Dorfin Localizes to Lewy Bodies and Ubiquitylates Synphilin-1*

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Running Title: Dorfin localizes to Lewy bodies and ubiquitylates synphilin-1

*This work was supported in part by a COE grant from the Ministry of Education, Culture, Sports, Science and Technology, and grants from the Ministry of Health, Labor and Welfare of Japan.

¶Research fellow of the Japan Society for the Promotion of Science for Young Scientists.

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Abstract

Parkinson’s disease (PD) is a neurodegenerative disease characterized by loss of nigral dopaminergic neurons. Lewy bodies (LBs) are a characteristic neuronal inclusion in PD brains. In this paper, we report that Dorfin, a RING-finger type ubiquityl ligase for mutant SOD1, was localized with ubiquitin in LBs. Recently, synphilin-1 was identified to associate with α-synuclein and to be a major component of LBs. We found that overexpression of synphilin-1 in cultured cells led to the formation of large juxtanuclear inclusions, but showed no cytotoxicity. Dorfin colocalized in these large inclusions with ubiquitin and proteasomal components. In contrast to full-length synphilin-1, overexpression of the central portion of synphilin-1 including ankyrin-like repeats, a coiled-coil domain and an ATP, GTP-binding domain, predominantly led to the formation of small punctate aggregates scattered throughout the cytoplasm and showed cytotoxic effects. Dorfin and ubiquitin did not localize in these small aggregates. Overexpression of the N- or C-terminus of synphilin-1 did not lead to the formation of any aggregates. Dorfin physically bound and ubiquitylated synphilin-1 through the central portion of synphilin-1, but ubiquitylated neither wild-type nor mutant α-synuclein. These results suggest that the central domain of synphilin-1 has an important role in the formation of aggregates and cytotoxicity, and that Dorfin may be involved in the pathogenetic process of PD and LB formation by ubiquitylation of synphilin-1.
Introduction

Parkinson’s disease (PD) is a neurodegenerative disease caused by loss of nigral dopaminergic neurons. The Lewy body (LB) is a characteristic neuronal inclusion in brains with PD (1-4). Although LBs are a prominent pathologic feature of PD, the underlying molecular mechanism accounting for LB formation is poorly understood. Several lines of evidence have suggested that derangements in the ubiquitin-proteasomal protein degradation pathway play a prominent role in the pathogenesis of PD (5). Ubiquitin and proteasome subunits colocalize in LB (6, 7), and biochemical studies have revealed reduced catalytic activities of proteasomes in the lesions of PD (8, 9). The gene product responsible for autosomal recessive juvenile parkinsonism (AR-JP), parkin (10), is an E3 ubiquityl ligase (11-13). Accumulation of target protein(s) due to loss of the ubiquitylation function of parkin may contribute to the development of AR-JP. In addition, a missense mutation in ubiquitin C-terminal hydrolase L1 (UCHL1) has been described in a family with PD (14). UCHL1 produces monomeric ubiquitin by cleaving polyubiquitin chains (15). Recently, ubiquityl ligase activity as well as the hydrolase activity of UCHL1 were also reported (16).

α-Synuclein is a 19-kDa presynaptic vesicular protein of unconfirmed function and one of the major components of LBs (17, 18). Mutations in α-synuclein (A30P and A53T) cause a rare autosomal dominant form of PD, which share many phenotypic findings with sporadic PD (19, 20). α-Synuclein aggregates deposit in LBs
in both autosomal dominant and sporadic PD (21, 22). In addition, it has been reported that transgenic flies and mice overexpressing human wild-type or mutant α-synuclein have abnormal cellular accumulation of α-synuclein and neuronal dysfunction and degeneration (23-30), indicating that α-synuclein has a role in the pathogenesis of both familial and sporadic PD.

Synphilin-1 was identified recently by yeast two-hybrid techniques to be a novel protein interacting with α-synuclein (31). α-Synuclein amino acid 1-65 region is sufficient for interaction, and the central portion of synphilin-1 (amino acids 349-555) is necessary and sufficient for interaction with α-synuclein (32). It has also been reported that the C-terminus of α-synuclein is closely associated with the C-terminus of synphilin-1 and a weak interaction occurs between the N-terminus of α-synuclein and synphilin-1 (33). Synphilin-1 is highly concentrated in presynaptic nerve terminals and its association with synaptic vesicles is modulated by α-synuclein (34). Coexpression of α-synuclein and synphilin-1 in transfected cells results in the formation of eosinophilic cytoplasmic inclusions that resemble LBs (31, 35), while transfection of synphilin-1 alone without expression of α-synuclein or parkin can also produce cytoplasmic inclusions in cultured cells (36, 37). Furthermore, synphilin-1 is ubiquitylated and degraded by proteasomes in human embryonic kidney 293 (HEK293) cells (37), and is localized as another major component of LB in the brains of patients with PD (38, 39). Thus, the process through which aggregations are formed by synphilin-1 may be important in the pathogenesis of PD.
Dorfin is a gene product, which we cloned from anterior horn tissues of the human spinal cord (40), that contains a RING-finger/IBR motif (41) at its N-terminus. It was reported that HHARI (human homologue of ariadne) and H7-AP1 (UbcH7-associated protein), both RING-finger/IBR motif-containing proteins, interact with ubiquitin-conjugating enzyme (E2) UbcH7 through the RING-finger/IBR motif, and that a distinct subclass of RING-finger/IBR motif-containing proteins represents a new family of proteins that specifically interact with distinct E2 enzymes (42, 43). Dorfin is a juxtanuclearly located E3 ubiquityl ligase, and may function in the microtubule organizing centers (MTOCs) (40). In the spinal cords of patients with sporadic and familial amyotrophic lateral sclerosis (ALS) with SOD1 mutation, Dorfin is colocalized with ubiquitin in hyaline inclusions (44). Dorfin physically bound and ubiquitylated various SOD1 mutants derived from familial ALS patients and enhanced their degradation (44). Thus, an important and interesting question is whether Dorfin is colocalized with ubiquitin in LBs of PD.

