Disulfide bond mediates aggregation, toxicity and ubiquitylation of familial amyotrophic lateral sclerosis-linked mutant SOD1

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Running Title: Disulfide-linking and ubiquitylation of mutant SOD1

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Mutations in the Cu/Zn-superoxide dismutase (SOD1) gene cause familial amyotrophic lateral sclerosis (ALS) through the gain of a toxic function; however, the nature of this toxic function remains largely unknown. Ubiquitylated aggregates of mutant SOD1 proteins in affected brain lesions are pathological hallmarks of the disease and are suggested to be involved in several proposed mechanisms of motor neuron death. Recent studies suggest that mutant SOD1 readily forms an incorrect disulfide bond upon mild oxidative stress in vitro and the insoluble SOD1 aggregates in spinal cord of ALS model mice contain multimers cross-linked via intermolecular disulfide bonds. Here we show that a non-physiological intermolecular disulfide bond between cysteines at positions 6 and 111 of mutant SOD1 is important for high molecular weight aggregate formation, ubiquitylation, and neurotoxicity; all of which were dramatically reduced when the pertinent cysteines were replaced in mutant SOD1 expressed in Neuro-2a cells. Dorfin is an ubiquityl ligase that specifically binds familial ALS-linked mutant SOD1 and ubiquitylates it, thereby promoting its degradation. We found that Dorfin ubiquitylated mutant SOD1 by recognizing the Cys6,111-disulfide cross-linked form and targeted it for proteasomal degradation.

INTRODUCTION

Cu/Zn superoxide dismutase (SOD1)1, a major intracellular antioxidant enzyme, metabolizes superoxide radicals to molecular oxygen and hydrogen peroxide (1,2). Since mutations in SOD1 linked to familial amyotrophic lateral sclerosis (ALS) were first identified (3), more than
100 mutations at over 70 residues in the 153 amino acid SOD1 protein have been reported (4). Most mutations are missense mutations, with a few causing early termination or frameshifts near the carboxyl terminus of the protein. SOD1 mutations account for about 20% of familial ALS, which is characterized by selective degeneration of motor neurons. SOD1 is primarily a cytosolic protein (5) and the active enzyme is a homodimer of two subunits (6). Each subunit contains four cysteine (Cys) residues at positions 6, 57, 111, and 146. An intramolecular disulfide bond between Cys$^{57}$ and Cys$^{146}$ of each subunit facilitates its correct folding and stabilizes the active homodimeric structure (7,8), but it is not known how the disulfide is formed in the reducing environment of the cytosol. While the endoplasmic reticulum (ER) is the specialized site for oxidative folding (9), there is no SOD1 localization to the ER (10). Most familial ALS-linked mutations render SOD1 more susceptible to intramolecular disulfide bond reduction (11) and accelerate the rate of protein turnover (12,13). Recent lines of evidence implicate the disulfide-reduced monomer as the common aggregation-prone, neurotoxic intermediate of mutant SOD1 proteins (8,11,14-16) and a significant fraction of the insoluble SOD1 aggregates in the spinal cord of mutant SOD1 transgenic mice contain high molecular weight-species cross-linked via intermolecular disulfide bonds (17). Hence, modulation of disulfide bond formation may be important in mutant SOD1-linked motor neuron-selective neurotoxicity.

ALS-linked mutant SOD1 proteins are turned over more rapidly than wild-type SOD1 and proteasome inhibitors increase the amount of mutant SOD1 (18,19). To date, two distinct ubiquityl ligases, Dorfin and NEDL1, have been reported to ubiquitylate mutant SOD1 (20,21). Dorfin is a RING-finger/IBR (in-between ring-finger) domain-containing ubiquityl ligase, which we previously identified from human spinal cord (22), and belongs to the RBR (RING-Between rings-RING) family of proteins (23). Dorfin physically binds and ubiquitylates various familial ALS-linked SOD1 mutants and subsequently targets them for proteasomal degradation, but it has no effect on the stability of wild-type SOD1 (20). Overexpression of Dorfin protects neuronal cells against the toxic effects of mutant SOD1 and reduces the number of aggregates composed of mutant SOD1 (20). However, the mechanism by which Dorfin discriminates between the normal and pathogenic status of SOD1 proteins remains unknown. There are numerous variants causing familial ALS, thus it seems reasonable that Dorfin recognizes a common protein modification among
mutant SOD1s that is not present in wild-type SOD1.

In this study, we generated SOD1 proteins with various combinations of the four Cys residues replaced by serines (Ser) and assessed their disulfide bond status, changes in the formations of their high molecular weight-species, and their neurotoxicity. Moreover, by studying the interaction between Dorfin and these engineered SOD1s, we investigated whether disulfide bonds are critical for Dorfin recognition and ubiquitylation of mutant SOD1s.

