

# Dorfin Localizes to the Ubiquitylated Inclusions in Parkinson's Disease, Dementia with Lewy Bodies, Multiple System Atrophy, and Amyotrophic Lateral Sclerosis

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**In many neurodegenerative diseases, the cytopathological hallmark is the presence of ubiquitylated inclusions consisting of insoluble protein aggregates. Lewy bodies in Parkinson's disease and dementia with Lewy bodies disease, glial cell inclusions in multiple system atrophy, and hyaline inclusions in amyotrophic lateral sclerosis (ALS) are representative of these inclusions. The elucidation of the components of these inclusions and the mechanisms underlying inclusion formation is important in uncovering the pathogenesis of these disorders. We hypothesized that Dorfin, a perinuclearly located E3 ubiquitin ligase, participates in the formation of ubiquitylated inclusions in a wide range of neurodegenerative diseases. Here, we report that affinity-purified anti-Dorfin antibody labeled ubiquitylated inclusions of Parkinson's disease, dementia with Lewy bodies disease, multiple system atrophy, and sporadic and familial ALS. A double-immunofluorescence study revealed that Dorfin shows a distribution pattern parallel to that of ubiquitin. Furthermore, by a filter trap assay, we detected that Dorfin is present in the ubiquitylated high-molecular weight structures derived from these diseases. These results suggest that Dorfin plays a crucial role in the formation of ubiquitylated inclusions of  $\alpha$ -synucleinopathy and ALS. However, because we failed to show the direct binding of  $\alpha$ -synuclein with Dorfin, future investigations into the binding partner(s) of Dorfin will be needed to deepen our understanding of the pathophysiology of  $\alpha$ -synucleinopathy and ALS. (*Am J Pathol* 2003, 163:609–619)**

Protein aggregates are formed when the cell fails to further degrade misfolded or mutated proteins. Protein

aggregates are generally difficult to unfold or degrade; their formation in cells is related to the pathogenesis of several common aging-related neurodegenerative diseases including Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), polyglutamine disease (Huntington's disease and spinocerebellar ataxias resulting from an expanded CAG repeat in their causative gene), and Alzheimer's disease.<sup>1,2</sup> These group of disorders are called conformational diseases, in which the underlying protein aggregation results from  $\beta$ -sheet linkages.<sup>1</sup> Furthermore, the characteristic intracellular inclusions composed of aggregated ubiquitylated protein surrounded by disorganized filaments are the common histopathological hallmark of many neurodegenerative diseases.<sup>3</sup> Lewy bodies (LBs) in PD and dementia with Lewy bodies (DLB), glial cell inclusions (GCIs) in multiple system atrophy (MSA), and hyaline and skein-like inclusions in ALS are representative of such inclusions.<sup>4–8</sup> To elucidate the mechanisms underlying inclusion body formation and neurodegeneration, it is important to know which protein components are involved.

We have reported previously that Dorfin is predominantly localized in neuronal hyaline inclusions found in familial ALS with *SOD1* mutation and in *SOD1*<sup>G93A</sup>-transgenic mice.<sup>9</sup> Dorfin is a gene product we cloned from the anterior horn tissues of the human spinal cord.<sup>10</sup> Its mRNA is ubiquitously expressed through the central nervous system, including the spinal cord. Dorfin contains a RING-finger/IBR (in-between ring-finger) domain<sup>11–13</sup> at its N-terminus and mediates E3 ubiquitin (Ub) ligase activity.<sup>10</sup> Dorfin physically binds and ubiquitylates various *SOD1* mutants derived from familial ALS patients and enhances their degradation, but it has no effect on the stability of wild-type *SOD1*.<sup>9</sup> Overexpression of Dorfin protects neural cells against the toxic effects of mutant *SOD1* and reduces *SOD1* inclusions.<sup>9</sup> Our previous results indicate that Dorfin protects neurons by recognizing

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and then ubiquitylating mutant SOD1 proteins, subsequently targeting them for proteasomal degradation.

Mutant SOD1 protein is fairly unstable compared to its wild-type, and toxic gain of function is thought to be related to this unstable conformation.<sup>14,15</sup> Recently, CHIP (carboxyl terminus of Hsc70-interacting protein), U-box type E3, has been shown to interact with Hsp90 or Hsp70 and to ubiquitylate unfolded proteins trapped by these molecular chaperones, thus acting as a quality control E3.<sup>16–18</sup> The physiological role of Dorfin remains unknown, but it may be regarded as another quality control E3 because it can discriminate between the normal and abnormal status of SOD1 proteins.<sup>9</sup> In cultured cells, Dorfin resides in the perinuclear region and forms aggregate-like structures.<sup>10</sup> Aggregates are perinuclear cytoplasmic inclusions containing misfolded ubiquitylated proteins that appear when the cell fails to further degrade such proteins.<sup>19,20</sup> Thus, an important and interesting question in this context is whether Dorfin plays a role in neurodegenerative diseases with cytosolic ubiquitylated inclusions other than familial ALS with SOD1 mutations through ubiquitylation of target proteins. To address this question, using immunohistochemical analysis of Dorfin, we examined various neurodegenerative diseases with ubiquitylated inclusion bodies, including  $\alpha$ -synucleinopathy (sporadic PD, DLB, and MSA) as well as motor neuron disease (sporadic and familial ALS). We here report that Dorfin co-localizes to the ubiquitylated inclusion bodies in these neurodegenerative diseases, and we suggest that Dorfin plays an important role in the disease process.

## Materials and Methods

### Tissue Samples

The participants of this study were five PD patients (age, 67 to 79 years; four men and one woman), five cases of DLB (age, 65 to 78 years; four men and one woman), five with MSA (age, 60 to 72 years; three men and two women), two men with sporadic ALS (SALS) (age, 68 and 69 years), one man with familial ALS (FALS) (57 years), and five controls without neurological disease (C; age, 61 to 78 years; four men and one woman). Diagnoses of all cases were confirmed by clinical and pathological diagnostic criteria for each disease.<sup>21–23</sup> The brain and spinal cord were removed at autopsy performed 4 to 12 hours postmortem. The midbrain for PD, cerebral cortex of the temporal lobe for DLB, putamen and midbrain for MSA, and the spinal cords for SALS and FALS were excised and subjected to extensive study for each disease. These tissues were fixed in 20% buffered formalin and embedded in paraffin.

