesis were *huchl3-C95S-s1* (5'-ATCAGCAATGCAATGCCTGTGGAAC AATTGGA-3'), *hchl3-C95S-as1* (5'-TCCAATGTTCCACAGGCATTGCTGAT-3'), *huchl3-D33A-s* (5'-CTAACT-GGCAATTCGTGCTGATATGGAATGGATCC TGAAC-3') and *huchl3-D33A-as* (5'-GTTCAGGATCCATCCATATA CAGCAACGAATGCCAGTTAG-3'). The human UHCL1 or GFP cDNA derived inserts were cloned into the XhoI and NotI site of the same retrovirus vector, as described previously (Sakurai et al., 2006). The production of retroviruses and cell lines were conducted as described elsewhere (Ogawa et al., 2002).

2.3. Immunoprecipitation

2.5×10^8 floating HeLa cells expressing FLAG-HA tagged hUCH-L3 were collected and washed with phosphate buffered saline (PBS) twice and the cell pellets were stored at -80 °C until all of the clones were ready for lysis. The floating HeLa cells were quickly thawed at 37 °C, suspended in 15 ml of ice-cold modified RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaVO₄, 1 mM NaF with Complete EDTA-free protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA)), and kept on ice for 30 min. After three repeated sonifications for 10 s with 30 s intervals, lysates were centrifuged at 12,000 rpm at 4 °C for 10 min using a swing-rotor type centrifuge. The supernatant was recovered for further purification and aliquots were kept at -80 °C as a pre-immunoprecipitation lysate. To the remaining supernatant, anti-FLAG M2 antibody-conjugated agarose (Sigma) in 600 μ l slurry

was added and gently rotated at 4 °C for 8 h. After rotation, the samples were poured into the empty column (Bio-Rad, Hercules, CA, USA), washed four times with 8 ml 0.1 B buffer (20 mM Tris-HCl (pH 8.0), 0.1 M KCl, 5 mM MgCl₂, 10% glycerol, 0.1% Tween-20, 10 mM β -mercaptoethanol, 0.2 mM PMSF), and briefly centrifuged to remove the excess washing buffer. After capping the bottom of the column, 600 μ l elution buffer (160 μ g/ml of 3 \times FLAG peptide in 0.1 B buffer) were added to the column, rotated at 4 °C for 1 h, and eluted into an eppendorf tube. This elution step was repeated and a total of 1.2 ml eluate was used for the second purification. The second purification was performed using the anti-HA antibody (clone 12CA5)-conjugated agarose. The steps were the same as for FLAG purification except that the scale was minimized with 100 μ l of elution (1 mg/ml HA peptide (Roche) in 0.1 B buffer). The resulting final eluates were kept at -80 °C until analysis.

2.4. Mass spectrometry analysis

After the negative staining (Nakalai Tesque Inc., Kyoto, Japan) of the SDS-PAGE gels, protein bands were sliced from the gel, reduced, S-alkylated, and subjected to in-gel trypsin digestion. The digested peptides were extracted with 50% acetonitrile and 0.1% trifluoroacetic acid and subjected to liquid-chromatography tandem mass spectrometry (LC-MS/MS) analysis.

2.5. Silver staining and Western blotting

Silver staining was done using the Daiichi Silver staining Kit (Daiichi, Tokyo, Japan). For the Western blotting of UCH-L3 or HA-FLAG tagged UCH-L3, 15% mini gels (DRC, Tokyo, Japan) were used. For the detection of mono- or di-Ub, or Nedd8, 15–20% gradient mini gels (DRC) were used. For 2D-Western blotting, samples were loaded on 4–11 N IPG strips (GE Healthcare UK Ltd., Buckinghamshire, England) using IPGphor and the second dimension was run on 15–20% gradient gels (DRC). The antibodies used were anti-HA antibody (clone 12CA5, 1:1,000), anti-FLAG M2 antibody (Sigma; 1:1000), anti-Ub antibody (Sigma; 1:1000 or Dako; 1:1000), anti-Nedd8 antibody (Cell signaling; 1:1000) anti-UCH-L1 antibody (Ultrasclone RA95101; 1:5000, England, UK) and anti-UCH-L3 antibody (Kwon et al., 2004; 1:1,000). HRP-conjugated secondary antibodies were from Pierce and signals were detected using a chemiluminescent Super Signal West Dura Extended Duration Substrate kit or West Femto Maximum Sensitivity Substrate kit (Pierce, Rochford, IL) and analyzed with a Chemilmager (Alpha Innotech, San Leandro, CA). The protein marker was purchased from Bio-Rad.