**EXPERIMENTAL PROCEDURES**

*Construction of the expression vectors - Construction of pcDNA3.1/MycHis-SOD1, pEGFP-N1-SOD1 and pcDNA4/HisMax-Dorfin vectors were described previously (20,22). Cys to Ser missense mutations were introduced into pcDNA3.1/MycHis-SOD1 and pEGFP-N1-SOD1 with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primer pairs for each Cys to Ser mutant were as follows: 5'-CGAAGGCGGTGCTCCGTGCTGAA GGGC-3’ and 5'-GCCCTTCAGCAGCAGGACAGGCC TTCG-3’ for C6S; 5'-GATAATACAGCAGGCTCTACTAC GTGCAGGTCC-3’ and 5'-GGACCTGCAGCAGCTTCAAGCTG

CTGTATTATC-3’ for C57S; 5'-CTCAGGAGACCATCCATCCATGG GCGCAC-3’ and 5'-GTGCGGCAATGATGGAATGTT CTCCTGAG-3’ for C111S; 5'-GGAAGTCTTGGCTCTGTTGT AATTGGGATCG-3’ and 5'-CGATCCCCATACACCAGAAGC CAAACGACTTCC-3 for C146S. Multiple Cys to Ser replaced vectors were obtained by repeatedly applying a mutagenesis.*

*Cell culture, transfection, and antibodies - Neuro-2a cells (American Type Culture Collection, Manassas, VA), a line derived from mouse neuroblastoma, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS), 5 U/ml penicillin and 50 µg/ml streptomycin. Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in the WST-1 assay, or Effectene Transfection Reagent (Qiagen, Valencia, CA) in other experiments according to the manufacturers’ instructions. To inhibit cellular proteasome activity, cells were treated with 1 µM (except as otherwise indicated) MG132 (Z-Leu-Leu-Leu-al; Sigma, St. Louis, MO) or Epoxomicin (Sigma) as indicated concentration for 24 h after overnight transfection. To differentiate Neuro-2a cells, they were changed to DMEM culture medium.*
containing 2% FCS and 20 µM retinoic acid and cultured for 48 h. Primary antibodies used were as follows: anti-Myc mouse monoclonal antibody (9E10; Sigma), anti-Myc rabbit polyclonal antibody (A-14; Santa Cruz Biotechnology, Santa Cruz, CA), anti-SOD1 rabbit polyclonal antibody (SOD100; Stressgen bioreagents, Victoria, Canada), anti-α Tubulin mouse monoclonal antibody (B-5-1-1; Sigma), anti-ubiquitin (Ub) mouse monoclonal antibody (4PD1; Santa Cruz Biotechnology), and anti-Xpress mouse monoclonal antibody (Invitrogen).

Transgenic mice – 17-week-old symptomatic B6SJL-TgN(SOD1-G93A)1Gur ALS mice overexpressing the human mutant SOD1G93A (The Jackson Laboratory, Bar Harbor, ME) were used. The experimental design of this study was fully approved by the Experimental Animal Ethical Committee of the Nagoya University Graduate School of Medicine. Tissues were homogenized in 10 volumes of lysis buffer (TNE) consisting of 50 mM Tris HCl, 150 mM NaCl, 1% Nonidet P-40 and 1 mM ethylenediaminetetraacetic acid (EDTA) with a protease inhibitor cocktail (Complete Mini, Roche Diagnostics). Protein concentrations were determined with a DC protein assay kit (Bio-Rad, Hercules, CA). Immunoprecipitation from the soluble fraction was performed with 2 µg anti-Myc or anti-Xpress antibodies and Protein A/G Plus-Agarose (Santa Cruz Biotechnology), and the precipitates were washed four times in TNE buffer. Cell lysates or immunoprecipitates were separated by SDS-PAGE (5%-20% gradient gel) and analyzed by Western blotting with ECL plus detection reagents (GE Healthcare Bio-Sciences, Piscataway, NJ). Non-reducing SDS-PAGE was conducted without 2-mercaptoethanol (2-ME) in the sample buffer. As omitting reducing agents from the protein samples

Immunoprecipitation and Western blotting analysis - 5×10^5 cells from a 6 cm dish were lysed on ice with 1 ml of TNE lysis buffer. The lysate was centrifuged at 1,000 × g for 15 min at 4°C to remove nuclei and cell debris. Denucleated cell lysates (crude fraction) were separated into supernatant (soluble fraction) and pellet fractions by centrifuging at 20,000 × g for 20 min at 4°C. The pellets were lysed (insoluble fraction) with 1 ml of TNES lysis buffer consisting of 50 mM Tris HCl, 150 mM NaCl, 1% Nonidet P-40, 2% SDS and 1 mM EDTA with a protease inhibitor cocktail (Complete Mini, Roche Diagnostics). Protein concentrations were determined with a DC protein assay kit (Bio-Rad, Hercules, CA).
can lead to adventitious air oxidation or disulfide scrambling, 100 mM iodoacetamide was added to the lysates to prevent these changes during sample preparation.