### Characterization of Anti-Dorfin Antibody

Polyclonal rabbit antiserum (Dorfin-30) was raised against a C-terminal portion (amino acids 678 to 690) of Dorfin as described.<sup>10</sup> A synthetic peptide, RKIHNRYEGKD-VSKHKRN (corresponding to amino acid sequence of residues 396 to 413 of Dorfin), was used for immunization

in rabbit and affinity-purified to raise another polyclonal antiserum against Dorfin (Dorfin-41). Brain (cerebral cortex and putamen) and spinal cord tissues from normal controls without neurological disease were homogenized in sodium dodecyl sulfate (SDS) lysis buffer (10 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 2% SDS) with a protease inhibitor mixture (Complete; Roche Diagnostics, Basel, Switzerland) and were fractionated by centrifugation at  $16,000 \times g$ . The protein concentration was determined with a DC protein assay kit (Bio-Rad, Hercules, CA), and supernatants were used for Western blotting analysis. Construction of an N-terminal Xpress-tagged Dorfin expression vector (pcDNA4/HisMax-Dorfin) and Myc-tagged Ub expression vector (pcDNA3.1Myc-Ub) was reported elsewhere.<sup>10</sup> A C-terminal Myc-tagged Dorfin expression vector was constructed from cDNA containing the entire coding region of Dorfin inserted in-frame into the *KpnI* and *XbaI* site of pcDNA3.1/MycHis(+) vector (Invitrogen, Carlsbad, CA). A N-terminal FLAG-tagged Dorfin expression vector was constructed from cDNA containing the entire coding region of Dorfin inserted in-frame into the *Clal* and *KpnI* site of pFLAG-CMV-2 vector (Sigma, St. Louis, MO). pcDNA3.1(+)-FLAG-CHIP and pcDNA3.1(+)-FLAG-parkin were kind gifts from Dr. Keiji Tanaka (Tokyo Metropolitan Institute of Medical Science) and Dr. Nobutaka Hattori (Juntendo University School of Medicine), respectively. Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Transfections were performed using the Effectene transfection reagent (Qiagen, Hilden, Germany). Cells were cultured for 24 hours and lysed in TNES lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Nonidet P-40, and 0.1% SDS) with a protease inhibitor mixture (Roche Diagnostics). The protein concentration was determined with a DC protein assay kit (Bio-Rad), and lysates were electrophoresed by SDS-polyacrylamide gel electrophoresis and transferred to Hybond-P polyvinylidene difluoride membrane (Amersham Pharmacia, Piscataway, NJ) for Western blotting. The membranes were blocked in 5% milk in Tris-buffered saline (TBS) containing 0.1% Tween-20, and incubated overnight at 4°C with Dorfin antiserum (1:5000 dilution). The blots were then washed three times for 10 minutes each in TBS with 0.1% Tween 20, followed by a 1-hour incubation in horseradish peroxidase coupled to secondary antibody (1:5000 dilution, Amersham Pharmacia). The blots were then washed three times for 10 minutes each in TBS with 0.1% Tween 20 before incubation in enhanced chemiluminescence reagent (Amersham Pharmacia) and exposure to film.

### Immunohistochemistry and Immunoelectron Microscopy

Immunohistochemistry was performed as described previously.<sup>24–26</sup> Four- $\mu$ m-thick sections were obtained from the paraffin-embedded midbrain, cerebral cortex, putamen, hippocampus, and spinal cord of the patients with PD, DLB, MSA, SALS, FALS, and controls, respectively. These sections were immunostained using the avidin-

biotin-peroxidase complex method with 3,3-diaminobenzidine tetrahydrochloride (Wako, Osaka, Japan) as a chromogen. The immunolabeled sections were lightly counterstained with hematoxylin. Dorfin antiserum (1:200 dilution in both Dorfin-30 and Dorfin-41), and anti-Ub (P4D1, 1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used. For staining with anti-Dorfin antiserum, the sections were pretreated with 99% formic acid (Wako) for 5 minutes at room temperature. Specificity of anti-Dorfin antibody on immunostaining for human tissue was assessed by the preabsorption of antibody with peptide antigen. To assess the co-localization of Dorfin and Ub, a double-labeling immunofluorescence study was performed on the selected sections with a combination of anti-Dorfin and anti-Ub antibodies. Anti-Dorfin antibody was visualized by anti-rabbit goat IgG coupled with Alexa Fluor 568 (Molecular Probes, Eugene, OR), and anti-Ub antibody was visualized with anti-mouse sheep IgG coupled with Alexa Fluor 488 (Molecular Probes), and observed under a LSM-510 confocal microscope (Carl Zeiss, Gottingen, Germany). To assess Dorfin immunoreactivity in ubiquitinated inclusions, serial sections were prepared for every two serial sections; one was stained with anti-Dorfin antibody and the other with anti-Ub antibody. The ratio of Dorfin-positive inclusion bodies among Ub-positive inclusions was evaluated by assessing 40 to 120 Ub-positive inclusion bodies in each case of PD, DLB, MSA, and at least 10 inclusion bodies in FALS and SALS.

Immunoelectron microscopy was performed as previously described.<sup>24,25</sup> The selected deparaffinized sections were immunostained with Dorfin-30 antibody in the same manner, then washed in phosphate buffer, post-fixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Ultrathin sections were prepared and examined under an H-7000 electron microscope (Hitachi, Tokyo, Japan). Each immunohistochemical reaction was ascertained by substituting normal rabbit or mouse sera for the primary antibodies, and no specific peroxidase reactions occurred in these control experiments.

### *Filter Trap Assay*

For this assay, ~100- to 200-mg tissues from the cerebral cortex of DLB, putamen of MSA, and the cerebral cortex and spinal cord of ALS and control were used. A filter trap assay was performed as previously described.<sup>27</sup> Tissues were homogenized in 10 vol of TBS. Homogenates were centrifuged at  $800 \times g$  for 10 minutes at 4°C and the supernatants were diluted with 10 vol of TBS with 0.1% SDS. Protein concentrations were determined with a DC protein assay kit (Bio-Rad) and, using a slot blot device (Bio-Rad), the supernatants were filtered under vacuum through 0.22- $\mu\text{m}$  cellulose acetate membranes (Sartorius, Gottingen, Germany) followed by two washes in TBS. The membranes were then incubated in 5% dry milk in TBS at room temperature for 1 hour, followed by an overnight incubation at 4°C with Dorfin-30 (1:5000 dilution), anti-Ub (1:1000 dilution; Zymed, San Francisco,

CA) or anti- $\alpha$ -synuclein (LB509, 1:1000 dilution; Zymed) antibody in TBS with 0.1% Tween 20. Horseradish peroxidase-conjugated second antibodies (1:5000, Amersham Pharmacia) were used and detected with enhanced chemiluminescence reagent (Amersham Pharmacia). To confirm equal loading of proteins, the same samples were filter trapped using 0.45- $\mu\text{m}$  nitrocellulose membranes (Bio-Rad) and were probed with anti- $\alpha$ -tubulin antibody (1:1000 dilution, Sigma).

