Dorfin Ubiquitylates Mutant SOD1 and Prevents Mutant SOD1-mediated Neurotoxicity*

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Amyotrophic lateral sclerosis (ALS) is a progressive paralytic disorder resulting from the degeneration of motor neurons in the cerebral cortex, brainstem, and spinal cord. The cytopathological hallmark in the remaining motor neurons of ALS is the presence of ubiquitylated inclusions consisting of insoluble protein aggregates. In this paper we report that Dorfin, a RING finger-type E3 ubiquitin ligase, is predominantly localized in the inclusion bodies of familial ALS with a copper/zinc superoxide dismutase (SOD1) mutation as well as sporadic ALS. Dorfin physically bound and ubiquitylated various SOD1 mutants derived from familial ALS patients and enhanced their degradation, but it had no effect on the stability of the wild-type SOD1. The overexpression of Dorfin protected against the toxic effects of mutant SOD1 on neural cells and reduced SOD1 inclusions. Our results indicate that Dorfin protects neurons by recognizing and then ubiquitylating mutant SOD1 proteins followed by targeting them for proteasomal degradation.

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† The abbreviations used are: ALS, amyotrophic lateral sclerosis; SOD1, copper/zinc superoxide dismutase; NHI, neuronal hyaline inclusion; Ub, ubiquitin; IBR, in-between RING finger; HCK293, human embryonic kidney 293; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; IP, immunopurified; PI, propidium iodide; GFP, green fluorescent protein; MIT, 3,4,5-dimethylthiazol-2-yl)-2,5-diphenylnitrazolium bromide; PD, Parkinson’s disease; CHIP, carboxyl terminus of Hsc70-interacting protein; Hsp, heat shock protein.
Fig. 1. Localization of Dorfin in inclusion bodies in spinal cord motor neurons of ALS and mutant SOD1-transgenic mice. The transverse sections of the spinal cord from familial ALS (FALS, panels A–C), SOD1 \textsuperscript{G93A}–transgenic mice (G93A-Tg, panel D), and sporadic ALS (SALS, panel E) were immunohistochemically stained with the anti-Dorfin antibody. The hyaline inclusions in familial ALS were stained with anti-SOD1 (α-SOD1, panels F and H) or anti-Dorfin (α-Dorfin, panels G and I) antibodies. Note that panels F and G or H and I are the identical sections doubly immunostained. Spinal cord sections from familial (panels J–L) and sporadic (panels M–O) ALS were double labeled by indirect immunofluorescence with anti-Dorfin antibody (panels J and M) and monoclonal antibody to Ub (α-Ub, panels K and N) and observed by a laser-scanning confocal microscope. Panel L is panels J and K merged; panel O is panels M and N merged. Magnification, ×225 (panels A–C, E and F–I) and ×675 (panels D and J–O).

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EXPERIMENTAL PROCEDURES

Immunohistochemistry—Immunohistochemical studies were carried out on 10% formalin-fixed, paraffin-embedded spinal cords filed in the Department of Neurology, Nagoya University Graduate School of Medicine. The specimens were obtained at autopsy from two familial ALS patients with mutant SOD1 \textsuperscript{G93A} (male aged 57 years and female aged 54 years), three sporadic cases of ALS (all males, aged 46, 59, and 67 years) and four age-matched, non-neurologic disease patients. The spinal cord specimens of these ALS cases and those of mutant SOD1 \textsuperscript{G93A}–transgenic mice (B6SJL-TgN(SOD1-G93A)1Gur; The Jackson Laboratory) were immunohistochemically stained with antibodies against Dorfin (14), SOD1 (SOD-100; StressGen Biotechnologies, La Jolla, CA), and Ub (P4D1; Santa Cruz Biotechnology). Double staining of identical sections was performed as described (31). In immunofluorescence microscopy, Alexa-488- and Alexa-546-conjugated secondary antibodies (Molecular Probes) were used. The human and animal studies described in this report were approved by the Ethics Review Committees of the Nagoya University Graduate School of Medicine.

Expression Plasmids, Cell Culture, and Transfection—Human wild-type SOD1 and mutant SOD1 \textsuperscript{G93A}, SOD1 \textsuperscript{G93A}–SOD1 \textsuperscript{G93A}, and SOD1 \textsuperscript{G93A}–dCasNs containing the entire coding region were inserted in-frame into the BamHI and XhoI site of pcDNA3.1+MycHis vector (Invitrogen) or into the XhoI and BamHI site of the pEGFP-N1 vector (CLONTECH). Construction of a pcDNA3.1+VFLAG-Ub vector and the Dorfin or Dorfin deletion mutant (Dorfin-N and Dorfin-C) pcDNA4/HisMax vector was reported elsewhere (14). Human embryonic kidney 293 (HEK293) cells 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). Cells were lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS) with a protease inhibitor mixture. To inhibit cellular proteasome activity, cells were treated with 0.5 μM MG132 (N-benzoyloxycarbonyl-Leu-Leu-leucinal, Sigma) for 16 h after overnight post-transfection.