In this study, we showed that Dorfin is colocalized with ubiquitin in LBs of PD. We found that Dorfin ubiquitylates synphilin-1 and that overexpression of synphilin-1 leads to ubiquitylated inclusions resembling LBs in cultured cells.
Experimental procedures

**Immunohistochemistry** -- Immunohistochemical studies were carried out on 20% buffered formalin-fixed, paraffin-embedded autopsied brains filed in the Department of Neurology, Nagoya University Graduate School of Medicine. Five PD brains (age: 67-69 years, four men and one woman) and five controls without neurological disease (age: 61-78 years, four men and one woman) were studied. The diagnosis of all cases was confirmed by clinical and pathological criteria. Immunohistochemistry was performed as described previously (45). Polyclonal rabbit antiserum was raised against a C-terminal portion of Dorfin, amino acid 678-690, as described previously (40). Dorfin antiserum (1:200 dilution) and monoclonal anti-ubiquitin antibody (P4D1, 1:400 dilution; Santa Cruz Biotechnology) were used. To assess the colocalization of Dorfin and ubiquitin, a double-labelling immunofluorescence study was performed on selected sections with a combination of anti-Dorfin and anti-ubiquitin antibodies. Anti-Dorfin antibody was visualized by anti-rabbit goat IgG coupled with Alexa Fluor 568 (Molecular Probes) and anti-ubiquitin antibody was visualized with anti-mouse sheep IgG coupled with Alexa Fluor 488 (Molecular Probes), and observed under an LSM-510 laser scanning confocal microscope (Carl Zeiss). For cultured cells, immunostainings were performed as follows. COS7 cells transiently expressing Synphilin-1-DsRed fusion protein in a 4-chamber slide (Nalge Nunc) coated with rat-tail collagen (Roche Diagnostics) were fixed with methanol at -20°C for 10 min, air-dried, and blocked with 5% goat serum for 30 min. Cells were then incubated.
overnight at 4°C with the appropriate primary antibody diluted in PBS. After washing with PBS three times, Alexa Fluor 488-conjugated secondary antibody (1:1000 dilution; Molecular Probes) was added for 1 h at room temperature. Samples were visualized under a BX51 epifluorescence microscope (Olympus). Primary antibodies against ubiquitin (P4D1, 1:200 dilution; Santa Cruz Biotechnology), Hsp70 (1:5000 dilution; StressGen), 20S proteasome core subunit (1:5000 dilution; Affiniti) and UbcH7 (1:100 dilution; Transduction Laboratories) were used.

**Expression Plasmids, Cell Culture and Transfection** -- Human synphilin-1 cDNA containing the entire coding region was amplified by *Pfu* turbo DNA polymerase (Stratagene) from human brain cDNAs, using 5’-GTCAGGATCCACCACCATGGGAAGCCCCTGAATACC-3’ as a forward primer and 5’-ATATCTCGAGTGCTGCCTTATTCTTTCCTTTG-3’ as a reverse primer, and inserted in-frame into the *Bam*HI and *Xho*I sites of pcDNA3.1/V5His vector (Invitrogen). Plasmid for DsRed-tagged synphilin-1 was constructed by polymerase chain reaction (PCR) amplification, using 5’-ATATCTCGAGACCACCATGGGAAGCCCCTGAATACC-3’ as a forward primer and 5’-GTCAGGATCCGCCTTTGCCTTATTCTTTCCTTTG-3’ as a reverse primer, and inserted in-frame into the *Xho*I and *Bam*HI sites of pDsRed-N1 vector (Clontech). A series of deletion mutants of synphilin-1 were prepared as synphilin-1-N (amino acid 1-348), synphilin-1-M (amino acid 349-555) and synphilin-1-C (amino acid 556-919). Synphilin-1-M is the central portion of synphilin-1, containing the ankyrin-like
repeat, the coiled-coil domain and the ATP/GTP binding domain (31). Primers pairs for each deletion mutant were as follows: 5’-GTCAGGATCCACACCACCATGGAAGCCCCTGAATACC-3’ and 5’-ATATCTCGAGTTCTCGTGGAATTTTGTCT-3’ for synphilin-1-N-V5; 5’-ATATCTCGAGACCACCACATGGAAGCCCCTGAATACC-3’ and 5’-GTCAGGATCCGCTTCGTCGTGAATTTTGTCTAG-3’ for synphilin-1-N-DsRed; 5’-GTCAGGATCCACCATGGAAGCCCCTGAATACC-3’ and 5’-ATATCTCGAGCTTGCCCTCTGATTTTCTGG-3’ for synphilin-1-M-V5; 5’-ATATCTCGAGACCACCACATGGAAGCCCCTGAATACC-3’ and 5’-GTCAGGATCCACCATGGAAGCCCCTGAATACC-3’ for synphilin-1-N-V5; 5’-ATATCTCGAGACCACCACATGGAAGCCCCTGAATACC-3’ and 5’-GTCAGGATCCACCATGGAAGCCCCTGAATACC-3’ for synphilin-1-N-DsRed; 5’-GTCAGGATCCACCATGGAAGCCCCTGAATACC-3’ and 5’-ATATCTCGAGACCACCACATGGAAGCCCCTGAATACC-3’ and 5’-GTCAGGATCCACCATGGAAGCCCCTGAATACC-3’ for synphilin-1-M-DsRed; 5’-GTCAGGATCCACCATGGAAGCCCCTGAATACC-3’ and 5’-ATATCTCGAGACCACCACATGGAAGCCCCTGAATACC-3’ and 5’-GTCAGGATCCACCATGGAAGCCCCTGAATACC-3’ for synphilin-1-M-DsRed; 5’-GTCAGGATCCACCATGGAAGCCCCTGAATACC-3’ and 5’-ATATCTCGAGACCACCACATGGAAGCCCCTGAATACC-3’ and 5’-GTCAGGATCCACCATGGAAGCCCCTGAATACC-3’ for synphilin-1-C-V5; and 5’-ATATCTCGAGACCACCACATGGAAGCCCCTGAATACC-3’ and 5’-GTCAGGATCCACCATGGAAGCCCCTGAATACC-3’ for synphilin-1-C-DsRed.