2.6. Pull-down assay

6HN-tagged human UCH-L3 and UCH-L1 proteins were prepared as described previously (Nishikawa et al., 2003). Pull-down assay was conducted as described previously with some modifications (Kabuta et al., 2008). In brief, 6 μ g 6HN-UCH and 4 μ g of either K48-linked di-Ub, K63-linked di-Ub, or linear di-Ub (all from Boston Biochem, Cambridge, MA, USA), were mixed in the presence of 0.1 μ g/ml BSA in tris-buffered saline (TBS) at 4 °C for 2 h. Fifty micro liters of TALON resin (Clontech, Palo Alto, CA, USA) were added to samples and incubated at 4 °C for 1 h. After washing the resin five times with PBS in the presence of 20 mM imidazole and 0.05% Tween-20, the bound proteins were eluted with SDS sample buffer.

2.7. Ubiquitin hydrolase activity assay

The Ub hydrolase activity assay, using recombinant UCH-L3 and Ub-AMC (both from Boston Biochem), was performed as described previously (Nishikawa et al., 2003), in the presence or absence of di-Ub.

3. Results

3.1. UCH-L3 but not UCH-L1 interacts with di-Ub

To identify the proteins that interact with UCH-L1 or UCH-L3, which might represent the potential substrates or the functional effectors, we performed immunoaffinity chromatography. Floating HeLa cells that stably expressed human UCH-L3 tagged with the FLAG-HA epitope at its N-terminus (L3 WT), UCH-L3 C95S (active site mutant of UCH-L3), UCH-L1 (L1 WT), UCH-L1 C90S (active site mutant of UCH-L1), GFP and Mock were constructed (Fig. 1A). Western blotting using anti-HA and anti-FLAG antibodies detected a single band at the appropriate molecular weight in each cell line indicating FLAG-HA tagged proteins were sufficiently expressed and at comparable levels (Fig. 1A). We detected two bands with the anti-UCH-L3 antibody in all of the cell lines; these bands correspond to the exogenously expressed and endogenously expressed UCH-L3 in HeLa cells (Fig. 1A).