*Filter trap assay* - Each of the various fractions from the cell lysates (crude, soluble, and insoluble fractions) was filtered under vacuum through 0.2 \( \mu \text{m} \) cellulose acetate membranes (Sartorius, Gottingen, Germany) followed by two washes in TBS. The membranes were then incubated with 5% milk powder in TBS at room temperature for 1 hour, followed by an overnight incubation at 4°C with anti-Myc antibody in TBS with 0.1% Tween 20. Primary antibodies were detected with HRP-conjugated secondary antibodies (GE Healthcare Bio-Sciences), which were then detected with ECL plus chemiluminescence reagent (GE Healthcare Bio-Sciences). To confirm equal loading of proteins, the same samples were blotted onto 0.45 \( \mu \text{m} \) nitrocellulose membranes (Bio-Rad) and probed with anti-Myc or anti-\( \alpha \) Tubulin antibodies.

*Neurotoxicity analysis and quantification of SOD1 aggregates* - 2\( \times 10^4 \) Neuro-2a cells were grown overnight on 4-chamber, collagen-coated slides (Nalge Nunc, Rochester, NY) and then transfected with 0.2 \( \mu \text{g} \) of pEGFP-N1-SOD1. After overnight incubation, the cells were differentiated in DMEM containing 2% FCS and 20 \( \mu \text{M} \) retinoic acid for 48 h. Inclusion bodies were counted in more than 100 randomly selected cells and the percentages of cells with such inclusions calculated. Data from three independent experiments were averaged. For the cell viability assay, 5\( \times 10^3 \) Neuro-2a cells were grown in 96-well collagen-coated plates overnight, and then transfected with 0.1 \( \mu \text{g} \) of pEGFP-N1-SOD1 or pcDNA3.1/MycHis-SOD1, with or without 0.1 \( \mu \text{g} \) pcDNA4/HisMax-Dorfin. pcDNA4/HisMax mock vector was used as a control. A 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H -5-tetrazolio]-1, 3-benzene disulfonate (WST-1)-based cell proliferation assay (Roche Diagnostics) was performed 48 h after differentiation. Absorbance was measured in a multiple plate reader (PowerscanHT, Dainippon Pharmaceutical, Japan). The assay was carried out in triplicate and statistically analyzed by one-way analysis of variance (ANOVA) or unpaired \( t \)-test.

*Quantitative analysis of gene expression levels* - Total RNA was extracted from Neuro-2a cells expressing SOD1-GFP and their Cys to Ser derivatives by using RNA Easy Kit (Qiagen), followed by cDNA synthesis primed with oligo-dT using Superscript II (Invitrogen). The gene expression level was examined by quantitative RT-PCR using primer sets.
specific to target genes and QuantiTect SYBR Green PCR kit (Qiagen). PCR was performed on iCycler system (Bio-Rad) under the manufacturer’s recommended conditions.

**Isolation of SOD1 aggregates** - Isolation of SOD1 inclusion bodies was carried out according to Lee et al. (24) with a slight modification. $5 \times 10^5$ Neuro-2a cells in a 60 mm-dish expressing SOD1-GFP were washed with cold phosphate-buffered saline (PBS) before addition of TNE buffer. After a 5-min incubation at room temperature, the supernatant containing Nonidet P-40-soluble proteins was carefully removed from dishes. After gentle washing of dishes with PBS, the Nonidet P-40-insoluble materials were scraped and incubated on ice for 5 min. The extract was then centrifuged at $80 \times g$ for 15 min. The pellet containing big inclusions was put onto a slide glass, sealed with a coverslip and observed under a BX51 epifluorescence microscope (Olympus, Tokyo, Japan).

**Cycloheximide chase analysis** - Neuro-2a cells grown on 6 cm dishes were transfected with 1 $\mu$g of pcDNA3.1/MycHis-SOD1 with or without 1 $\mu$g pcDNA4/HisMax-Dorfin. Twenty-four hours after transfection, cycloheximide (50 $\mu$g/ml) was added to the culture medium and the cells were harvested at the indicated time points. The samples were subjected to SDS-PAGE and analyzed by Western blotting with anti-Myc antibody. The intensities of the bands were quantified by ImageGauge software (Fuji Film, Tokyo, Japan). The assay was carried out in triplicate and statistically analyzed by one-way ANOVA or unpaired t-test.

**RESULTS**

Proteasome inhibition increases SDS-resistant disulfide-linked species as well as insoluble ones of ALS-linked mutant SOD1 - Mutant SOD1 is a fairly unstable protein and the increased turnover of mutant SOD1 is mediated by the ubiquitin-proteasome pathway (18,19). Thus, we first examined the effect of proteasome inhibition on mutant SOD1 proteins. When cellular proteasome activity was blocked by the proteasome inhibitor MG132, the level of soluble mutant SOD1$^{G85R}$ and SOD1$^{G93A}$ increased in a dose-dependent manner (Fig. 1B, arrow head) and an SDS-resistant mutant SOD1 dimer appeared (Fig. 1B, arrow). The increase in the amount of wild-type SOD1 was much smaller than that of mutant SOD1 (Fig. 1B, arrow head). Detergent-insoluble, sedimentable mutant SOD1 also increased as proteasome activity was inhibited (Fig. 1C). Interestingly, as the proteasome activity was inhibited, aberrant high molecular weight SDS-resistant disulfide-linked
mutant SOD1$_{G85R}$ and SOD1$_{G93A}$ became more abundant (Fig. 1A, asterisk). There were almost no SDS-resistant disulfide-linked species of the wild-type SOD1. Same findings were obtained when blots were probed with anti-SOD1 antibody (Supplemental Fig. S1A). These results were also confirmed with epoxomicin, a selective and irreversible proteasome inhibitor (Supplemental Fig. S1B). Thus, intermolecular disulfide bond-linked mutant SOD1 is unstable and prone to degradation by the proteasome.