Construction of pcDNA4/HisMax-Dorfin, pcDNA3.1(+)-FLAG-ubiquitin and pcDNA3.1/MycHis(+) SOD1 vectors was described elsewhere (40, 44). α-Synuclein cDNA was amplified by PCR from human brain cDNAs and cloned into the EcoRV site of pcDNA3.1/MycHis(+) (Invitrogen). To generate the mutant α-synuclein expression vector, A30P and A53T mutations were introduced into the pcDNA3.1/MycHis(+) α-synuclein with a QuickChange site-directed mutagenesis kit (Stratagene) following the method of Lee et al. (46). COS7, HEK293 and Neuro2a
cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Transfections were performed using the Effectene transfection reagent (Qiagen), according to the manufacturer’s instructions. To inhibit cellular proteasome activity, cells were treated with 0.5 μM MG132 (Z-Leu-Leu-Leu-als; Sigma) for 16 h overnight after transfection.

**Immunoprecipitation and Western Blotting Analysis** -- Cells were lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS) with a protease inhibitor mixture (Complete; Roche Diagnostics). Immunoprecipitation from transfected cell lysates was performed with 2 μg antibody and Protein A/G Plus-Agarose (Santa Cruz Biotechnology), and the immunoprecipitate was then washed four times in lysis buffer. Anti-V5 antibody (Invitrogen) for synphilin-1-V5 fusion proteins and anti-Myc antibody (A-14; Santa Cruz Biotechnology) for α-synuclein-Myc or SOD1-Myc fusion proteins were used. Immunoprecipitates were subjected to SDS-PAGE and analyzed by Western blotting with ECL detection reagents (Amersham Pharmacia).

**In Vitro Ubiquitylation Assay** -- Immunopurified (IP)-Xpress-Dorfin bound to anti-Xpress antibody (Invitrogen) with Protein A/G Plus-Agarose (Santa Cruz Biotechnology) was prepared from lysates of HEK293 cells transfected with pcDNA4/HisMax-Dorfin. IP-synphilin-1-V5 was prepared with anti-V5 antibody bound to Protein A/G Plus-Agarose from lysates of HEK293 cells transfected with pcDNA3.1/V5His-synphilin-1. IP-α-synuclein-Myc and IP-SOD1-Myc were prepared
with anti-Myc antibodies from lysates of pcDNA3.1/MycHis(+)-%-synuclein and pcDNA3.1/MycHis(+)–SOD1 transfected HEK293 cells, respectively. Slurries of IP-Xpress-Dorfin were mixed with IP-synphilin-1-V5, IP-%-synuclein-Myc or IP-SOD1-Myc and incubated at 30 °C for 90 min in 50 µl of reaction buffer containing ATP (4 mM ATP in 50 mM Tris-HCl pH 7.5, 2 mM MgCl$_2$ and 2 mM DTT), 100 ng of rabbit E1 (Calbiochem), 2 µg of UbcH7 (Affiniti), and 2 µg His-ubiquitin (Calbiochem). The reaction was terminated by adding 20 µl of 4× sample buffer, and 20 µl aliquots of the reaction mixtures were subjected to SDS-PAGE followed by Western blotting with anti-His antibody (Novagen).