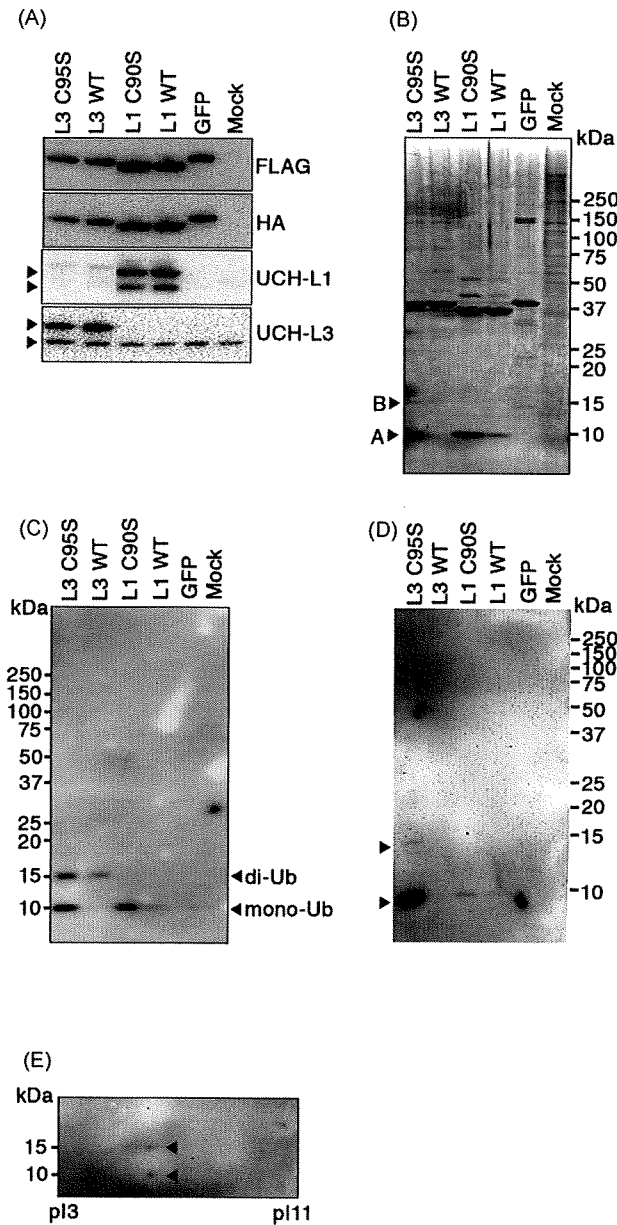


Fig. 1. Di-Ub interacts with UCH-L3. (A) Floating HeLa cells stably expressing FLAG-HA double-tagged UCH-L3 or UCH-L1 were constructed. The expression of exogenous UCH-L3 or UCH-L1 was confirmed by anti-FLAG, anti-HA, anti-UCH-L1 and anti-UCH-L3 antibodies. Anti-UCH-L1 antibody showed two bands (indicated by the arrows), where upper band corresponds to the predicted molecular weight of UCH-L1 including FLAG-HA tag at the N-terminal. The lower band may indicate the N-terminally processed form of UCH-L1, which lack FLAG-HA tag. Anti-UCH-L3 antibody also showed two bands, where lower band represent the endogenous UCH-L3 expressed in HeLa cells. (B) Silver staining of proteins, which are obtained by the sequential immunoaffinity purification using anti-FLAG antibody-conjugated agarose and anti-HA antibody-conjugated agarose. The bands, which are indicated by the arrow A and B were excised and processed for LC-MS/MS analysis. (C) Western blotting of the proteins as in (B) with anti-Ub antibody from Sigma-Aldrich. Note the presence of anti-Ub antibody immunoreactive bands at about 10 kDa and 15 kDa, which represent mono- and di-Ub. (D) Western blotting of the proteins as in (B) with anti-Nedd8 antibody from Cell Signaling. Note the presence of anti-Nedd8 antibody immunoreactive bands at about 10 kDa and 15 kDa, which may represent mono- and di-Nedd8. (E) 2D-Western blotting of the proteins, which is obtained by the sequential immunoaffinity purification of UCH-L3 C95S expressing HeLa cell lysate as in (B). Anti-Ub antibody from Sigma-Aldrich is used.

UCH-L1- or UCH-L3-containing complexes were affinity purified from stable cells using anti-FLAG antibody-conjugated agarose, followed by anti-HA antibody-conjugated agarose and each elution was conducted under native conditions using 3×

Table 1

The amino acid sequences of fragments detected by the mass spectrometric analysis of bands A and B from Fig. 1B.

Band	Amino acid sequences
A	MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ
	QRLIFAGKQL EDGRTLSDYN IQKESTLHLV LRLRGG
B	MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ
	QRLIFAGKQL EDGRTLSDYN IQKESTLHLV LRLRGG

The bands, from the immunoaffinity-purified complex of C95S mutant corresponding to A and B of Fig. 1B, are excised and subjected to LC-MS/MS analysis as described in the Section 2, and the amino acid sequences in red are detected fragments by LC-MS/MS analysis.

FLAG peptide or HA peptide. After the second immunoaffinity chromatography, proteins associated with UCH-L3 WT, UCH-L3 C95S, UCH-L1 WT and UCH-L1 C90S were identified by SDS-PAGE followed by silver staining (Fig. 1B). The deduced amino acid sequences, which were obtained from the LC-MS/MS analysis, indicate that the bands with a molecular weight of about 9 kDa (arrow A) in Fig. 1B correspond to the free mono-Ub (Table 1). Consistently, the immunoblotting of immunoaffinity-purified proteins with anti-Ub antibody, which recognizes both free Ub and ubiquitylated proteins, revealed that these bands represent free mono-Ub (Fig. 1C). The finding that UCH-L1 and the UCH-L1 C90S mutant have strong affinity for mono-Ub is consistent with our previous finding that UCH-L1 interacts with and stabilizes free mono-Ub *in vivo* (Osaka et al., 2003). The strong detection of mono-Ub with the UCH-L3 C95S mutant complex but not with the UCH-L3 WT complex indicates that C95S mutation leads to either the conformational change resulting in the strong interaction with mono-Ub, or the lack of hydrolase activity that results in the interaction with the substrate.

The molecule with molecular weight of about 15 kDa (arrow B in Fig. 1B) identified for the UCH-L3 WT and UCH-L3 C95S immunoaffinity-purified proteins, and which had Ub sequence within this molecule, was of interest because this molecule was specific to UCH-L3 and not UCH-L1. Although the molecular weight is greater than for free mono-Ub, LC-MS/MS analysis of this band only revealed Ub (Table 1). It is known that free di-Ub has a size of about 15 kDa. To confirm whether the bands at 15 kDa (arrow B in Fig. 1B) are, in fact, free di-Ub, we performed 2D-Western blotting of the C95S immunoaffinity-purified complex using the anti-Ub antibody. The *pI* of di-Ub should be similar to that of mono-Ub. As shown in Fig. 1E, the 15 kDa molecule (upper arrow in Fig. 1E) had a *pI* similar to that of free mono-Ub (lower arrow in Fig. 1E), indicating that the band may represent di-Ub.