_Free Cys$^6$ and Cys$^{111}$ are important for generating disulfide bond-linked species and insoluble, sedimentable forms of mutant human SOD1._ We examined the role of Cys residues in the formation of aberrant disulfide-bond linked high molecular weight species. Various combinations of the four Cys residues at positions 6, 57, 111 and 146 replaced with serines (Ser) were introduced into SOD1 protein-expression vectors using site-directed mutagenesis. The effects of amino acid replacement at one of the four Cys residues, at two of the four Cys residues, and at all four Cys residues on wild-type and two familial ALS-linked SOD1 mutants, SOD1$_{G85R}$ and SOD1$_{G93A}$ were investigated. We used MycHis-tagged SOD1 expression vectors and an antibody against the tag peptide to detect SOD1 protein so as to avoid possible reduced detection of SOD1 with multiple amino acids changes by the anti-SOD1 antibody. Interestingly, none of the Cys residue replacements generated disulfide-linked species in wild-type SOD1 proteins (Fig. 2, left panel). Under reducing conditions, replacement of Cys$^6$ had a stronger effect on the formation of disulfide-linked species of mutant SOD1 than did the other three Cys residue replacements (Fig. 2, middle and right panels, asterisk). Combinations of replacing Cys$^6$ and one of the other Cys residues further attenuated the aberrant disulfide-linking of mutant SOD1 seen with the single substitution of Cys$^6$ (Fig. 2, filled circle). Under usual reducing conditions, the same reduced oligomerization of mutant SOD1 was observed when combinations of Cys$^6$ and other Cys residues were replaced (Fig. 2, arrow). The detergent-insoluble, sedimentable form of mutant SOD1 was also reduced especially if both Cys$^6$ and Cys$^{111}$ were replaced (Fig. 2, filled square). Replacement of all four Cys residues completely abolished the disulfide-linked species in the non-reducing condition and the oligomeric, detergent-insoluble form of mutant SOD1 in the reducing condition (Fig. 2, lane 4xCS). Because simultaneous substitutions of Cys$^6$ and Cys$^{111}$ had the strongest effects on the formation of aberrant species of mutant SOD1 in both non-reducing and reducing conditions, we compared C6S, C111S
Substituting both Cys\textsuperscript{6} and Cys\textsuperscript{111} greatly reduces high molecular weight aggregate formation and ubiquitylation of mutant SOD1 - In studies of polyglutamine disorders, it has been demonstrated that high molecular weight aggregates of mutant proteins are retained by filtration through cellulose acetate (25,26). Cellulose acetate membranes usually bind protein very poorly and are used to trap high molecular weight structures from complex mixtures through filtration. This assay was also successfully applied to detect mutant SOD1 aggregation (27). Thus we used a cellulose acetate filter trap assay to investigate whether SOD1 proteins with Cys substitutions are retained in high molecular weight aggregates from lysates of SOD1-MycHis expressing Neuro-2a cells. Cells were lysed in TNE buffer, fractionated into crude denucleated, soluble, and insoluble fractions, and each fraction was then filtered through a 0.22 µm cellulose acetate membrane. Subsequent staining with anti-Myc antibody revealed trapped SOD1 proteins (Fig. 3A, upper panel). Interestingly, high molecular weight aggregates were abundantly detected in mutant SOD1\textsuperscript{G85R}, SOD1\textsuperscript{G93A} and their C57S, C146S derivatives. Replacements of Cys\textsuperscript{6} and Cys\textsuperscript{111} greatly reduced high molecular weight structures of mutant SOD1. No high molecular weight aggregates were present in either wild-type SOD1 or their Cys-substituted mutants.