### *Fractionation of Normal and Diseased Brain Tissues*

Approximately 100- to 200-mg tissues of cingulate gyrus from normal or DLB brains, 200-mg tissues of putamen from MSA brains, or 400-mg ALS spinal cord were homogenized in 10 vol of lysis buffer A (50 mmol/L Tris-HCl at pH 7.5, 500 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, and 10 mmol/L NaF) with a protease inhibitor mixture (Complete, Roche Diagnostics) and centrifuged at  $16,000 \times g$  for 30 minutes at 4°C. Resulting pellets were sequentially extracted by homogenization in Triton X-100 (buffer A containing 1% Triton X-100), and urea (50 mmol/L Tris-HCl, 8 mol/L urea, 1 mmol/L EGTA) followed by centrifugation at  $100,000 \times g$ .

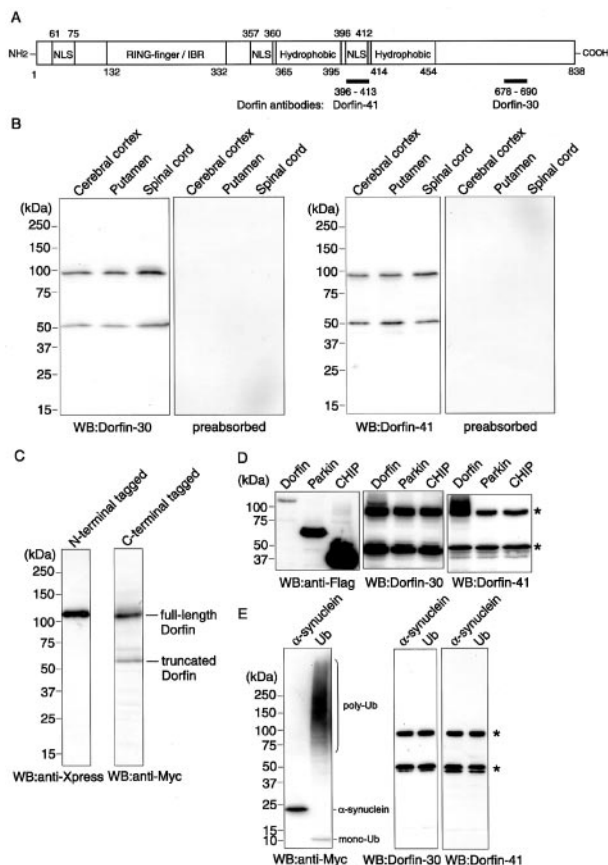
### *Immunoprecipitation*

$\alpha$ -Synuclein cDNA was amplified by polymerase chain reaction from human brain cDNAs and cloned into the EcoRV site of pcDNA3.1/MycHis(+) (Invitrogen). To generate the mutant  $\alpha$ -synuclein expression vector, A30P and A53T mutations were introduced into the pcDNA3.1/MycHis- $\alpha$ -synuclein with a QuickChange site-directed mutagenesis Kit (Stratagene, La Jolla, CA) according to Lee and colleagues.<sup>28</sup> Construction of pcDNA3.1/MycHis-wild-type and G85R mutant SOD1 vector was previously described.<sup>9</sup> Xpress-tagged Dorfin (pcDNA4/His-Max-Dorfin) and Myc-tagged  $\alpha$ -synuclein or SOD1 were transiently expressed using the Effectene transfection reagent (Qiagen) in HEK293 cells. Cells were lysed in TNES lysis buffer with a protease inhibitor mixture (Roche Diagnostics). To inhibit cellular proteasome activity, cells were treated with 0.5  $\mu\text{mol/L}$  MG132 (Z-Leu-Leu-Leu-al; Sigma) for 16 hours after overnight posttransfection. Immunoprecipitation from the transfected cell lysates was performed with 2  $\mu\text{g}$  of anti-Xpress antibody (Invitrogen) and protein A/G Plus agarose (Santa Cruz Biotechnology), and then washed four times in lysis buffer. Immunoprecipitates were analyzed by Western blotting with enhanced chemiluminescence detection reagents (Amersham Pharmacia).

## **Results**

### *Specificity of Anti-Dorfin Antibody*

To examine the pathophysiological role of Dorfin, we raised affinity-purified antisera to two separate regions of human Dorfin (Figure 1A) and characterized their speci-



**Figure 1.** Characterization of affinity-purified antibody to human Dorfin. **A:** Schematic diagram of human Dorfin and peptide antibodies. IBR, in-between ring-finger; NLS, nuclear localization signal-like sequence. **B:** SDS-polyacrylamide gel electrophoresis and Western blotting analysis of the extracted protein from human central nervous tissues. Soluble extracts from normal adult brains (cerebral cortex and putamen) and spinal cord (50  $\mu$ g each) were used and probed with antibodies to Dorfin. Note that antiserum preabsorbed with excess peptide antigen shows no staining. **C:** SDS-polyacrylamide gel electrophoresis and Western blotting analysis of lysates of HEK293 cells expressing Xpress-Dorfin (left) or Dorfin-Myc (right). Note that only C-terminal Myc-tagged Dorfin shows truncated fragment. **D:** Specific binding of anti-Dorfin antibodies to Dorfin proteins. Lysates of HEK293 cells expressing FLAG-tagged Dorfin, parkin, and CHIP were analyzed by Western blotting with antibodies to Dorfin. Both Dorfin-30 and Dorfin-41 recognize only Dorfin fusion proteins. **E:** Anti-Dorfin antibodies do not cross-react with  $\alpha$ -synuclein and Ub. Lysates of HEK293 cells expressing Myc-tagged  $\alpha$ -synuclein and Ub were analyzed by Western blotting with antibodies to Dorfin. Both Dorfin-30 and Dorfin-41 recognize only endogenous Dorfin. **Asterisks** on the right indicate endogenous Dorfin.