To confirm the direct interaction of di-Ub and UCH-L3, we performed pull-down assays using recombinant mono-Ub or di-Ub and UCH-L3. As shown in Fig. 2B, UCH-L3 can form physiological complexes not only with di-Ub but also with mono-Ub. However, the affinity of UCH-L3 to mono-Ub is weak compared to di-Ub (see Fig. 1B, C and Fig. 2A).

Nedd8, an Ub-like protein with 57% identity to Ub, is a previously reported UCH-L3 and UCH-L1 interacting protein (Wada et al., 1998; Hemelaar et al., 2004). Although we did not detect any derivatives of Nedd8 on LC-MS/MS analysis, Western blotting analysis of immunoaffinity-purified complexes using the anti-Nedd8 antibody revealed several bands, which presumably represent mono-Nedd8 (lower arrow in Fig. 1D) and di-Nedd8 (upper arrow in Fig. 1D).

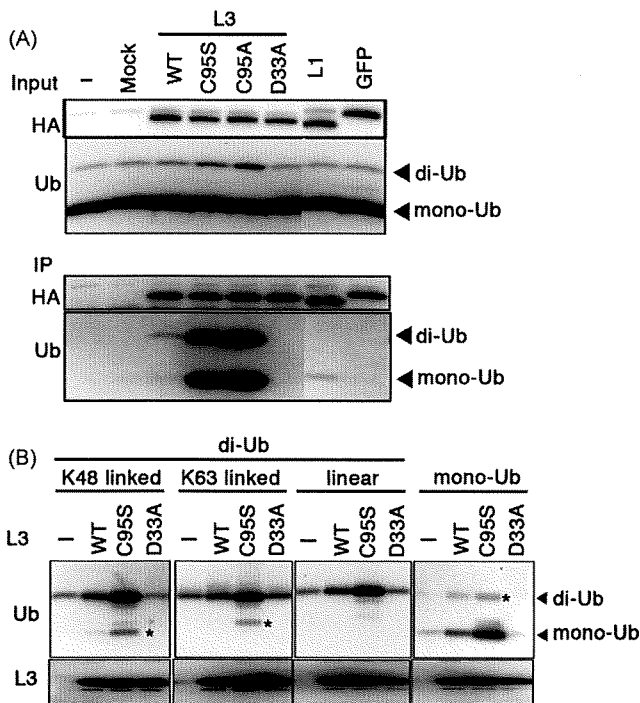


Fig. 2. C95 and D33 of UCH-L3 are involved in the interaction with K48-linked, or K63-linked or linear di-Ub. (A) UCH-L3 interacting proteins were co-immunoprecipitated from HeLa cells expressing FLAG-HA tagged UCH-L3 mutants. Cell lysates were immunoprecipitated using anti-HA antibody and co-immunoprecipitates were analyzed by anti-Ub antibody from Sigma–Aldrich. Note the presence of di-Ub in UCH-L3 WT or C95S mutant or C95A mutant immunoprecipitates but not in UCH-L3 D33A mutant or UCH-L1 immunoprecipitates. (B) *In vitro* pull-down assays using recombinant UCH-L3 and recombinant di-Ub or mono-Ub. The pulled-down samples were analyzed by anti-UCH-L3 antibody and anti-Ub antibody from DAKO. UCH-L3 WT and C95S mutant interacted with K48-linked, or K63-linked or linear di-Ub or mono-Ub while these interactions were diminished with D33A mutant. The bands with the asterisks are unidentified molecules, which is presumably derived from the contaminants of di-Ub or mono-Ub.

3.2. Di-ubiquitin interacts directly with UCH-L3 via hydrolytic active site

NMR studies and X-ray studies of UCHs complexed with Ub derivatives indicated that D33 of UCH-L3 is important for its affinity to Ub (Wilkinson et al., 1999; Misaghi et al., 2005). To confirm whether this amino acid is also responsible for the interaction of di-Ub with UCH-L3, we performed an immunoprecipitation assay using mutant UCH-L3. FLAG-HA tagged UCH-L3 WT, C95S, C95A, D33A mutants and UCH-L1 expressing HeLa cells were immunoprecipitated with anti-HA antibody-conjugated agarose. As shown in Fig. 2A, the D33A mutation diminished the interaction of di-Ub with UCH-L3 while the C95S and the C95A mutations enhanced the interaction.