In Vitro Ubiquitination Assay and Pulse-Chase Analysis—Immunopurified (IP) Xpress-Dorfin bound to anti-Xpress antibody (Invitrogen) with protein G beads (Amersham Biosciences) was prepared from lyses of HEK293 cells transfected with 1 μg of pcDNA3.1+MycHis wild-type or mutant SOD1. Slurries of IP-Xpress-Dorfin were mixed with IP-Myc-SOD1 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, and 2 mM MgCl\textsubscript{2}), 100 ng of rabbit E1 (Calbiochem), 2 μg of Ubch7 (Affiniti, Exeter, United Kingdom), and 2 μg of Ub-His (Calbiochem). The reaction was terminated by adding 20 μl of 4x sample buffer, and 20-μl aliquots of the reaction mixtures were subjected to SDS-PAGE followed by Western blotting. Pulse-chase analysis of SOD1-transfected HEK293 cells was performed as described previously (30). Pulse labeling was performed with 250 μCi/ml [35S]SOS for 45 min. After washing in phosphate-buffered saline (PBS), the cells were chased for the indicated time intervals in complete medium. Samples were immunoprecipitated with anti-Myc antibody, separated on 5–20% SDS-PAGE, and analyzed by phoshorimaging (LAS2000; Fujix, Tokyo, Japan).

Neurotoxicity Analysis and Quantification of SOD1 Aggregates—4 × 10\textsuperscript{4} Neuro2a cells were grown overnight on 2-well collagen-coated slides. They were transfected with 0.4 μg of pcDNA3.1+MycHis-SOD1, 0.4 μg of pcDNA4/HisMax-Dorfin, and 0.4 μg of pEGFP-C3 vector (CLONTECH). pcDNA4/HisMax-LacZ was used as control instead of Dorfin. Cells were incubated for 16 h, and the medium was then replaced with serum-free medium. Cell death was determined by propidium iodide (PI)-stained preparations at 48 h after serum deprivation. The ratio of dead cells was expressed as the percentage of PI- and GFP-positive cells in GFP-positive cells. For cell viability assay, 5 × 10\textsuperscript{3} Neuro2a cells were grown in 96-well collagen-coated plates overnight. They were then transfected with 0.1 μg of pcDNA3.1+MycHis-SOD1 and 0.1 μg of pcDNA4/HisMax-Dorfin. pcDNA4/HisMax-LacZ was used as control instead of Dorfin. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based cell proliferation assay was then performed using CellTiter 96 (Promega) at 0, 24, and 48 h after incubation. The assay was carried out in triplicate. Absorbance at 490 nm was measured in a multiple plate reader. For quantification of SOD1 aggregates, Neuro2a cells transfected with pEGFP-N1-SOD1 and pcDNA4/HisMax-Dorfin or -LacZ were examined using a confocal microscope (Radiance; Bio-Rad). All cells were counted in fields selected at random from four different quadrants of the culture well. Counting was performed by an investigator blind to the experimental condition.

RESULTS

Dorfin Is Localized in the Inclusion Bodies in ALS Motor Neuron—Immunohistochemical analysis revealed that Dorfin was predominantly localized in the NHI found in familial ALS (Fig. 1, A–C) and mutant SOD1 \textsuperscript{G93A}–transgenic mice (Fig. 1D) as well as in sporadic ALS (Fig. 1E). About 50% of NHIs were positively stained for Dorfin. Dorfin immunoreactivity was concentrated either at the periphery of the inclusion (Fig. 1A) or throughout it (Fig. 1B). Some aggregates localized in neuronal processes (Fig. 1C). Furthermore, Dorfin colocalized not only with SOD1-positive inclusions (Fig. 1, F–I) but also with Ub in NHI in both familial (Fig. 1, J–L) and sporadic (Fig. 1, M–O) ALS. Neither neural tissues nor tissues other than central nervous tissue were stained with Dorfin. Only weak staining of Dorfin was diffusely observed throughout motor neurons in normal human spinal cords without neu-
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**Fig. 2. Association of Dorfin with mutant SOD1 but not wild-type SOD1 in HEK293 cells.** A, various Myc-tagged mutant SOD1s as indicated were co-transfected with Xpress-tagged Dorfin. After immunoprecipitation was performed with anti-Xpress antibody, the resulting precipitates and the cell lysate were analyzed by Western blotting with anti-Myc-HRP or anti-Xpress-HRP antibodies. Arrowheads on the right indicate the position of SOD1 or Dorfin. B, schematic representation of Dorfin and deletional mutants of Dorfin (i.e. Dorfin-N and Dorfin-C) used in this study. C, binding of mutant SOD1 to the C-terminal portion of Dorfin. After Myc-tagged mutant SOD1G85R and Xpress-tagged full-length Dorfin or Dorfin-mutants were transfected, immunoprecipitation and Western blotting were performed as described in A.