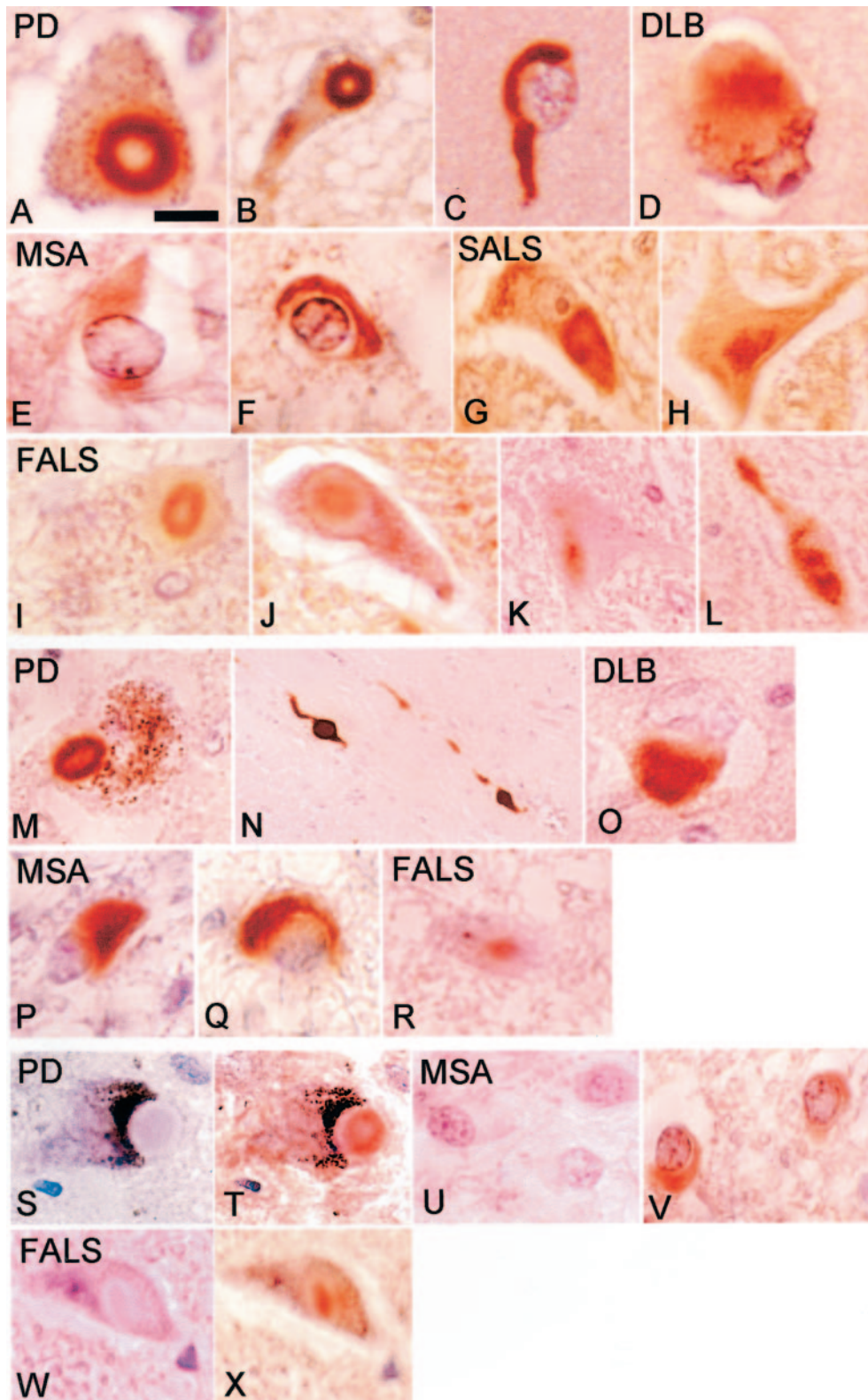
ficity. First, we tested Dorfin antisera by Western blotting under reduced conditions against brain and spinal cord tissues from a control subject without neurological disease. In this assay, both Dorfin-30 (amino acids 678 to 690) and Dorfin-41 (amino acids 396 to 413) recognized these tissues' cognate antigens in a specific manner, and recognized ~98-kd and ~52-kd protein of human brain extracts as well as those of spinal cord extracts (Figure 1B, left). Their reactivities were abolished by preabsorption with the excess of peptide antigens (Figure 1B, right). The ~98-kd protein probably corresponds to the full-length Dorfin protein because the calculated molecular weight of Dorfin is ~91 kd. The ~52-kd band is thought to be a truncated C-terminal fragment of Dorfin recognized by Dorfin antiserum. To confirm this inference, we next

examined the size of N-terminal Xpress-tagged and C-terminal Myc-tagged Dorfin fusion proteins expressed in the cultured cell line HEK293 by Western blotting. Both anti-Xpress and anti-Myc antibody recognized ~102-kd full-length proteins, but only anti-Myc antibody to the C-terminal tag revealed the ~60-kd truncated C-terminal fragment (Figure 1C). Similar results have been reported by another laboratory, using various mouse tissues with anti-XYbp antiserum (*XYbp* is a mouse orthologue of *Dorfin*) in Western blotting analysis.<sup>29</sup> Furthermore, we determined the selective specificity of our antibodies to Dorfin. We assessed whether our antibodies recognize other E3 ligases, although two epitope regions, we have chosen to create anti-Dorfin antibodies, show no homology to other proteins with BLASTp searches in the database of National Center for Biotechnology Information. We chose E3 ligases, parkin, and CHIP as controls, because parkin has RING-finger/IBR domain,<sup>11</sup> localizes in the LBs,<sup>30</sup> and ubiquitylates unfolded protein(s)<sup>31</sup> as well as Dorfin, and CHIP is also E3 ligase for abnormal proteins as the so-called protein quality control ligase<sup>16-18</sup> and interacts with parkin to enhance its activity.<sup>32</sup> N-terminal FLAG-tagged fusion proteins of Dorfin, parkin, and CHIP were examined by Western blotting with Dorfin-30 and Dorfin-41 antibodies. Both anti-Dorfin antibodies recognized ~102-kd full-length fusion proteins and endogenous Dorfin, but did not recognized parkin and CHIP at all (Figure 1D). In addition, we confirmed that anti-Dorfin antibodies do not cross-react with  $\alpha$ -synuclein and Ub (Figure 1E).

### *Dorfin Localizes to Ubiquitylated Inclusion Bodies of PD, DLB, MSA, and ALS*

To assess Dorfin immunoreactivity in the involved lesions of the central nervous system of neurodegenerative diseases, we examined the inclusion body-rich regions of the brain and spinal cord sections from sporadic PD, DLB, MSA, sporadic and familial ALS, and normal controls by light and electron microscopic immunohistochemistry.

In PD, both anti-Dorfin antisera, Dorfin-30 and Dorfin-41, labeled LBs of various types existing inside and outside the substantia nigra. More intense immunoreactivity was observed in LBs with Dorfin-41 antibodies. The peripheral rims of typical LBs, either round or elongated, in neuronal cell bodies and in processes were strongly stained, whereas the central cores remained unstained or only weakly stained (Figure 2; A, B, and M). The pale body, Lewy neuritis, axonal spheroids in substantia nigra, and the glial inclusions were also immunostained by both anti-Dorfin antibodies (Figure 2, C and N). Ubiquitin was predominantly seen in rims of LBs, but sometimes in the core of LB. Most, if not all,  $\alpha$ -synuclein-positive LBs are also ubiquitin-positive<sup>33</sup> and we have previously shown that Dorfin co-localized with ubiquitylated hyaline inclusions in ALS.<sup>9</sup> Thus, we counted Dorfin-positive inclusions in comparison with ubiquitin. Serial sections stained with anti-Dorfin and anti-Ub antibodies showed that  $40.9 \pm 12.1\%$  of Ub-positive LBs were positive for Dorfin-30, and  $92.2 \pm 11.8\%$  of them were positive for Dorfin-41