The first methionine and seven internal lysine residues of Ub are known to form isopeptide bonds with the carboxy terminus glycine of an adjacent Ub molecule (Kirkpatrick et al., 2005; Kirisako et al., 2006). Thus, eight various forms of di-Ub may exist. However, only K48 and K63-linked di-Ub are presumed to exist with relative abundance *in vivo* with the former being identified *in vivo* (van Nocker and Vierstra, 1993; Hochstrasser, 2006). In addition, previous studies raised the possibility that tandemly conjugated di-Ub may form after processing of the Ub gene products by the DUBs (Wilkinson, 1997). Thus, we assessed whether these di-Ubs (linear, K48-linked and K63-linked) can interact with UCH-L3 *in vitro*. The pull-down analysis of di-Ub with 6HN-tagged UCH-L3 indicated that the K48-linked, K63-linked and linear di-Ub all interacted with UCH-L3 (Fig. 2B). In addition, the

pull-down analysis of di-Ub using the D33A mutant abolished the interaction, while the C95S mutant increased the interaction. These results also indicate that D33 and C95 of UCH-L3 are amino acid residues that are involved in the interaction with di-Ub.

3.3. Exogenously expressed UCH-L3 does not hydrolyze di-Ub in cells

We have shown that UCH-L3 interacts with free di-Ub. The previous *in vitro* studies using recombinant human UCH-L3 showed that UCH-L3 neither have the activity toward hydrolyzing tandemly conjugated di-Ub or K48-linked di-Ub though it can hydrolyze Ub conjugates with small adducts less than 20 amino acids or nucleophiles such as glutathione (Larsen et al., 1998). To confirm that di-Ubs are not the substrate of UCH-L3, we also performed hydrolase assays using K48-linked, K63-linked and linear di-Ub as the substrates *in vitro*. After incubation for 24 h, UCH-L3 did not produce mono-Ub from these three forms of di-Ub (data not shown), which is consistent with previous reports (Larsen et al., 1998). Although UCH-L3 did not show hydrolase activity toward the K48-linked, K63-linked or linear di-Ub, there exists the possibility that other forms of di-Ub (presumably K11-linked, K27-linked, K29-linked and K33-linked di-Ubs) may be hydrolyzed by UCH-L3. To eliminate this possibility, we analyzed stable cell lines expressing several functional mutants of UCH-L3 and determined whether di-Ub accumulates in the absence of UCH-L3 hydrolase activity.

Two immortalized 3T3 cell lines derived from two different UCH-L3 knockout mouse embryonic fibroblasts were generated and used to make stable cell lines either expressing WT UCH-L3, its mutants C95S, D33A, UCH-L1 or GFP. The same retrovirus systems were used as in Fig. 1, resulting in the attachment of FLAG-HA tags to each exogenous protein. Cell lysates were immunoblotted using anti-HA, anti-UCH-L1, and anti-UCH-L3 antibodies (Fig. 3A). The absence of endogenous UCH-L1 or UCH-L3 and the presence of exogenous proteins at comparable levels were assessed in these cells (Fig. 3A). The immunoprecipitation assays of several UCH-L3 mutants indicated that the D33A mutant lost the affinity to interact with di-Ub while the C95S mutant retained this function (Fig. 2A). In this condition, immunoblotting with the anti-Ub antibody was performed. We presumed that, if UCH-L3 functions to hydrolyze free di-Ub *in vivo*, the free di-Ub would accumulate in cells not expressing the WT UCH-L3. However, cells expressing WT UCH-L3 showed increasing levels of free di-Ub compared with the Mock- or GFP-expressing cells (Fig. 3A and B). Moreover, the D33A mutant-expressing cells did not show any signs of free di-Ub accumulation (Fig. 3A and B). In addition, lack of UCH-L3 expression in the tissues or MEF cells derived from Uchl3 knockout mice indicated no signs of di-Ub accumulation (Fig. 3C and data not shown). These results indicate that UCH-L3 does not function to hydrolyze free di-Ub.

3.4. Endogenous UCH-L3 does not show stabilizing effects on mono or di-Ub in cells or tissues

We have already shown that UCH-L1 interacts with and stabilizes mono-Ub *in vivo* (Osaka et al., 2003). The increased levels of free mono-Ub and di-Ub in the cells expressing WT and C95S mutant UCH-L3 in the immortalized 3T3 KO cells (Fig. 3A and B) prompted us to speculate that UCH-L3 might stabilize mono- and di-Ub *in vivo*. Thus, we compared the protein levels of mono- and di-Ub in various tissues from wild-type mice and knockout mice using Western blotting analysis with the anti-Ub antibody. As shown in Fig. 3C, tissues (including the testis where UCH-L3 expression is most abundant) of knockout mice did not show reduced mono-Ub or di-Ub levels. This result indicates that, at least in the tissues analyzed, UCH-L3 is not involved in the stabilization of di-Ub *in vivo*.