Neurologic disease and non-transgenic littermate mice (data not shown). These findings suggest that Dorfin is involved in inclusion body formation via the ubiquitylation of substrate(s) yet to be identified in NHI and that SOD1 is a plausible target for ubiquitylation by Dorfin in familial ALS.

**Dorfin Interacts with Mutant SOD1 but Not Wild-type SOD1**—We examined whether Dorfin interacts with SOD1 in vivo. To this end, Xpress-tagged Dorfin was coexpressed with C-terminal Myc-tagged wild-type or various mutant forms of SOD1 in HEK293 cells. Western blotting analysis revealed that Dorfin co-immunoprecipitated with all mutant SOD1s examined here but not with wild-type SOD1 (Fig. 2A). However, Dorfin failed to bind either of the androgen receptors with normal (Q24) and extended (Q97) polyglutamine tracts or wild-type and mutant (A30P, A53T) α-synuclein (data not shown). Dorfin has a unique primary structure containing a RING finger/IBR motif at its N terminus and can be structurally divided into two parts, i.e. 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) (Fig. 2B). We found that Dorfin-C but not Dorfin-N specifically bound mutant SOD1G85R (Fig. 2C), indicating that Dorfin binds to the mutant SOD1 via its C-terminal region.

**Dorfin Ubiquitylates Mutant SOD1 in Vitro and Promotes Ubiquitylation and Degradation of SOD1 in Vitro**—Physical interaction between Dorfin and mutant SOD1 prompted us to investigate whether SOD1 itself is ubiquitylated by Dorfin. We first examined whether SOD1 is ubiquitylated in a culture cell model. C-terminal Myc-tagged wild-type or mutant SOD1 was co-transfected with FLAG-tagged Ub in HEK293 cells. When SOD1 was immunoprecipitated after treatment with the proteasome inhibitor MG132, all mutant SOD1s, unlike wild-type SOD1, were found to be polyubiquitylated (Fig. 3A). In addition, ectopic expression of Dorfin increased the ubiquitylation of mutant SOD1G85R without affecting the wild-type SOD1 (Fig. 3B).

We next examined whether Dorfin directly ubiquitylates SOD1 in vitro. For this purpose, we prepared IP Xpress-Dorfin or its deletion mutants and IP wild-type or mutant SOD1G85R. Myc without proteasome inhibitor after transfection into HEK293 cells, independently. When these immunoprecipitates were incubated with recombinant E1, E2 (UbcH7), His-tagged Ub, and ATP, high molecular weight ubiquitylated bands were observed in the presence of IP-Xpress-Dorfin with mutant SOD1G85R, whereas no signal was noted in wild-type SOD1 or mutant SOD1G85R in the absence of either Dorfin, E1, or E2 (Fig. 3C). Deletion mutants of Dorfin (Dorfin-N or Dorfin-C) did not show a significant activity upon ubiquitylation against mutant SOD1G85R (Fig. 3C), indicating that both E2-recruiting N-terminal and substrate-binding C-terminal portions are required for Dorfin-mediated ubiquitylation. Further in vitro studies using other mutants showed that Dorfin also ubiquitylated SOD1G77R and SOD1G93A significantly and ubiquitylated SOD1G148R as well, although to a lesser extent (Fig. 3D).

Mutant SOD1 protein has a short half-life compared with wild-type SOD1 as shown previously by pulse-chase experiments (30, 32, 33). By blocking the ubiquitin-proteasome pathway, its half-life can be elongated (30, 33). We examined whether the in vivo stability of wild-type and mutant SOD1 is affected by Dorfin. We used pulse-chase analysis to evaluate the stability of wild-type and mutant Myc-tagged SOD1 expressed in HEK293 cells in the presence or absence of Dorfin. Pulse-chase experiments revealed that 35S-labeled SOD1G85R was fairly unstable compared with its wild-type version, and the degradation of SOD1G85R was greatly accelerated when Dorfin was overexpressed, whereas the stability of wild-type SOD1 was unaffected (Fig. 3E).