**Neurotoxicity Analysis and Quantification of Synphilin-1 Aggregates** – One×10^4 COS7 cells were grown overnight on 4-chamber well collagen-coated slides (Nalge Nunc). They were transfected with 0.2 µg of pDsRed-N1-synphilin-1 or its deletion mutants. To inhibit cellular proteasome activity, cells were treated with 0.5 µM MG132 (Z-Leu-Leu-Leu-als; Sigma) for 16 h overnight after transfection. The number of inclusions was counted in more than 100 cells randomly selected and data were averaged from three independent experiments. For cell viability assay, 5×10^3 Neuro2a cells were grown in 96-well collagen coated plates overnight. They were then transfected with 0.1 µg of pcDNA3.1/V5His-synphilin-1 or deletion mutants of synphilin-1. pcDNA3.1/V5His-LacZ was used as a control. Next, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)-based cell proliferation assay was performed using CellTiter 96
(Promega) at 24 h after serum deprivation. The assay was carried out in triplicate. Absorbance at 490 nm was measured in a multiple plate reader.
Results

**Dorfin Localizes to Lewy Bodies of PD** -- We first examined whether LBs contain Dorfin. Immunohistochemical analysis revealed that Dorfin was predominantly localized in LBs found in PD (Fig. 1A). The peripheral rim of a typical LB in a neuronal cell body was strongly stained, while the central core remained unstained (Fig. 1B). Dorfin was also localized in Lewy neurites (Fig. 1C), which are a pathological hallmark in addition to LBs of degenerating neurons in the brains of patients suffering from PD (47). Anti-Dorfin antibody did not stain any abnormal structures in normal brains (data not shown). Double-labeled immunofluorescence study revealed that Dorfin was colocalized with ubiquitin in LB (Fig. 1D-F). Serial sections stained with anti-Dorfin and anti-ubiquitin antibodies showed that about 90% of ubiquitylated LBs were positive for Dorfin immunoreactivity. The staining profile of Dorfin was very similar to that of α-synuclein (48), which is predominantly located in the peripheral rim of LBs, but was different from that of parkin, which localizes predominantly in the core of LBs (49).

**Expression of Synphilin-1 Induced LB-like Large Juxtanuclear Inclusions, and Dorfin Localized to These Inclusions** -- To investigate the relationships of Dorfin to components of LBs other than ubiquitin, we first examined the subcellular localization of α-synuclein or synphilin-1 in cultured cells. We created wild-type or mutant α-synuclein-GFP and α-synuclein-Myc fusion constructs, but there was no evidence of α-synuclein aggregation in transfected COS7 cells in the presence or absence of
proteasome inhibitor (data not shown). We created a synphilin-1-DsRed fusion construct by fusing red fluorescent protein DsRed to the C-terminus of synphilin-1, and performed transient transfection in COS7 cells with this construct. Large juxtanuclear inclusions were spontaneously formed in the transfected COS7 cells in the absence of proteasome inhibitor (Fig. 2A-O). We also constructed synphilin-1 fusion proteins with a smaller V5/6×His tag, which formed identical inclusions when overexpressed in COS7 cells, although to a lesser extent than synphilin-1-DsRed fusion proteins (data not shown). Immunostaining with anti-ubiquitin and anti-Dorfin antibodies revealed that most large juxtanuclear inclusions of synphilin-1 contain ubiquitin (Fig. 2A-C) and Dorfin (Fig. 2D-F). Immunohistochemical studies of human LBs have previously shown that LBs are stained with proteasomal subunits (6) and molecular chaperones, such as Hsp40 and Hsp70 (24). Thus, we next examined if the inclusion bodies in COS7 cells contain the 20S core subunit of proteasome and 70-kDa heat shock protein (Hsp70). We found both the 20S subunit of proteasome and Hsp70 to be colocalized with synphilin-1 inclusion bodies (Fig. 2G-L). Dorfin binds specifically to UbcH7 as an E2 through the RING-finger/IBR domain (40). UbcH7 was also localized with Dorfin in these inclusions (Fig. 2M-O). These observations suggest that large juxtanuclear inclusions formed by synphilin-1 in our cell culture system have many characteristic features of LBs, and that synphilin-1 can aggregate when overexpressed, and that this process may be associated with its ubiquitylation.
Expression of the Central Portion of Synphilin-1 Induces Large Juxtanuclear Inclusions as Full-length Proteins, but Small Punctate Aggregates Are Also Formed -- To further analyze which part of synphilin-1 is related to aggregation formation, we prepared a series of deletion mutants of synphilin-1. We divided synphilin-1 into three parts, the N-terminus of synphilin-1 (synphilin-1-N) containing amino acids 1-348, the central portion of synphilin-1 (synphilin-1-M) containing amino acids 349-555, and the C-terminus of synphilin-1 (synphilin-1-C) containing amino acids 556-919, and fused them to DsRed at their C-terminus (Fig. 3A and B). Inclusions were not seen with overexpression of DsRed alone, synphilin-1-N or synphilin-1-C in COS7 cells (Fig. 3C-E). Expression of synphilin-1-M, however, resulted in the production of two types of inclusions: large juxtanuclear inclusions (Fig. 3F-K) and small punctate aggregates scattered throughout the cytoplasm (Fig. 3L-Q). The large inclusions were stained with ubiquitin (Fig. 3F-H) and Dorfin (Fig. 3I-K), as were inclusions induced by full-length synphilin-1. However, neither ubiquitin nor Dorfin was colocalized with the small punctate aggregates scattered throughout the cytoplasm (Fig. 3L-Q).