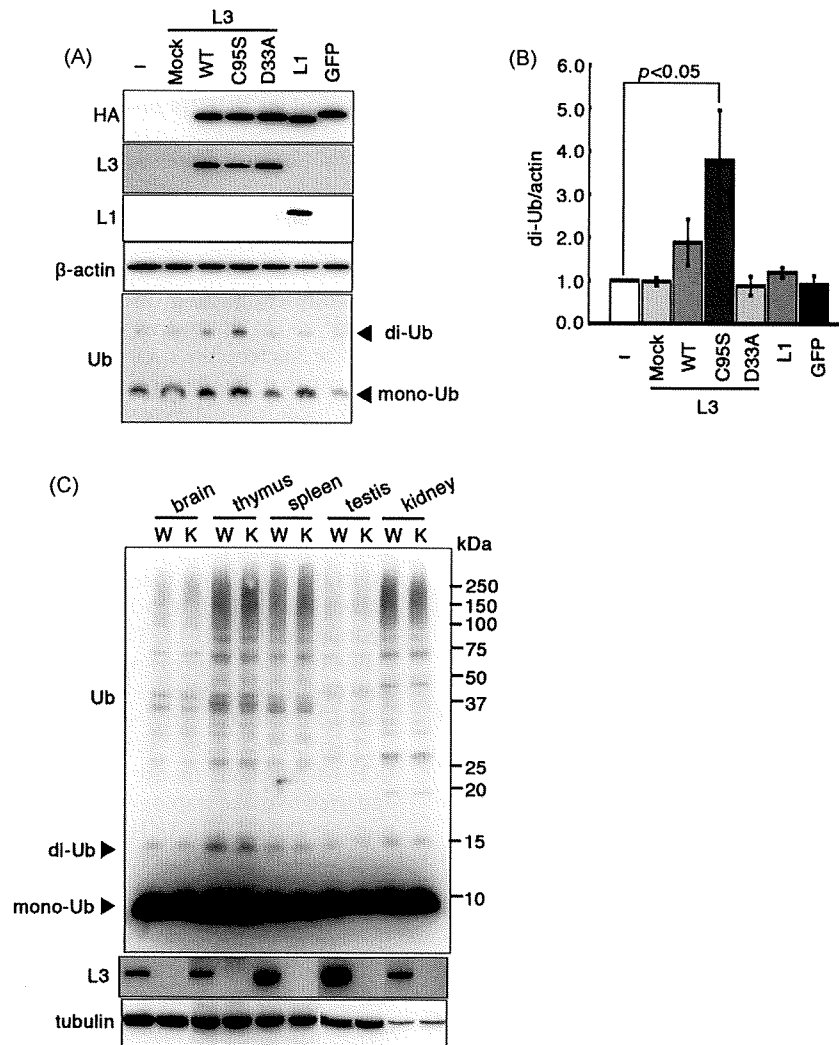


Fig. 3. UCH-L3 does not hydrolyze nor stabilize di-Ub *in vivo*. (A) The Western blotting analysis of immortalized MEF cells derived from *Uchl3* knockout mice, which express FLAG-HA tagged UCH-L3 mutants. Note that these cells lack the expression of endogenous UCH-L3. (B) The quantitative analysis of bands corresponding to di-Ub in (A). The experiments were done three times and the statistical analysis was conducted by ANOVA followed by Dunnett's test. Data are mean \pm S.E.M, $P < 0.05$. (C) Western blotting analysis of the tissues (whole brain, thymus, spleen, testis and kidney) from *Uchl3* knockout (KO) mice and his wild-type littermate (WT) using anti-Ub antibody from Dako. Testis and spleen were included because these tissues express UCH-L3 most abundantly.

3.5. Di-Ub and mono-Ub inhibit the hydrolase activities of both UCH-L3 and UCH-L1

As shown in Fig. 2A and B, D33 and C95 of UCH-L3 are involved in the interaction with di-Ub. C95 is part of the active site triad of proteases and D33 is an essential amino acid for the interaction with Ub-aldehyde or Ub-vinylmethylsulfone (Wilkinson et al., 1999; Misaghi et al., 2005). Thus, we speculated that di-Ub might inhibit the enzymatic activity of UCH-L3. We performed *in vitro* hydrolase activity assays using Ub-AMC as the model substrate in the presence and absence of K48-linked, K63-linked and tandemly linked di-Ub and mono-Ub. As shown in Fig. 4A–C, the hydrolase activity of UCH-L3 is significantly inhibited in the presence of all forms of di-Ub and mono-Ub with the most prominent effect observed with the K48-linked di-Ub (about 60% inhibition). Meanwhile, mono-Ub as well as K48-linked di-Ub showed a significant inhibitory effect on the hydrolase activity of UCH-L1 (about 60% and 45% inhibition, respectively) (Fig. 4D).