**Figure 2.** Light microscopic Dorfin immunohistochemistry in neuronal and glial inclusions of PD, DLB, MSA, SALS, and FALS. Immunostainings with Dorfin-30 antibody (**A–L, P–X**) and Dorfin-41 antibody (**M–O**) are shown. LBs in the substantia nigral neurons (**A** and **M**) and in the Edinger-Westphal nucleus (**B**) of PD patients are heavily Dorfin-immunoreactive. GCIs (**C**) and Lewy neuritis (**N**) of PD patients are also Dorfin-immunoreactive. Cortical LBs in the temporal cortex of the DLB patient are Dorfin-positive (**D** and **O**). Glial inclusions of the oligodendroglia in MSA patients in the putamen are Dorfin-immunoreactive (**E, F, P**, and **Q**). Dorfin is also localized in the cytoplasmic inclusions of the spinal motor neurons of SALS patients (**G** and **H**). Dorfin is localized in the LB-like inclusions (**I, J, K**, and **L**) and skein-like inclusions (**K** and **L**) in the remaining motor neurons of the FALS spinal cord. Each inclusion was strongly immunostained by Dorfin (**T, V**, and **X**), whereas preabsorbed antibody abolished most immunoreactivity (**S, U**, and **W**). **S** and **T**: LBs in pigmented neuron of the substantia nigra in PD. **U** and **V**: GCIs of the putamen in MSA. **W** and **X**: LB-like inclusion of hypoglossal neuron of a FALS case. Scale bar in **A** is equivalent to: 20  $\mu\text{m}$  (**A, B, G, J, L**, and **M**); 10  $\mu\text{m}$  (**C, D, I**, and **O**); 5  $\mu\text{m}$  (**E, F, P**, and **Q**); 16  $\mu\text{m}$  (**H, K**, and **R**), 52  $\mu\text{m}$  (**N**), 12  $\mu\text{m}$  (**S–V**); 8  $\mu\text{m}$  (**W** and **X**).

**Table 1.** Dorfin and Ubiquitin Immunoreactivity in Neuronal and Glial Inclusions of PD, DLB, MSA, and ALS

Antibody	Dorfin (+) inclusions (assessed number)	Ubiquitin (+) inclusions (assessed number)	Dorfin (+)/ubiquitin (+) (%)
PD ( <i>n</i> = 5)			
Dorfin-30	33.8 ± 11.4	68.6 ± 10.5	40.9 ± 12.1
Dorfin-41	52.4 ± 6.5	57.2 ± 4.1	92.2 ± 11.8
DLB ( <i>n</i> = 5)			
Dorfin-30	34.2 ± 12.8	53.6 ± 17.8	62.6 ± 13.7
Dorfin-41	52.4 ± 4.3	64.0 ± 8.0	85.4 ± 9.2
MSA ( <i>n</i> = 5)			
Dorfin-30	85.0 ± 18.5	125.0 ± 28.9	70.9 ± 20.5
Dorfin-41	69.0 ± 13.5	73.8 ± 9.2	92.9 ± 7.5
SALS ( <i>n</i> = 2)			
Dorfin-30	7	17	41.2
Dorfin-41	4	17	23.5
FALS ( <i>n</i> = 1)			
Dorfin-30	15	31	48.4
Dorfin-41	14	31	45.2

(+), Immunoreactive.

Numbers of Dorfin- and ubiquitin-immunoreactive inclusions were assessed on 10 consecutive sections. Values are shown as mean ± SD for samples indicated in parentheses.

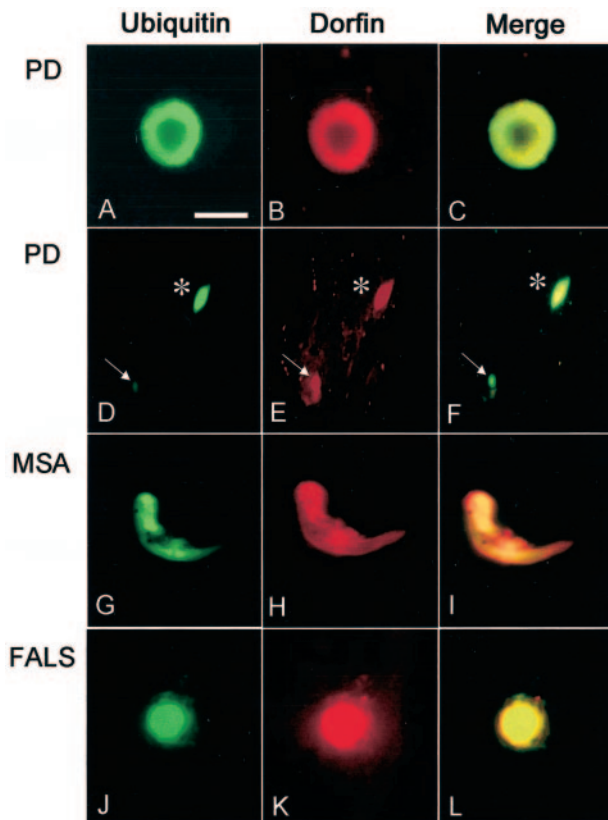
(Table 1). In DLB, Dorfin-30 and Dorfin-41 labeled the cortical type LB (Figure 2, D and O). Lewy neurites in the hippocampal CA2-3 region and glial inclusions were also immunolabeled by anti-Dorfin antibody as they were in PD (data not shown). The 62.6 ± 13.7% of the Ub-positive cortical LBs were immunoreactive for Dorfin-30, and the 85.4 ± 9.2% of them were immunoreactive for Dorfin-41 (Table 1). In MSA, GCIs observed in the oligodendroglia were immunostained by anti-Dorfin antibody. Dorfin-immunoreactive inclusions appear as flame-shaped (Figure 2, E and P) or sickle-shaped (Figure 2, F and Q) in glial cells. The 70.9 ± 20.5% of ubiquitin-positive GCIs were labeled by Dorfin-30 antibody, and 92.2 ± 11.8% were by Dorfin-41 antibody (Table 1). Only a few of the neuronal cytoplasmic inclusions and neuronal and glial nuclear inclusions were positive for Dorfin (data not shown). In SALS and FALS, eosinophilic, Ub-positive cytoplasmic inclusions and skein-like inclusions were found in motor neurons of the spinal cord.<sup>7</sup> LB-like hyaline inclusions (LBHIs) were also ubiquitylated.<sup>7</sup> Dorfin-30 and Dorfin-41 labeled some of these inclusions, such as the ring-, round-, irregular round-, filamentous rod-, and loose ball-shaped types (Figure 2; G to L and R). Dorfin immunoreactivity was strongly present in the central core of the round- or irregular round-shaped inclusions, and in the middle layer of the ring-shaped inclusions, whereas the outer layers of these inclusions were less immunoreactive (Figure 2; H to J). The Bunina body, which was negative for anti-Ub antibody, was not stained with Dorfin-30 and Dorfin-41 antibody (data not shown). Dorfin-30 was positive at 48.4% of Ub-positive inclusions in SALS and 41.2% in FALS, and Dorfin-41 was positive at 23.5% in SALS and 45.2% in FALS (Table 1). These inclusions were only weakly stained by Dorfin-41. Preabsorbed anti-Dorfin antibody abolished most immunoreactivity (Figure 2; S to X), indicating that our anti-Dorfin antibody specifically recognizes Dorfin-epitopes on the inclusions of these tissues. In normal control, no Dorfin-immunoreactive structure was detected (data not shown).