**Dorfin Protects Neuronal Cells from Mutant SOD1-mediated Neurotoxicity through Its E3 Activity and Reduces SOD1 Inclusion Bodies**—Based on these observations, we inferred that Dorfin protects cells against mutant SOD1-mediated neurotoxicity. To study neuronal cell death induced by mutations in SOD1, we used a mouse neuroblastoma cell line (Neuro2a) transiently transfected with wild-type and a mutant (G85R and G93A). These cells are maintained as nondifferentiated dividing cells but can be induced to differentiate to be neural cells by serum deprivation (34). We transfected SOD1 with Dorfin or LacZ into Neuro2a cells and induced neural differentiation by serum deprivation after overnight post-transfection. Co-expression of Dorfin significantly reduced the percentages of PI-positive dead cells in both mutant SOD1G85R- and SOD1G93A-transfected cells compared with those in cells co-expressing LacZ (Fig. 4A). In contrast, Dorfin-N and Dorfin-C had no protective activity, indicating that the E3 activity of Dorfin is essential for the suppression of mutant SOD1-induced neuronal cell death. We also found a similar Dorfin-dependent protective effect against the loss of neuronal cell viability evoked by mutant SOD1 using the MTT assay (Fig. 4B). One hypothesis argues that toxicity results from the tendency of mutant SOD1 to aggregate into the cytoplasmic inclusion bodies (35) that are evident in cultured spinal motor neurons (36) and COS7 cells (37) expressing mutant SOD1 cDNA or in motor neurons from SOD1 transgenic mice (38, 39). In our experimental model, the expression of mutant SOD1G85R induced perinuclear intracytoplasmic inclusion bodies in Neuro2a cells (Fig. 4C, left panel). The overexpression of Dorfin significantly reduced the number of aggregates in SOD1G85R-transfected Neuro2a cells (Fig. 4C, right panel, and 4D).
DISCUSSION

In the present study, we showed for the first time that mutant SOD1s are selectively degraded through a Dorfin-mediated Ub-proteasome pathway and that Dorfin protects neuronal cells against the toxic effects of mutant SOD1. Whereas Dorfin can ubiquitylate mutant SOD1s, probably because of their fragile or misfolded conformation, the constant production of high amounts of impaired SOD1 in familial ALS becomes a burden on the protein degradation process through the Ub-proteasome pathway, eventually overwhelming the capacity of the proteasome to degrade toxic SOD1 and subsequently leading to the accumulation of ubiquitylated SOD1 and motor neuron death. Consistent with this scenario, recent studies reported that impairment of the Ub-proteasome system is caused by protein aggregation (40). Thus, up-regulation or exogenous expression of Dorfin may be therapeutically beneficial in familial ALS. Our results also showed that Dorfin colocalized with ubiquitylated inclusions not only in familial ALS but also in sporadic ALS. Based on this finding, it is conceivable that familial and sporadic forms of ALS share a common mechanism