Expression of the Central Portion of Synphilin-1 Compromises Cell Viability -- We examined the frequency of the inclusion formation by synphilin-1 or its deletion mutants. The number of inclusions was counted with and without the proteasome inhibitor MG132 in COS7 cells (Fig. 4A). Both synphilin-1-N and synphilin-1–C formed almost no inclusions in either the presence or absence of MG132. Full-length
synphilin-1 and synphilin-1-M produced inclusions with high frequency even in the absence of MG132, and the number of cells with inclusions induced by full-length synphilin-1 was significantly greater than that induced by synphilin-1-M (Fig. 4A). Treatment with MG132 significantly increased the number of inclusions. We next measured the ratio of cells that contain small punctate aggregates among total cells bearing all inclusions (Fig. 4B), because two types of aggregates, large juxtanuclear inclusions and small punctate scattered aggregates, were observed. In contrast to full-length synphilin-1, the inclusions induced by overexpression of synphilin-1-M were predominantly small punctate aggregates scattered through the cytoplasm (Fig. 4B). Treatment with MG132 decreased the ratio of small aggregates induced by synphilin-1-M (Fig. 4B).

The effects of synphilin-1 expression on cell viability are poorly understood. O'Farrell et al. reported that cells transfected with synphilin-1 were more viable than cells transfected with LacZ (36). On the other hand, Lee et al. reported that synphilin-1 compromised cell viability (37). Thus, we examined the effects of synphilin-1 or its deletion mutants on cell viability using MTS assay in a neuronal cell line, Neuro2a (Fig. 4C). We found that synphilin-1-M had a cytotoxic effect, whereas overexpression of full-length synphilin-1, or N-terminal or C-terminal deletion mutants of synphilin-1, did not (Fig. 4C). We used synphilin-1-V5 fusion constructs, but synphilin-1-DsRed fusion constructs gave the same results (data not shown).
Dorfin Interacts with Synphilin-1 -- We examined whether Dorfin interacts with synphilin-1, because Dorfin localizes in LBs and cytoplasmic juxtanuclear inclusions formed by synphilin-1. To identify which portion of synphilin-1 binds to Dorfin, we expressed a series of deletion mutants of V5-tagged synphilin-1 and Xpress-tagged Dorfin in COS7 cells (Fig. 5A). Co-immunoprecipitation confirmed that Dorfin bound to full-length synphilin-1 (Fig. 5B) and interacted with synphilin-1-M strongly and synphilin-1-N weakly, but Dorfin failed to bind synphilin-1-C (Fig. 5C). Thus, Dorfin interacts with synphilin-1 mainly through its central portion, which contains the ankyrin-like repeat, the coiled-coil domain and the ATP/GTP binding domain. Dorfin has a unique primary structure containing a RING-finger/IBR motif at its N-terminus and can be structurally divided into two parts, the N-terminal region containing a RING-finger/IBR motif (Dorfin-N) that interacts with E2, and the C-terminal region with no similarity to any other known proteins (Dorfin-C) (40) (Fig. 5A). We found that Dorfin-C, but not Dorfin-N, specifically bound synphilin-1, indicating that Dorfin binds to synphilin-1 via its C-terminal region (Fig. 5D).

Dorfin Ubiquitylates Synphilin-1 Through the Central Domain of Synphilin-1 In Vitro – The physical interaction between Dorfin and synphilin-1 prompted us to investigate whether synphilin-1 itself is ubiquitylated by Dorfin. We first examined whether synphilin-1 is ubiquitylated in a culture cell model. V5-tagged full-length or deletion mutants of synphilin-1 were co-transfected with FLAG-tagged ubiquitin in HEK293 cells. When full-length or deletion mutants of synphilin-1 were
immunoprecipitated after treatment with a proteasome inhibitor, MG132, full-length synphilin-1 and synphilin-1-M were found to be polyubiquitylated, but synphilin-1-N and –C were not (Fig. 6A). Wild-type and mutant α-synuclein were not found to be polyubiquitylated, while, as previously reported (44), mutant SOD-1 was polyubiquitylated (Fig. 6A).

We next examined whether Dorfin is involved in the ubiquitylation of synphilin-1 in vitro. For this purpose, we immunopurified Xpress-Dorfin and synphilin-1-V5 independently after transfection into HEK293 cells. When these immunopurified proteins were incubated with recombinant E1, E2 (UbcH7), Histagged ubiquitin, and ATP, high molecular weight ubiquitylated bands were observed in the presence of Xpress-Dorfin with synphilin-1, while no signal was noted in synphilin-1 in the absence of either E1 or E2 (Fig. 6B). Dorfin ubiquitylated mutant SOD1 in vitro, as previously reported (44). Dorfin ubiquitylated neither wild-type nor mutant α-synuclein (Fig. 6B). In vitro ubiquitylation assay of a series of synphilin-1 deletion mutants by Dorfin revealed synphilin-1-M was ubiquitylated, whereas synphilin-1-N or synphilin-1-C were not ubiquitylated at all (Fig. 6C).
Discussion

Several lines of evidence have suggested that derangements in the ubiquitin-proteasomal protein degradation pathway may have a prominent role in the pathogenesis of PD (5). Our present study shows that Dorfin, an E3 ubiquityl ligase, is colocalized with ubiquitin in LBs of PD and physically binds to ubiquitylate synphilin-1, which is known to be a major component of LBs (31, 38, 39).