A double-labeled immunofluorescence study revealed that Dorfin-30 and Ub were co-localized in LBs (Figure 3; A to C). Pale bodies, which have been considered to be the precursors of LB,<sup>34</sup> and Lewy neurites were also immunolabeled by anti-Dorfin and anti-Ub antibodies (Figure 3; D to F). GCIs in MSA (Figure 3; G to I), and the hyaline inclusions in FALS (Figure 3; J to L) were also Dorfin- and Ub-immunoreactive.

At the immunoelectron microscopic level, Dorfin-30 immunoreactivity was localized on filamentous structures, particularly the halo of the LBs that was composed of the radially arranged intermediate filaments associated with granular materials and vesicular structures. These radial filaments and associated structures were strongly immunostained by anti-Dorfin antibody, whereas the central core was not stained (Figure 4, A and B). Thus, the staining profile of Dorfin was very similar to that of Ub and  $\alpha$ -synuclein,<sup>33</sup> but different from that of parkin, which localizes predominantly to the core of LBs.<sup>30</sup> Dorfin immunoreactivity in GCI of MSA was composed of randomly arranged tubules or filamentous structures associated with granular materials (Figure 4, C and D). In FALS, the thicker, granule-coated filaments were decorated by the Dorfin immunoreactive deposit and formed the core, whereas the thinner filaments without a granular coating were not recognized by anti-Dorfin antibody and formed the halo (Figure 4, E and F).

#### *Dorfin Accumulation in Ubiquitylated High-Molecular Weight Complexes*

In studies of polyglutamine disorders, it has been demonstrated that high-molecular weight aggregates of mutant proteins are retained by filtration through cellulose acetate.<sup>35,36</sup> In a *SOD1*-transgenic mice ALS model, this assay is also applied to detect mutant *SOD1* aggregation.<sup>27</sup> Cellulose acetate membranes usually bind protein very poorly and are used to filter high-molecular weight structures from complex mixtures.<sup>35</sup> Thus we investi-



**Figure 3.** Co-localization of Dorfin-30 immunoreactivity with Ub in neuronal and glial inclusions. Sections were doubly labeled with Dorfin antiserum and an antibody against Ub and analyzed with a laser-scanning confocal microscope. Panels at **left (green)** correspond to Ub, **middle panels (red)** correspond to Dorfin, and panels at **right** correspond to merged images; structures in yellow indicate co-localization. Dorfin is co-localized with Ub in ubiquitylated inclusions in the nigral neurons of PD (**A–F**), in glial cells in the putamen of MSA (**G–I**), and in spinal motor neurons of FALS (**J–L**). Co-localization of Dorfin and Ub is also seen in the pale body (**arrow**) and Lewy neurite (**asterisk**) in (**D–F**). Scale bar in **A** is equivalent to: 20  $\mu\text{m}$  (**A–C**) and 100  $\mu\text{m}$  (**D–F**); 5  $\mu\text{m}$  (**G–I**).

gated whether Dorfin is retained in high-molecular weight aggregates from  $\alpha$ -synucleinopathy and ALS tissues by cellulose acetate filter trap assay. Homogenates of brain and spinal cord tissues from DLB, MSA, SALS, and controls were solubilized in TBS with 0.1% SDS, then filtered through a 0.22- $\mu\text{m}$  cellulose acetate membrane. We have chosen the cerebral cortex for DLB, putamen for MSA, and the cerebral cortex and spinal cord for SALS for this assay, because these regions exhibit the most prominent pathology in each disease. Subsequent staining with Dorfin antiserum revealed trapped proteins (Figure 5A, top), which were also recognized by antibodies to Ub (Figure 5A, top middle). Furthermore, in DLB and MSA brains,  $\alpha$ -synuclein was trapped, as expected (Figure 5A, bottom middle). Interestingly, high-molecular weight aggregates were difficult to detect in extracts of the cerebral cortex of SALS, in which pathological changes are less severe compared to those in the spinal cord. No high-molecular weight aggregate was present in either brain or spinal cord samples from normal control.

Sequential detergent extraction methods have been successfully applied to detect high-molecular weight complexes in brain of patients with  $\alpha$ -synucleinopa-

thies.<sup>37,38</sup> We used this method to detect insoluble Dorfin molecules in inclusion-rich tissues. Buffer- and Triton X-100-soluble  $\sim$ 98-kd and  $\sim$ 52-kd Dorfin were detected in brains from normal control as well as from DLB, MSA, and ALS patients (Figure 5B). In contrast to urea extracts of normal brain that were devoid of Dorfin immunoreactivities,  $\sim$ 98-kd full-length and  $\sim$ 52-kd truncated Dorfin were found in urea extracts from DLB, MSA, and ALS patients (Figure 5B). Bands,  $\sim$ 200 kd,  $\sim$ 45 kd and  $\sim$ 35 kd bands, were also detected (Figure 5B). These Dorfin-immunoreactive bands were not recognized by anti-Ub antibody (data not shown), and higher molecular weight bands may represent dimeric forms of Dorfin and smaller molecular weight species may be processed products of Dorfin.