4. Discussion

Our immunoaffinity purification assay coupled with LC-MS/MS analysis of HeLa cells stably expressing exogenous UCH-L3 and

UCH-L1 revealed that UCH-L3, but not its closest isozyme UCH-L1, interacts with di-Ub. Interaction with mono-Ub, an important physiological interacting partner of UCH-L1, was not observed with WT UCH-L3 in cells (Fig. 1). Structure analysis by X-ray crystallography and NMR spectroscopy indicated that important amino acids for the interaction of Ub-aldehyde or Ub-vinylmethyl-ester, with UCH-L1 and UCH-L3 are all conserved in these two enzymes (Wilkinson et al., 1999; Misaghi et al., 2005). However, these enzymes share only 53% amino acid identity with different affinities for mono- and di-Ub (Figs. 1 and 2). In addition, the enzymatic activity toward the artificial Ub substrate (Ub-AMC) differs significantly between UCH-L1 and UCH-L3; UCH-L1 activity (K_{cat}) is less than 1/200 that of UCH-L3 (Liu et al., 2002). The amino acid sequences, which are not conserved among these UCHs should be responsible for these different characteristics.

Analysis of the UCH-L3 mutant revealed that D33 and C95 are essential for the interaction with di-Ub (Fig. 2). The previous kinetic analysis, NMR analysis and X-ray analysis revealed that C95 is an active site and D33 reside within the enzymatic catalytic pocket of UCH-L3 (Larsen et al., 1996; Johnston et al., 1997; Wilkinson et al., 1999; Misaghi et al., 2005). The carboxy terminal moiety of Ub in the ubiquitinated substrate penetrates this catalytic pocket of UCH-L3 and D33 of UCH-L3 forms a salt bridge

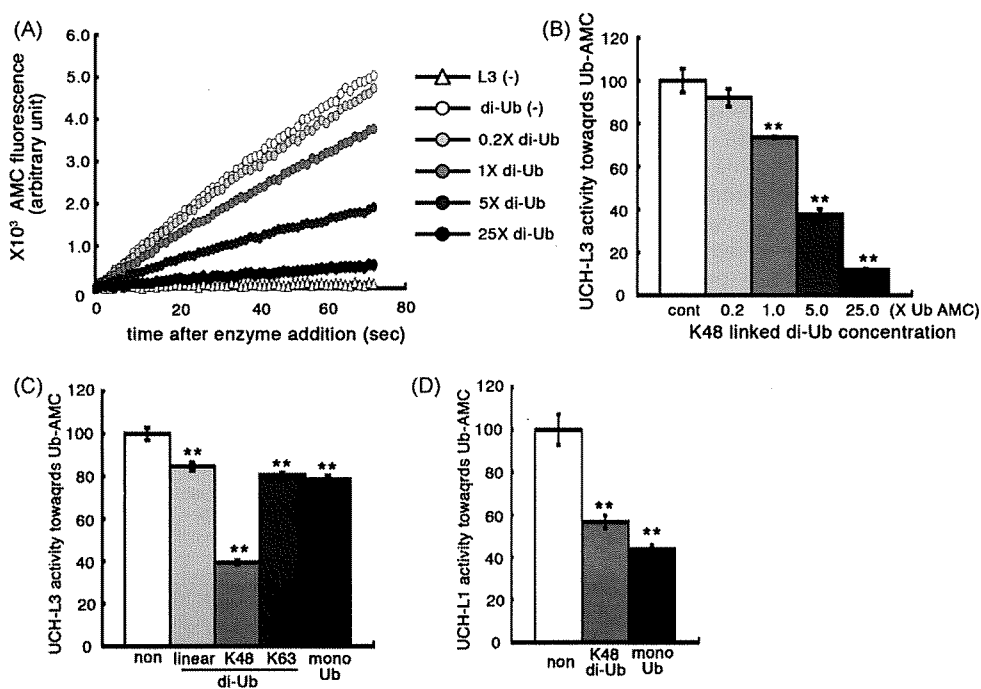


Fig. 4. Di-Ub inhibits UCH-L3 deubiquitinating activity while mono-Ub inhibits UCH-L1 deubiquitinating activity *in vitro*. (A) The kinetic curves of UCH-L3 in the presence or absence of K48-linked di-Ub. 0.2X or 1X or 5X or 25X moles of di-Ub compared to Ub-AMC substrate were included in the reaction. (B) The kinetic analysis of (A). (C) UCH-L3 deubiquitinating activity in the presence or absence of K48-linked or K63-linked or linear di-Ub or mono-Ub as in (B). 5X moles of di-Ub or mono-Ub compared to Ub-AMC substrate were included in the reaction. (D) UCH-L1 deubiquitinating activity in the presence or absence of K48-linked di-Ub or mono-Ub as in (B). 5X moles of di-Ub or mono-Ub compared to Ub-AMC substrate were included in the reaction. All statistical analyses were conducted by ANOVA followed by Dunnett's test. Data are mean \pm S.E.M. ($n = 4$) ** $p < 0.001$ vs controls.

with R72 of Ub. The active site cysteine and an acidic residue corresponding to C95 and D33 of UCH-L3 are conserved among all UCH family proteins including UCH-L1 (Wilkinson et al., 1999; Misaghi et al., 2005). Thus, the interactions of di-Ub with UCH-L3 and mono-Ub with UCH-L1 occupy the catalytic centers of UCH-L3, and UCH-L1, respectively.