Mutant, but not wild-type, SOD1 is conjugated to a mult ubiquitin chain and degraded at the proteasome (20,28). To assess whether SOD1 proteins are ubiquitylated, we carried out an \textit{in vivo} ubiquitylation analysis by expressing SOD1\textsuperscript{WT}, SOD1\textsuperscript{G85R}, SOD1\textsuperscript{G93A} and their Cys to Ser mutants in Neuro-2a cells in the presence of the proteasome inhibitor MG132. When SOD1 was then immunoprecipitated, mutant SOD1s, but not wild-type SOD1, were polyubiquitylated (Fig. 3B, lane 1). Replacement of both Cys\textsuperscript{6} and Cys\textsuperscript{111} abolished ubiquitylation of mutant SOD1, while replacement of Cys\textsuperscript{57} and Cys\textsuperscript{146} did not affect the ubiquitylation status of mutant SOD1 (Fig. 3B, lane 2 vs. lane 3). Wild-type SOD1 and its Cys-replacement mutants were not ubiquitylated at all. Replacing only one of the four Cys residues attenuated neither the formation of high molecular weight-species nor the ubiquitylation of mutant SOD1 (data not shown). Thus, the presence of both Cys\textsuperscript{6} and Cys\textsuperscript{111} is important for high molecular weight aggregate formation and ubiquitylation of mutant SOD1. Disulfide bond formation at Cys\textsuperscript{6} or Cys\textsuperscript{111} is critical step for ubiquitylation of mutant SOD1.
Formation of disulfide-linked species of mutant SOD1 strongly correlates with visible aggregate formation and neurotoxicity - Expression of mutant, but not wild-type, SOD1 induces large perinuclear intracytoplasmic aggregates in differentiated Neuro-2a cells and reduces cellular viability (20). We analyzed the role of mutant SOD1 Cys residues in aggregate formation and neurotoxicity in Neuro-2a cells. Replacements of Cys\textsuperscript{6} and Cys\textsuperscript{111} significantly reduced the percentage of mutant SOD1\textsuperscript{G85R} and SOD1\textsuperscript{G93A} cells with visible aggregates (Fig. 4A). To further demonstrate the extent of aggregate formation, we isolated SOD1 aggregates with a procedure according to Lee et al. (24). Differentiated Neuro-2a cells bearing SOD1-GFP aggregates were extracted with 1% Nonidet P-40 in the culture dish, and the Nonidet P-40-soluble proteins were gently removed. Under this condition, the soluble monomeric SOD1 was completely removed, and the aggregates remained in the culture dish due to their association with unknown structures (24). The remaining Nonidet P-40-insoluble portion was then scraped and centrifuged at 80 \( \times \) g. After the centrifugation at 80 \( \times \) g for 15 min, the pellet fraction was found to contain exclusively the large inclusion bodies. Replacements of Cys\textsuperscript{6} and Cys\textsuperscript{111} markedly reduced the number of inclusion bodies in G93A mutant SOD1-GFP (Fig. 4B). Mutant SOD1\textsuperscript{G85R} and SOD1\textsuperscript{G93A}, but not wild-type SOD1, are toxic in differentiated Neuro-2a cells as previously described (20). However, replacement of the Cys\textsuperscript{6} and Cys\textsuperscript{111} residues markedly reduced this neurotoxicity (Fig. 4C), which was not affected by replacing the Cys\textsuperscript{57} and Cys\textsuperscript{146} residues. There were no significant differences among the expression levels of all the constructs (Fig. 4D). Thus, changes in inclusion formation and toxicity are not due to differences in altered expression. These results provide evidence of direct links among intermolecular disulfide bonding, ubiquitylated complex formation, visible aggregate formation, and neurotoxicity.

Preferential occurrence of disulfide-crosslinked mutant SOD1 in the affected lesions of ALS model mice - Although mutant SOD1 is expressed at similar levels in both neuronal and non-neuronal tissues, the aggregated and ubiquitylated forms are selectively found in the pathological lesions of patients and mutant SOD1-transgenic mice (29,30). Thus, we next examined whether mutant SOD1 is aberrantly disulfide-linked in various tissues from symptomatic mutant SOD1 transgenic mice. Western blotting analysis, using anti-SOD1 antibody under reducing and non-reducing (omitting reducing agent 2-ME) conditions, demonstrated that the expression levels of
mutant SOD1 were nearly the same in all tissues examined. Each of the tissues showed some of the disulfide-linked mutant SOD1 species; however, in the brain stem and spinal cord, the areas predominantly affected in mutant SOD1-linked ALS, there was increased formation of intermolecular disulfide-linked species of mutant SOD1 (Fig. 5). Thus, intermolecular disulfide-linked species are implicated as the aggregation-prone and neurotoxic intermediate of mutant SOD1 in vivo.

**Affects of Cys\(^6\)- and Cys\(^{111}\)-mediated disulfide-linking on the rate of mutant SOD1 degradation** - To determine whether replacement of Cys residues affects the degradation of SOD1 proteins, we examined the stability of mutant SOD1 proteins expressed in Neuro-2a cells (Fig. 6A, B). Chase experiments with cycloheximide, which halts all cellular protein synthesis, demonstrated that replacement of Cys residues did not influence the stability of wild-type SOD1 protein (Fig. 6A). By contrast, although mutant SOD1 showed the enhanced degradation compared with wild-type proteins previously described (18-20), when both Cys\(^6\) and Cys\(^{111}\) were replaced with Ser, the degradation of mutant SOD1 was markedly increased (Fig. 6B). Replacement of Cys\(^{57}\) and Cys\(^{146}\) did not significantly change the rate of degradation compared to Cys-native mutant SOD1 protein.