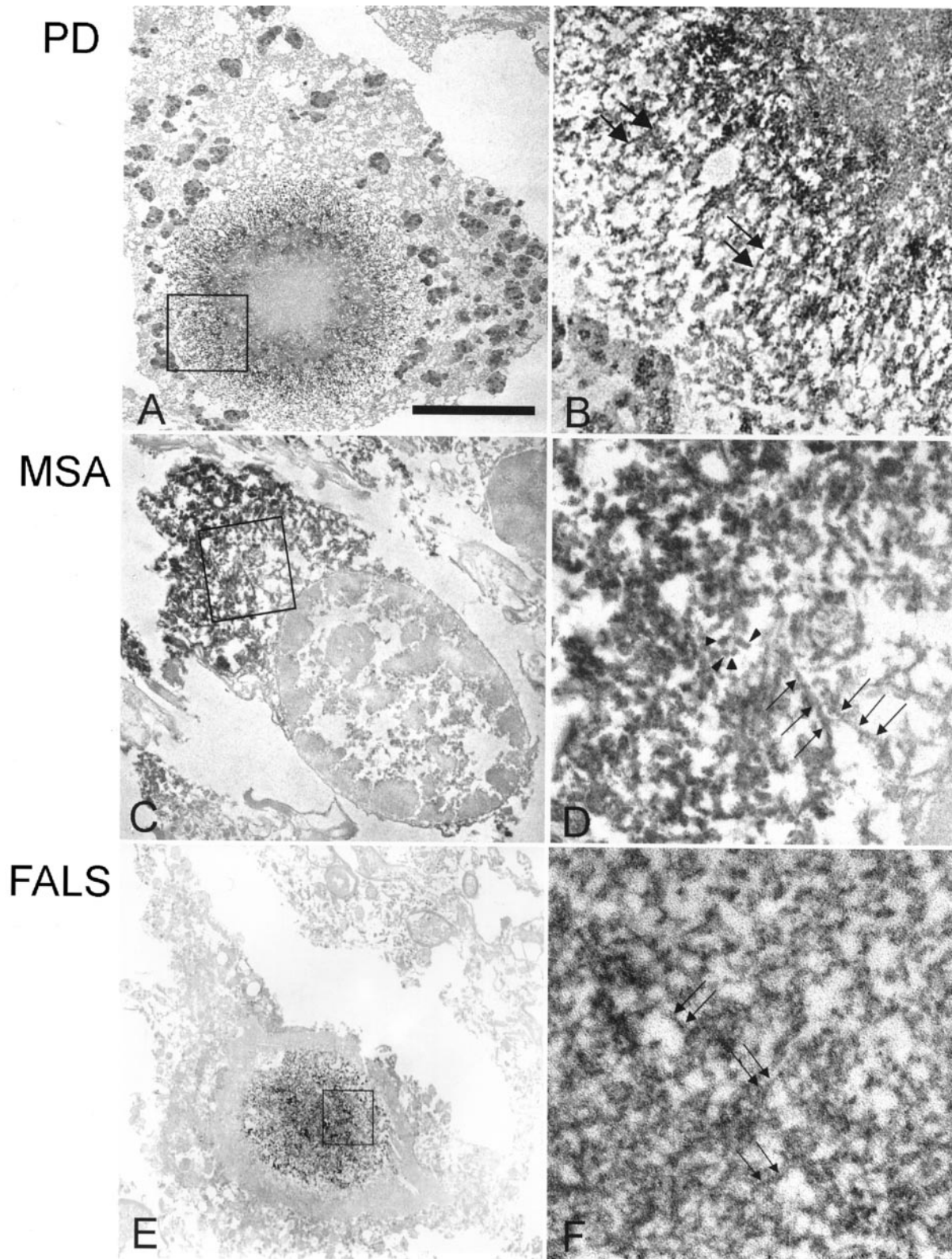
These observations indicated that Dorfin is present in high-molecular weight structures in the tissues of  $\alpha$ -synucleinopathy and ALS, suggesting that Dorfin is a major component of aggregated and ubiquitylated proteins forming inclusion bodies in these neurodegenerative disorders. Full-length Dorfin antigen is present in abnormal inclusions, but Triton-insoluble-urea-soluble higher molecular weight Dorfin and processed fragments seem to be major building blocks for inclusion bodies.

### *Dorfin Does Not Bind to Wild-Type and Mutant $\alpha$ -Synuclein*

$\alpha$ -Synuclein is the main structural component of the insoluble protein aggregates that form the LBs of PD and DLB as well as the GCIs of MSA<sup>37–39</sup> and it has been shown to be ubiquitylated by an E3 Ub ligase, parkin.<sup>40</sup> As demonstrated, Dorfin is another major component of inclusion bodies in  $\alpha$ -synucleinopathy, and because it has E3 activity,<sup>9,10</sup> we examined whether it interacts with  $\alpha$ -synuclein *in vivo*. To this end, Xpress-tagged Dorfin was co-expressed with Myc-tagged wild-type or mutant forms of  $\alpha$ -synuclein in HEK293 cells (Figure 6). In our experimental system, exogenously expressed  $\alpha$ -synuclein was not phosphorylated (data not shown). Western blotting analysis after immunoprecipitation revealed that Dorfin binds with neither wild-type nor mutant  $\alpha$ -synuclein. However, it strongly bound with mutant SOD1 (Figure 6), as we reported previously.<sup>9</sup> *In vitro* ubiquitylation assay using immunoprecipitated  $\alpha$ -synuclein from transformed HEK293 cells, Dorfin did not ubiquitylate wild-type and mutant  $\alpha$ -synuclein (data not shown).

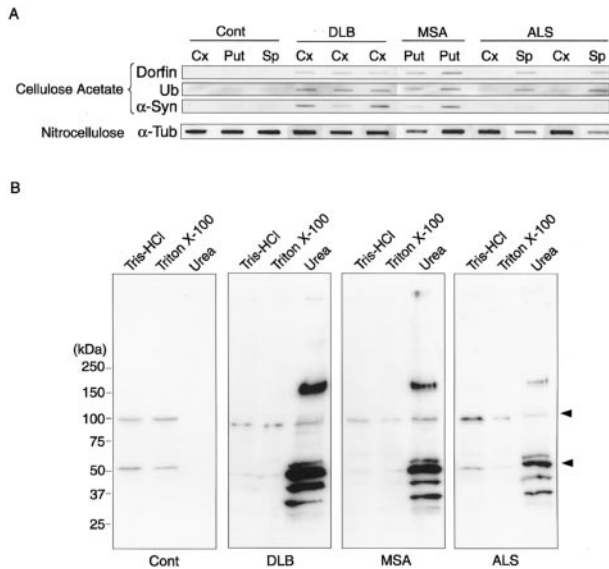
### *Discussion*

In the present study, we showed that Dorfin co-localizes to the ubiquitylated inclusions in common neurodegenerative diseases, including LBs in PD and DLB, GCIs in MSA, and hyaline and skein-like inclusions in ALS. Moreover, filter-trapped high-molecular weight structures contained Dorfin, indicating that it is a major constituent of these inclusions irrespective of the different disease etiologies and different morphological features of these inclusion bodies.