For the analysis of LB formation by synphilin-1, various cell culture models have been reported (31, 35-37). In our cell culture model, overexpression of synphilin-1 alone induced large juxtanuclear cytoplasmic inclusions. In these large inclusions, Dorfin was colocalized with ubiquitin-proteasome pathway-related proteins, such as ubiquitin, the 20S core subunit of proteasome, and Hsp70, just as Dorfin in LBs. The central portion of synphilin-1 contains the ankyrin-like repeat, the coiled-coil domain and the ATP/GTP binding domain (31). This region is reported to be necessary for interaction with α-synuclein (32). We found that the central portion of synphilin-1 also bound with Dorfin and overexpression of this region alone led to inclusion body formation, while neither the N-terminal nor C-terminal regions induced aggregates. Overexpression of this central portion of synphilin-1 produced small punctate aggregates scattered throughout the cytoplasm as well as large juxtanuclear inclusions, but the former predominated. The small punctate aggregates colocalized with neither ubiquitin nor other proteasome pathway-associated proteins, and had cytotoxic effects as revealed by MTS assays. Recently, Lee et al. reported that overexpression of α-
synuclein in culture cells produced two distinct types of aggregates: large juxtanuclear inclusions and small punctate aggregates scattered throughout the cytoplasm (50). The juxtanuclear inclusion bodies were filled with amyloid-like $\alpha$-synuclein fibrils, whereas the small aggregates contained non-fibrillar spherical aggregates (50). They suggested that these aggregates appear sequentially, with the smallest population appearing the earliest and the fibrillar inclusions the latest, and that the small spherical aggregates are the cellular equivalents of protofibrils (50). Protofibrils are recognized to be more important in the terms of cytotoxicity than mature fibrils in A$\beta$ (51, 52) and $\alpha$-synuclein (53, 54). In our cell culture model, overexpression of synphilin-1 produced two distinct types of aggregates, very closely resembling two types of $\alpha$-synuclein aggregates (50). Thus, the small punctate aggregates scattered throughout the cytoplasm induced by the central portion of synphilin-1 might have characteristic similar to those of protofibrils. Our cell culture system will allow detailed characterization of LB formation and cytotoxic processes in further studies.

We reported previously that Dorfin localizes in the inclusion bodies of familial ALS with SOD1 mutations as well as in those of sporadic ALSs, and ubiquitylates various SOD1 mutants derived from familial ALS patients (44). Based on these findings, it is conceivable that familial and sporadic forms of ALS share a common mechanism involving the dysfunction of the ubiquitin-proteasome pathway, despite having distinct etiological mechanisms. In sporadic ALS, unknown substrate(s) of Dorfin might play a role in the pathogenesis of the disease and accumulate in
ubiquitylated inclusion bodies. The following results support the view that Dorfin plays an important role in the formation of LBs of PD: (i) the presence of Dorfin in LBs and large juxtanuclear inclusions of synphilin-1 in our cell culture model, (ii) the parallel distribution patterns of ubiquitin and Dorfin in LBs and inclusion bodies induced by synphilin-1 in cultured cells, and (iii) the E3 function of Dorfin to ubiquitylate synphilin-1. Dorfin ubiquitylated neither wild-type nor mutant α-synuclein; however, our results can not exclude the possibility that post-translational modification, such as glycosylation (55) or phosphorylation (56, 57), of α-synuclein may be necessary for it to become a substrate for Dorfin, because overexpressed α-synuclein in our cell culture system was not phosphorylated (data not shown). The relation between Dorfin and PD shows striking similarities to the relation between Dorfin and ALS. Our findings raise the possibility that PD 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.

Parkin has been shown to have E3 ubiquityl ligase activity (10-12). It was recently demonstrated that an O-glycosylated α-synuclein (55) and synphilin-1 (35) are the substrates of parkin, and that parkin localizes to LBs of sporadic PD (49). The link between sporadic and familial PD through α-synuclein, synphilin-1 and parkin sheds new light on underlying common molecular pathogenetic mechanisms in PD. What roles, then, do Dorfin and parkin play with respect to each other in the
pathogenesis of PD and/or LB formation? Both proteins have a RING-finger/IBR domain and E3 ubiquitin ligase activities. Parkin interacts both with \( \alpha \)-synuclein (55) and synphilin-1 (35), whereas Dorfin binds and ubiquitylates only synphilin-1. Parkin resides in the core of LBs (49), while Dorfin predominantly localizes to the rim. Impaired function of parkin as an E3 ubiquityl ligase is responsible for one of the most common forms of familial PD, AR-JP (9, 10). However, there has been no analysis as to whether Dorfin gene mutation causes familial PD. Recently, Valente et al. identified a locus, PARK6, on chromosome 1p35-1p36 that is involved in the autosomal recessive form of parkinsonism (58, 59). Interestingly, a human paralog of Dorfin (Dj1174N9.1) has been mapped at 1p34.1-1p35.3 (60). Furthermore, Dorfin was identified by a phage display system to be one of the binding proteins with 2-metylnorharman, an analog of the parkinsonism-inducing toxin, 1-metyl-4-phenylpyridinium cation (MPP+) (61). These findings suggest the utility of analyzing Dj1174N9.1 or Dorfin mutation for potential involvement in familial PD. Production of Dorfin knockout mice will also answer the question of whether Dorfin is essential for pathogenesis and/or ubiquitylated inclusion body formation in PD.
Acknowledgments

We thank Dr. Miya Kobayashi (Kinjo Gakuin University) for her helpful comments.