**FIG. 3.** Ubiquitylation of mutant SOD1 by Dorfin. A, mutant SOD1s were ubiquitylated in HEK293 cells, which were co-transfected with Myc-tagged wild-type SOD1 or SOD1 mutants (as indicated) and FLAG-tagged Ub. These cells were treated with 0.5 μM MG132 for 16 h after overnight post-transfection. Immunoprecipitates prepared by an anti-Myc antibody were used for immunoblotting with the anti-FLAG antibody. The high molecular-mass ubiquitylated SOD1s are shown as SOD1-(Ub)n on the right (upper panel). Asterisks indicate IgG light and heavy chains. The blot shown is a representative blot from three independent experiments. Total lysate was used for Western blot with the anti-Myc antibody (lower panel). Asterisk on the right indicates the position of SOD1. B, increased ubiquitylation of SOD1G85R by overexpression of Dorfin. HEK293 cells were co-transfected with Myc-tagged wild-type SOD1 or SOD1G85R mutants and FLAG-tagged Ub in the presence of Dorfin. Immunoprecipitated Dorfin and Myc-tagged SOD1 were transferred into HEK293 cells independently. Immunoprecipitation and immunoblotting were performed as described in A. C, in vitro ubiquitylation assay of SOD1G85R by Dorfin. Xpress-tagged Dorfin and Myc-tagged SOD1 were transfected into HEK293 cells independently. Immunoprecipitated Dorfin (IP Xpress-Dorfin) and SOD1 (IP-SOD1-Myc) were prepared and mixed in an assay mixture for ubiquitylation. For this assay, wild-type and mutant SOD1G85R and full-length Dorfin, Dorfin-N, and Dorfin-C were used. After a 90-min incubation at 30°C, SDS-PAGE was performed followed by Western blotting for SOD1 with the anti-Myc antibody. Monomeric, dimeric, and the high molecular mass-ubiquitylated SOD1 are shown on the right. Note that only mutant SOD1 showed SDS-resistant dimeric banding indicated by (SOD1)2. D, in vitro ubiquitylation assay of various SOD1 mutants by Dorfin. The ubiquitylation assay was carried out as described in C except that various SOD1 mutants were used as indicated, and Western blotting was also conducted with an anti-Myc antibody (left panel) for the detection of SOD1 as well as anti-Ub antibody (right panel). E, accelerated degradation of mutant SOD1G85R by Dorfin. HEK293 cells transiently expressing wild-type (open circles and open squares) or mutant SOD1G85R (closed circles and closed squares) were pulse-labeled with [35S]Cys for 45 min and chased for the time intervals indicated under the overexpression of Dorfin (open squares and closed squares) or LacZ (open circles and closed squares). Data are mean values of three independent experiments.
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**Fig. 4.** Dorfin protects neural cells against mutant SOD1-mediated toxicity. A, cell death assay using PI staining. Neuro2a cells were grown on collagen-coated 2-chamber well slides and transfected with Myc-tagged SOD1 (wild-type, SOD1GR85S, and SOD1GR93A) and Xpress-tagged Dorfin (full-length, red bars; Dorfin-N, yellow bars; Dorfin-C, green bars). Xpress-tagged LacZ (white bars) was used as control. The pEGFP-C3 vector was also transfected as a marker of transfected cells. After 48 h of serum withdrawal, the proportions of PI- and GFP-positive cells among the GFP-positive cells were counted. Data are mean ± S.D. values of triplicate assays. Statistical analyses were carried out by one-way analysis of variance (ANOVA). *p < 0.001. B, the reduction of mutant SOD1 aggregates by Dorfin. Neuro2a cells grown on collagen-coated 2-chamber well slides were transfected with a GFP-tagged wild-type or mutant SOD1GR85S in the presence of Dorfin (red bars) or LacZ (white bars). After 48 h of serum withdrawal, slides were examined using a laser-scanning confocal microscope. Panel C shows a typical example in which the overexpression of Dorfin reduces SOD1GR85S aggregate-bearing Neuro2a cells. The percentages of aggregate-positive cells among the GFP-positive cells were determined in D. Data are the mean ± S.D. values of triplicate assays. Statistical analyses were carried out by Mann-Whitney's U test. *p < 0.01.

involving the dysfunction of the Ub-proteasome pathway despite distinct etiological mechanisms.

Studies of parkin have provided new insights into the importance of the ubiquitin-proteasome pathway in the neuronal degeneration of PD. Parkin was shown to have E3 activity (19–21). Recently, an O-glycosylated form of α-synuclein and synphilin-1 were shown to be substrates of parkin (41, 42). Both α-synuclein and synphilin-1 are major components of Lewy bodies, which are ubiquitylated inclusion bodies characteristic of sporadic PD. A link between sporadic and familial PD through a-synuclein, synphilin-1, and parkin suggests that common molecular pathogenic mechanisms underlie PD. The accumulation of toxic or undesired proteins in neurons may result from the failure of degradation systems and could subsequently lead to neurodegeneration. From this point of view, in sporadic ALS post-translational modified SOD1 (43) or other unknown substrates might accumulate in the ubiquitylated form and play a role in the pathogenesis of the disease.

Our results raise an important question related to the function of Dorfin; what is the biological role of Dorfin and how does Dorfin recognize the abnormal mutant SOD1? Because we used IP-Dorfin and IP-mutant SOD1 for an ubiquitylating assay here, it is possible that Dorfin interacts with mutant SOD1 indirectly. Recently CHIP (carboxyl terminus of Hsc70-interacting protein), an U-box type E3, has been shown to interact with Hsp90 or Hsp70 and ubiquitylate unfolded proteins captured by these molecular chaperones in a selective manner, thus acting as a ‘quality control E3’ (44, 45). Likewise, because Dorfin can discriminate between the normal and abnormal status of SOD1 proteins, it can be regarded as another quality control E3. However, preliminary results showed that CHIP did not ubiquitylate mutant SOD1s (data not shown); therefore, Dorfin possesses a distinct way for recognition of the abnormality of SOD1. It is possible that a novel mechanism for trapping the target in a direct or indirect manner. Further studies should be designed to determine this mechanism, which should enhance our understanding of Dorfin function in vivo and its relationship to ALS pathogenesis.

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