One of the physiological functions of UCH-L1 is to interact with mono-Ub and to protect mono-Ub from degradation (Osaka et al., 2003). This function was confirmed using *gad* mice and nm3419 mutant mice, which lack UCH-L1 protein expression. The protein level of free mono-Ub in the brain stem or the sciatic nerve, the site where *gad* mice are most severely affected, was prominently decreased in the *gad* mice compared with controls. In addition, cerebrum of nm3418 mutant mice also contained decreased levels of mono-Ub compared to that of control mice (Walters et al., 2008). Likewise, UCH-L3 might interact with di-Ub to protect it from degradation. To confirm this possibility, we compared the protein level of di-Ub in several tissues of UCH-L3 knockout mice with that of wild-type mice. In contrast with our expectations, none of the tissues derived from the knockout mice showed lower di-Ub levels (Fig. 3C). However, the exogenous expression of UCH-L3 in 3T3 immortalized mouse embryonic fibroblast, derived from the UCH-L3 knockout mice, revealed that its expression up-regulated the protein level of di-Ub, while the D33A mutant, a mutant that lacks the ability to interact with di-Ub, did not show this effect (Fig. 3A and B). Thus, we cannot exclude the possibility that, under some conditions, UCH-L3 may interact with di-Ub to protect it from degradation.

Previous biochemical analysis indicated that UCH-L3 is unable to hydrolyze tandemly conjugated di-Ub or K48-linked di-Ub (Larsen et al., 1998). To confirm that UCH-L3 is not capable of hydrolyzing di-Ub, we also performed *in vitro* enzymatic assays using K48-linked, and K63-linked di-Ub. We choose these two forms of di-Ub, among the eight possible linkages because these two forms of di-Ub are known to exist at relatively high levels *in*

vivo (van Nocker and Vierstra, 1993; Hochstrasser, 2006). Consistent with the previous reports (Larsen et al., 1998), neither form of di-Ub was hydrolyzed by UCH-L3 even after 24 h incubation (data not shown). Thus, di-Ub is unlikely to be a substrate of UCH-L3.

Because the mutant analysis indicated that the catalytic pocket of UCH-L3 is affected by the interaction with di-Ub, another functional explanation of di-Ub interaction with UCH-L3 is that it might inhibit enzymatic activity. Indeed, the addition of K48-linked, K63-linked, and tandemly conjugated di-Ub, as well as mono-Ub showed inhibitory effects on hydrolyzing Ub-AMC (Fig. 4C). Of these three forms of di-Ub and mono-Ub, K48-linked di-Ub showed the most prominent inhibitory effect toward UCH-L3. A previous report indicated that nonhydrolyzable di-Ub analogues can inhibit Ub hydrolase activity of UCH-L3 where its activity was monitored by Ub-AMC (Yin et al., 2000). UCH-L3 was inhibited approximately equally by mono-Ub and di-Ub analogues linked through residues 11, 29, 48 (Kis of 200, 151, 199, and 107 μ M, respectively) and the dimer analogue linked through residues 63 and 76 (head-to-head tandem conjugation) with a Ki of 13 μ M (Yin et al., 2000). In addition, it has been reported that K48-linked di-Ub is the most abundant of all eight forms of di-Ub in tissues and cells. Thus, our results indicate that K48-linked di-Ub can function as an endogenous UCH-L3 inhibitor. Furthermore, we also found that addition of mono-Ub as well as K48-linked di-Ub inhibited the enzymatic activity of UCH-L1 (Fig. 4D). As UCH-L1 preferentially interacts with mono-Ub and the protein level of mono-Ub is high compared to di-Ubs in the cell, mono-Ub can function as endogenous inhibitor of UCH-L1. Together, these results indicate that di-Ub and mono-Ub regulate the enzymatic activity of UCH-L3 and UCH-L1, respectively.

In addition to Ub, UCH-L1 and UCH-L3 show affinity for Nedd8, an Ub-like protein, which shares 63% amino acid identity with Ub. Thus, these enzymes are thought to possess dual enzymatic activity toward the Ub and Nedd8 substrates. Although we could