**Ubiquityl ligase Dorfin ubiquitylates and promotes degradation of disulfide-linked mutant SOD1** - We have previously shown that Dorfin physically binds and ubiquitylates various familial ALS-linked SOD1 mutants and enhances their degradation (20). Thus, we examined whether Cys residues on SOD1 affect the binding and ubiquitylating activities of Dorfin. To this end, Dorfin was co-expressed with wild-type or mutant SOD1 in Neuro-2a cells. Dorfin co-immunoprecipitated with G85R and G93A mutant SOD1s and their Cys\(^{57,146}\)-replaced derivatives (Fig. 7A). However, Dorfin interacted with Cys\(^{6,111}\)-replaced mutant SOD1 only very weakly and failed to bind to mutant SOD1 when all four Cys residues were replaced (Fig. 7A). Dorfin did not bind at all to wild-type SOD1. Using an in vivo ubiquitylation assay, we further examined whether co-expressed Dorfin enhances the ubiquitylation of Cys-substituted mutant SOD1 (Fig. 7B). When Cys-native or Cys\(^{57,146}\)-replaced mutant SOD1s were co-expressed with Dorfin, ubiquitylation of mutant SOD1s were increased; however, co-expression of Dorfin with mutant SOD1 in which Cys\(^{6,111}\) or all four Cys residues were replaced did not promote ubiquitylation of these mutant SOD1s (Fig. 7B). Chase experiments with cycloheximide in the presence or
absence of Dorfin demonstrated that degradation of Cys-native and Cys$^{57,146}$-replaced mutant SOD1$^{G93A}$ was greatly accelerated when Dorfin was overexpressed, whereas the stability of Cys$^{6,111}$- or all four Cys-replaced mutant SOD1$^{G93A}$ were unaffected (Fig. 7C). We have previously shown that Dorfin exerts neuro-protective effects by promoting degradation of mutant SOD1 through its ubiquityl ligase activities (20). Co-expression of Dorfin improved the viability of Neuro-2a cells expressing Cys-native and Cys$^{57,146}$-replaced mutant SOD1$^{G93A}$ (Fig. 7D).

DISCUSSION
Mutations in the SOD1 gene cause familial amyotrophic lateral sclerosis through the gain of a toxic function, however, the nature of this toxic function remains largely unknown (31). Ubiquitylated aggregates of mutant SOD1 proteins in affected lesions are a pathological hallmark of the disease (32) and suggest their relation to neurotoxicity. Recent biochemical studies suggest that the immature disulfide-reduced forms of the familial ALS mutant SOD1 proteins play a critical role in this neurotoxicity; in vitro, these forms tend to misfold, oligomerize, and readily undergo incorrect disulfide bond formation upon mild oxidative stress (16,33). Among the more than 100 ALS-associated human SOD1 mutants, some cannot intrinsically form the essential intramolecular disulfide bonds. One of the conserved Cys residues, Cys$^{146}$, is missing in some of the mutants, such as the Leu126 del TT (stop at 131) and Gly127 ins TGGG (stop at 133); however, it has been reported that minute quantities of SOD1 aggregates can cause the disease in mice expressing the truncated mutant, Gly127 ins TGGG (stop at 133) (34). Furthermore, a significant fraction of the insoluble SOD1 aggregates in the spinal cord of ALS model mice contain multimers cross-linked via intermolecular disulfide bonds (17,35). In the present study, we showed that non-physiological intermolecular disulfide bonds involving Cys$^{6}$ and Cys$^{111}$ of the mutant SOD1 were important for high molecular weight aggregate formation, ubiquitylation, and neurotoxicity in vivo, all of which were dramatically reduced in Neuro-2a cells when these residues were replaced with serines.

Human SOD1 has two free cysteine residues, Cys$^{6}$ and Cys$^{111}$ (36). Cys$^{6}$ is located adjacent to the dimer interface pointed toward the interior of the β-barrel and is solute inaccessible in the native, folded conformation. Cys$^{111}$ is located near the surface and is solute accessible and reactive, often becoming blocked during purification (37). Replacement of the free Cys residues increased the resistance to thermal inactivation (38). Increased resistance of
mutant SOD1s is due to increased resistance to irreversible unfolding and relatively unaffected by changes in conformational stability (39). Our data showing that aggregate formation of mutant SOD1 is reduced when Cys\textsuperscript{6} and Cys\textsuperscript{111} are replaced with serines, is compatible with these observations. Mutations of the Cys\textsuperscript{6} residue (C6F and C6G) still result in familial ALS (4), and in a transgenic mouse expressing mouse SOD1 retaining Cys\textsuperscript{6} but lacking Cys\textsuperscript{111} and with a G86R mutation corresponding to G85R mutation in human SOD1, degeneration of motor neurons in the spinal cord has been observed (40). These results imply that if one of either the Cys\textsuperscript{6} or Cys\textsuperscript{111} residues is present, it can still be a disease-causing SOD1. Our data here also revealed that replacement of only one of the Cys residues at positions 6 or 111 had modest effects on the formation of aggregates (Fig. 2).