Abbreviations

1The abbreviations used are: PD, Parkinson's disease; LB, Lewy body; E3, ubiquityl ligase; AR-JP, autosomal recessive juvenile parkinsonism; UCHL1, ubiquitin C-terminal hydrolase L1; HEK293, human embryonic kidney 293; IBR, in-between-RING-finger; E2, ubiquitin-conjugating enzyme; MTOC, microtubule organizing center; ALS, amyotrophic lateral sclerosis; SOD1, superoxide dismutase 1; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; IP, immunopurified; Hsp, heat shock protein.
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Figure legends

Fig. 1. Colocalization of Dorfin and ubiquitin in Lewy bodies of Parkinson’s disease. Substantia nigra tissue of Parkinson's disease (PD) cases was immunohistochemically stained with anti-Dorfin antibody. A, Lewy bodies (LBs) (arrow) in neurons are strongly stained. B, the peripheral rim of a typical LB is predominantly stained with anti-Dorfin antibody. C, Lewy neurites (arrow head) are also Dorfin-immunoreactive. The scale bar in A is equivalent to 100 µm in A and C, and 12 µm in B. D-F, laser scanning confocal microscopy images of double-labelling immunofluorescence study of LB. Frozen sections prepared from substantia nigra tissue of PD were incubated with rabbit anti-Dorfin IgG and labeled with Alexa Fluor 568-conjugated anti-rabbit antibodies (red in D), and mouse monoclonal anti-ubiquitin and Alexa Fluor 488-conjugated anti-mouse antibodies (green in E). F shows a merged image of the double-stained LB (D and E), and regions of overlapping between Dorfin and ubiquitin immunoreactivities are shown in yellow. The scale bar in D is 10 µm, and also applies to E and F.

Fig. 2. Formation of large juxtanuclear inclusions by overexpression of synphilin-1. Full-length synphilin-1 was overexpressed in COS7 cells as DsRed fusion protein. After 2 days post-transfection, cells were fixed and immunostained with the indicated antibodies. Large juxtanuclear inclusions of synphilin-1 are formed spontaneously without proteasome inhibition. Cells with large juxtanuclear inclusions were co-
stained with ubiquitin (A-C), Dorfin (D-F), Hsp70 (G-I), 20 S proteasome core subunit (J-L) or UbcH7 (M-O). Regions of overlap between synphilin-1 (red) and immunoreactivities of the indicated proteins (green) are shown in yellow. Nuclei are stained with Hoechst 33342 (blue). Scale bar, 10 μm.

**Fig. 3. Formation of two types of aggregates by the central portion of synphilin-1.**

COS7 cells were transfected with expression vectors for DsRed alone or DsRed fusion proteins of deletion mutants of synphilin-1. Two days after transfection, cells were analyzed by Western blotting and immunocytochemistry. A, schematic representation of DsRed fusion protein of synphilin-1 deletion mutants used in this study. B, Western blotting analysis of lysates from transfected cells. DsRed alone (C), synphilin-1-N (D), or synphilin-1-C (E) form no aggregate, whereas overexpression of the central portion of synphilin-1 (synphilin-1-M) induced two types of inclusions: large juxtanuclear inclusions (F and I) and small punctate aggregates scattered throughout the cytoplasm (L and O). Large juxtanuclear inclusions are ubiquitin-positive (F-H) and colocalize with Dorfin (I-K), whereas small punctate aggregates are ubiquitin-negative (L-N) and do not colocalize with Dorfin (O-Q). Regions of overlap between synphilin-1 (red) and immunoreactivities of indicated proteins (green) are shown in yellow. Nuclei are stained with Hoechst 33342 (blue). Scale bar, 10 μm.
Fig. 4. The central portion of synphilin-1 produces predominantly small punctate aggregates and compromises cell viability. A, the frequency of inclusion-bearing cells transfected with synphilin-1 and its deletion mutants. COS7 cells were grown on collagen-coated 4-chamber well slides and transfected with expression vectors for synphilin-1-DsRed fusion proteins. Two days after transfection, cells were fixed and percentages of inclusion-positive cells among DsRed-positive cells were determined. For proteasome inhibition, cells were treated with 0.5 μM MG132 for 16 h before fixation. B, the frequency of cells bearing small punctate aggregates scattered through the cytoplasm among all inclusion-positive cells. Experimental conditions were same as described in A. Data are the mean ± SD values of triplicate assays. Statistical analyses were carried out with Mann-Whitney's U test. *, p < 0.01. C, the cytotoxic effect of synphilin-1-M expression on an MTS assay. Neuro2a cells were grown on collagen-coated 96 wells and transfected with V5-tagged synphilin-1 or its deletion mutants. After changing to a serum-free medium, MTS assays were performed after 24 h of incubation. Viability of cells was measured as the level of absorbance at 490 nm. Data are mean ± SD values of triplicate assays. Statistical analyses were carried out by one-way analysis of variance. *, p < 0.01.