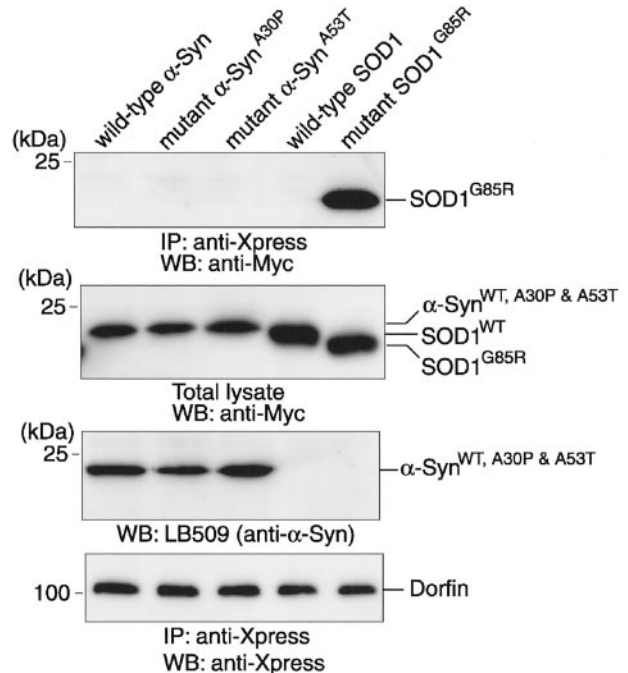
**Figure 4.** Immunoelectron microscopy of the neuronal and glial inclusions of PD, MSA, and FALS using the Dorfin-30 antibody. **A** and **B**: Typical LB immunostained with anti-Dorfin antibody in a pigmented neuron of substantia nigra in PD. The peripheral radiating filaments are strongly stained, and the central core is not Dorfin-immunoreactive. The immunoreactive filaments labeled with anti-Dorfin-30 antibody in **A** are shown at higher magnification (**arrows** in **B**) from area in the **square** in **A**. **B** shows that granular and fibrous materials are strongly positive for Dorfin, and are arranged radially. **C** and **D**: GCI of the oligodendrocyte is positive for Dorfin immunoreactivity in MSA. The granular materials (**arrowheads** in **D**) and the fibrous structures (**arrows** in **D**) are strongly stained with the Dorfin antibody from area in the **square** in **C**. **E** and **F**: LB-like hyaline inclusion in the spinal motor neurons in FALS. The filamentous structures (**arrows** in **F**) in the central core are strongly stained with anti-Dorfin antibody, but the filaments in the halo are not. **F** shows higher magnification of the central core region (**square**) in **E**. The granule-associated thick filaments are decorated. Scale bar in **A** is equivalent to: 5.5  $\mu\text{m}$  (**A** and **E**); 2.5  $\mu\text{m}$  (**C**); 0.8  $\mu\text{m}$  (**B**); 0.83  $\mu\text{m}$  (**D**); 0.85  $\mu\text{m}$  (**F**).





**Figure 5.** Dorfin accumulation in ubiquitylated high-molecular weight complexes. **A:** Detection of Dorfin in high-molecular weight structures in DLB, MSA, and SALS. Homogenates from brain and spinal cord of these diseases were filtered through cellulose acetate membranes and detected with Dorfin-30 antibody (**top**), anti-Ub antibody (**top middle**), and anti- $\alpha$ -synuclein antibody (**bottom middle**). Cx, cerebral cortex; Put, putamen; Sp, spinal cord. Nitrocellulose slot-blot probed with anti- $\alpha$ -tubulin antibody was used to confirm loading of equal amounts of samples (**bottom**). These results were replicated in duplicate experiments with different DLB, MSA, SALS, and control cases. **B:** Sequential extraction of Dorfin. Brains (normal, DLB, and MSA) and spinal cord (ALS) samples were sequentially extracted based on their solubility into Tris-HCl, Triton X-100, and urea buffers. Dorfin immunoreactivities were recovered in Tris and Triton fractions as ~98-kd and ~52-kd bands. In addition to these bands, Dorfin-30 antibody detected ~200-kd, ~45-kd, and ~35-kd bands in DLB, MSA, and ALS urea extracts, but not in normal brain urea extract. The **arrowheads** indicate ~98-kd full-length and ~52-kd truncated Dorfin bands.

The following among our reported observations support the view that Dorfin plays an important role in the formation of ubiquitylated inclusion bodies in  $\alpha$ -synucleinopathy and ALS: 1) the presence of Dorfin in the inclusion bodies of these diseases, 2) the parallel distribution patterns of Ub and Dorfin, 3) the perinuclear aggresome-like localization of Dorfin in cultured cells,<sup>10</sup> and 4) the E3 Ub ligase function of Dorfin and the resultant generation of mutant SOD1-(Ub)<sub>n</sub> conjugates.<sup>9,10</sup> The relation of Dorfin to  $\alpha$ -synucleinopathies and ALS shows striking similarities to the relation between parkin and PD. Parkin, a gene product responsible for one of the most common forms of familial PD,<sup>41</sup> was shown to have E3 Ub ligase activity.<sup>31,40,42-45</sup> It was recently demonstrated that an O-glycosylated  $\alpha$ -synuclein is the substrate of parkin,<sup>40</sup> and that parkin localizes to the LBs of sporadic PD and DLB.<sup>30</sup> A link between sporadic and familial PD through  $\alpha$ -synuclein and parkin suggests that common molecular pathogenetic mechanisms underlie PD. The accumulation of toxic or undesired proteins in neurons may result from a primary failure of degradation systems, and could subsequently lead to neurodegeneration. Alternatively, the constant production of high levels of impaired proteins may become a burden on the protein degradation process through the Ub-proteasome pathway, gradually overwhelming the capacity of the proteasome to degrade



**Figure 6.** Dorfin binds to mutant SOD1 but not to  $\alpha$ -synuclein. Myc-tagged wild-type and mutant  $\alpha$ -synucleins were co-transfected with Xpress-tagged Dorfin into HEK293 cells. After immunoprecipitation with anti-Xpress antibody, the resulting precipitates were analyzed by Western blotting. Myc-tagged wild-type SOD1 and mutant SOD1<sup>G85R</sup> were used for negative and positive controls, respectively.

toxic proteins and subsequently leading to the accumulation of ubiquitylated proteins and eventual neuronal cell death. Such a scenario is consistent with a recent report that impairment of the Ub-proteasome system is caused by protein aggregation.<sup>46</sup>

From this perspective, it is conceivable that familial and sporadic forms of ALS also share a common pathogenetic mechanism with PD involving the dysfunction of the Ub-proteasome pathway. In sporadic ALS, posttranslationally modified unknown substrates of Dorfin other than mutant SOD1 might accumulate in ubiquitylated form and play a role in the pathogenesis of the disease. Therefore, it is important to identify the protein(s) that is (are) the substrate(s) of the E3 activity of Dorfin for an understanding of the pathogenetic mechanism of sporadic ALS. In addition, substrates of Dorfin other than  $\alpha$ -synuclein may play an important role in the pathogenesis of sporadic PD, DLB, and MSA, or a posttranslational modification (eg, glycosylation,<sup>40</sup> phosphorylation<sup>47,48</sup>) of  $\alpha$ -synuclein may be necessary to be a substrate for Dorfin, because we failed to show the interaction between Dorfin and nonmodified  $\alpha$ -synuclein overexpressed in HEK293 cells in this report. Furthermore, our findings raise the possibility that PD, DLB, MSA, and ALS are etiologically distinct, but share a biochemically common metabolic pathway through Dorfin leading to the formation of ubiquitylated inclusion bodies and to neuronal cell degeneration.

The generation of Dorfin knockout mice may determine whether Dorfin is essential to form ubiquitylated inclusion bodies and is indispensable to prevent neurons from the

toxic insult of protein aggregation. It may reveal what specific roles and relationships Dornin and parkin have with one another as members of an E3 Ub ligase family containing a RING-finger/IBR domain.

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