Cytoplasmic proteins are degraded mainly via two pathways, the ubiquitin-proteasome pathway (6) and via autophagy (7). Previous studies have shown that mutant SOD1 proteins are turned over more rapidly than wild-type SOD1 (12,18,19). Two distinct ubiquitin ligases, Dorfin and NEDL1, were reported to specifically ubiquitylate mutant but not wild-type SOD1 (20,21). These studies suggest that mutant SOD1 is degraded by the ubiquitin-proteasome pathway and that the accelerated turnover of mutant SOD1 is mediated in part by this pathway. Impairment of the proteasome activities may contribute to ALS pathogenesis (28,41,42). We showed here that proteasome inhibition led to a dose-dependent accumulation of aberrant disulfide-linked high molecular weight mutant SOD1 (Fig. 1), suggesting that disulfide-linking mediates ubiquitylation of mutant SOD1. In fact, we found that Dorfin ubiquitylated mutant SOD1 by recognizing the Cys\textsuperscript{6}, Cys\textsuperscript{111} disulfide cross-linked form and targeted it for proteasomal degradation (Fig. 7). Mutant SOD1, in which the Cys\textsuperscript{6} and Cys\textsuperscript{111} were replaced, was not ubiquitylated (Fig. 3) and its rate of degradation was not affected in the presence of Dorfin (Fig. 7). It is possible that mutant SOD1 lacking Cys\textsuperscript{6} and Cys\textsuperscript{111} may be degraded directly by the proteasome without ubiquitylation (43) or by autophagy (44), but further studies are needed to address this issue.

The appearance of mutant SOD1 aggregates in motor neurons of familial ALS patients and mouse models has suggested that aggregation plays an important role in neurotoxicity (31). However, conflicting results have been reported on the correlation between aggregate formation and cell death. One report showed that aggregate formation of mutant SOD1\textsuperscript{A4V} and SOD1\textsuperscript{V148G} does not correlate with cell death (45), while
another study using live cell imaging techniques reported that the ability of mutant SOD1$^{G85R}$ and SOD1$^{G93A}$ proteins to form aggregates directly correlates with neuronal cell death (46). These controversies also exist in other neurodegenerative diseases (47,48). In this study, we clearly showed a direct link among intermolecular disulfide bond-mediated high molecular weight complex formation, visible aggregate formation, and neurotoxicity (Figs. 2, 3 and 4).

Furukawa et al. reported that formation of disulfide-linked multimers need not involve the nonconserved Cys residues, Cys$^6$ and Cys$^{111}$, and that the conserved Cys residues, Cys$^{57}$ and Cys$^{146}$, play an important role in the apo-form of SOD1 multimerization upon oxidative stress (16). Our results underscore the importance of Cys$^6$ and Cys$^{111}$ for high molecular weight aggregate formation, ubiquitylation and neurotoxicity in Neuro-2a cells. This discrepancy may result from differences in experimental conditions; we studied human SOD1 proteins expressed in Neuro-2a cells and Furukawa et al. used the purified apo-form of human SOD1 from *Escherichia coli*. Further studies will clarify the roles of each of the Cys residues of the mutant SOD1 protein in the ALS pathogenesis in vivo by generating transgenic mice bearing mutant SOD1 lacking Cys$^6$, Cys$^{111}$ or Cys$^{57}$, Cys$^{146}$.

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FOOTNOTES

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1 The abbreviations used are: Cu/Zn superoxide dismutase, superoxide dismutase 1; ALS, amyotrophic lateral sclerosis; ANOVA, analysis of variance; ER, endoplasmic reticulum; 2-ME, 2-mercaptoethanol; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.
FIGURE LEGENDS

Fig. 1. Proteasome inhibition leads to the accumulation of intermolecular disulfide bond-linked mutant SOD1. Neuro-2a cells expressing wild-type (WT), G85R and G93A mutant SOD1-MycHis were treated with MG132 for 24 h at the indicated concentrations. Soluble fractions were analyzed by SDS-PAGE in the absence (A) or presence (B) of 2-ME. Insoluble fractions were analyzed by SDS-PAGE in the presence of 2-ME (C). Arrow, a soluble SDS-resistant dimer; arrowhead, a soluble monomeric SOD1; asterisk, disulfide-linked high molecular weight species of SOD1. (D) Anti-α tubulin as loading control.