Fig. 5. Association of Dorfin with synphilin-1 in COS7 cells.

A, schematic representation of Xpress-tagged Dorfin, deletion mutants of Dorfin (i.e. Dorfin-N and Dorfin-C), V5-tagged synphilin-1 and deletion mutants of synphilin-1
(i.e. synphilin-1-N, synphilin-1-M and synphilin-1-C) used in this study. 

B, Dorfin binds with synphilin-1. V5-tagged synphilin-1 or LacZ were co-transfected with Xpress-tagged Dorfin in COS7 cells. After immunoprecipitation was performed with anti-Xpress antibody, the resulting precipitates and cell lysate were analyzed by Western blotting with anti-V5-HRP or anti-Xpress-HRP antibodies. 

C, Dorfin binds with synphilin-1 mainly through its central portion. After V5-tagged deletion mutants of synphilin-1 and Xpress-tagged Dorfin were transfected, immunoprecipitation and Western blotting were performed as described in B. 

D, binding of synphilin-1 to the C-terminal portion of Dorfin. After V5-tagged synphilin-1 and Xpress-tagged deletion mutants of Dorfin were transfected, immunoprecipitation and Western blotting were performed as described in B.

**Fig. 6. Ubiquitylation of synphilin-1 by Dorfin.** A, synphilin-1 were ubiquitylated in HEK293 cells. V5-tagged synphilin-1 or its deletion mutants were co-transfected with FLAG-tagged ubiquitin in HEK293 cells and treated with 0.5 μM MG132 for 16 h overnight after transfection. Myc-tagged α-synuclein or SOD1 were co-transfected with FLAG-ubiquitin and were treated as mentioned above. Immunoprecipitates prepared by anti-V5 or anti-Myc antibodies were used for immunoblotting with anti-FLAG antibody. 

B, *in vitro* ubiquitylation assay of synphilin-1 by Dorfin. Xpress-tagged Dorfin and V5-tagged synphilin-1 were transfected into HEK293 cells independently. Immunopurified Dorfin (IP-Xpress-Dorfin) and synphilin-1 (IP-
synphilin-1-V5) were prepared and mixed in an assay mixture for ubiquitylation. For this assay, Myc-tagged wild-type and mutant α-synuclein, and Myc-tagged mutant SOD1G85R were also used instead of synphilin-1. After a 90-min incubation at 30 °C, SDS-PAGE was performed followed by Western blotting for His-tagged ubiquitin with anti-His antibody. C, in vitro ubiquitylation assay of various synphilin-1 deletion mutants by Dorfin. V5-tagged deletion mutants of synphilin-1 were transfected into HEK293 cells, immunopurified and mixed with IP-Xpress-Dorfin in an assay mixture for ubiquitylation as described in B. The reaction products were analyzed by Western blotting with anti-His antibody for ubiquitin (left panel) and with anti-V5 antibody for synphilin-1 (right panel). The high molecular weight ubiquitylated synphilin-1 or synphilin-1-M are shown as (Ub)n on the right. Asterisks indicate IgG light and heavy chains.
Fig. 1 (Ito et al.)
Fig. 2 (Ito et al.)
Fig. 3 (Ito et al.)

A

synphilin-1 (1-919)
synphilin-1-N (1-348)
synphilin-1-M (349-555)
synphilin-1-C (556-919)

B

(kDa)

WB: anti-DsRed

DsRed
synphilin-1-N
synphilin-1-C
synphilin-1-M
synphilin-1-C

C
D
E

Dorfin
merge

F
G
H

merge

synphilin-1-M
Ub

I
J
K

merge

synphilin-1-M
Dorfin

L
M
N

merge

synphilin-1-M
Ub

O
P
Q

merge

synphilin-1-M
Dorfin
Fig. 4 (Ito et al.)

A

B

C

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http://www.jbc.org/Downloaded from
Fig. 5 (Ito et al.)

A  

B  

C  

D
Fig. 6 (Ito et al.)

### A

| synphilin-1-V5 | α-synuclein-Myc | SOD1-Myc |
|---------------|----------------|----------|
| E1            | +             | +        |
| E2            | +             | +        |

**IP: anti-V5**

**WB: anti-FLAG**

**WB: anti-V5**

**WB: anti-Myc**

### B

**E1**

- +
- +
- +
- +
- +
- +

**E2**

- +
- +
- +
- +
- +
- +

**IP-Xpress-Dorfin**

**WB: anti-His**

**WB: anti-V5**

**WB: anti-V5**

**WB: anti-Myc**

### C

- synphilin-1
- synphilin-1-N
- synphilin-1-M
- synphilin-1-C

**WB: anti-His**

**WB: anti-V5**

**WB: anti-V5**
Dorfin localizes to Lewy bodies and ubiquitylates synphilin-1
Takashi Ito, Jun-ichi Niwa, Nozomi Hishikawa, Shinsuke Ishigaki, Manabu Doyu and Gen Sobue

J. Biol. Chem. published online May 15, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302763200

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