Fig. 2. Free Cys\textsuperscript{6} and Cys\textsuperscript{111} are important for generating intermolecular disulfide-linked species and insoluble, sedimentable forms of mutant human SOD1. Various combinations of replacing Cys with Ser were introduced into wild-type (WT) and mutant (G85R and G93A) SOD1-MycHis. Neuro-2a cells expressing SOD1-MycHis were treated with 2 µM MG132 for 24 h. Soluble fractions were analyzed by SDS-PAGE in the absence (upper panels) or presence (middle panels) of 2-ME. Insoluble fractions were analyzed by SDS-PAGE in the presence of 2-ME (lower panels). Asterisk, a disulfide-linked high molecular weight species; arrow, an SDS-resistant dimer of mutant SOD1. Filled circles, marked reduction of an SDS-resistant dimer with a Cys\textsuperscript{6}-replacement of mutant SOD1; filled squares, further reduction of the detergent-insoluble, sedimentable form of mutant SOD1 with simultaneous Cys\textsuperscript{6} and Cys\textsuperscript{111}-replacements. nr, SOD1 without replacement in cysteine residue; 4×CS, all four cysteines replaced by serines.

Fig. 3. Replacing both Cys\textsuperscript{6} and Cys\textsuperscript{111} greatly reduces high molecular weight aggregate formation and ubiquitylation of mutant SOD1-MycHis. (A) Crude, soluble, and insoluble fractions of cell lysates were analyzed by filter trap assay (upper panel). Nitrocellulose dot-blots probed with anti-Myc (middle panel) and anti-α Tubulin (lower panel) antibodies were used as loading controls. (B) In vivo ubiquitylation assay. Western blotting of SOD1-MycHis immunoprecipitates with anti-Ub antibody demonstrated polyubiquitylation of mutant SOD1s and their C57, 146S derivatives. Replacement of Cys\textsuperscript{6} and Cys\textsuperscript{111} abolished polyubiquitylation of mutant SOD1. nr, SOD1 without replacement in cysteine residue; 4×CS, all four cysteines replaced by serines.

Fig. 4. Formation of disulfide-linked species of mutant SOD1 strongly correlates
with visible aggregate formation and neurotoxicity. (A) The frequency of inclusion-bearing cells transfected with wild-type (WT), G85R and G93A mutant SOD1-GFP and their Cys to Ser derivatives. (B) G93A mutant SOD1-GFP inclusion bodies in 80 × g pellet. Lower panels are a high magnification image of the portin on upper panels showing the whole pellet. The scale bar is equivalent to 10 mm in upper panels, and 200 µm in lower panels. (C) Change in the neurotoxic effect of mutant SOD1-GFP by Cys replacements to Ser. Cell viability was measured by the WST-1-based assay. (D) All the constructs have equal expression. Transcription levels of SOD1-GFP in Neuro-2a cells expressing WT, G85R and G93A mutant SOD1 and their Cys to Ser derivatives were examined by quantitative RT-PCR. Data were normalized with GAPDH expression and then represent relative expression levels compared with levels in cells expressing WT SOD1-GFP. Data are mean ± SD values of triplicate assays. Statistical analyses were carried out by ANOVA. *, p < 0.01. nr, SOD1 without replacing cysteine residues; 4×CS, all four cysteines replaced by serines.

Fig. 5. Preferential occurrence of disulfide-crosslinked mutant SOD1 in the affected lesion of ALS model mice. Western blotting of tissue samples from two 17-week-old symptomatic G93A mutant SOD1-transgenic mice under non-reducing (upper panel) and reducing (lower panel) conditions. Cx, cerebral cortex; Cbl, cerebellum; Bs, brain stem; Sc, spinal cord; L, liver.

Fig. 6. Affects of disulfide-linking at Cys⁶ and Cys¹¹¹ on the rate of mutant SOD1 degradation. Cycloheximide chase analysis on Neuro-2a cells expressing (A) wild-type (WT) and (B) G93A mutant SOD1 and their Cys to Ser derivatives. Western blots showing levels of SOD1 protein at various times after the cycloheximide chase are on the left panels. Quantitative data on the right are mean ± SD values of three independent experiments. Statistical analyses were carried out by ANOVA. *, p < 0.01. nr, SOD1 without cysteine residue replacement; 4×CS, all four cysteines replaced by serines.

Fig. 7. Ubiquityl ligase Dorfin binds, ubiquitylates, and promotes degradation of disulfide-linked mutant SOD1. (A) Replacement of Cys⁶ and Cys¹¹¹ nearly eliminated the interaction of Dorfin with mutant SOD1. Various SOD1-MycHis were co-transfected with Xpress-Dorfin. After immunoprecipitation with anti-Xpress antibody, the resulting precipitates and cell lysates were analyzed by Western blotting with anti-Myc antibody. (B) In vivo, Dorfin failed to promote ubiquitylation of mutant SOD1 with the Cys⁶, Cys¹¹¹-replacement. Western blotting of SOD1-MycHis
immunoprecipitates with anti-Ub antibody. (C) Dorfin failed to promote degradation of mutant SOD1 with both Cys\textsuperscript{6} and Cys\textsuperscript{111} replaced. Cycloheximide chase analysis of G93A mutant SOD1 with Cys\textsuperscript{6} and Cys\textsuperscript{111}-replacements (left panel) or with Cys\textsuperscript{57} and Cys\textsuperscript{146}-replacements (right panel) in the presence or absence of overexpressed Xpress-Dorfin. (D) Dorfin prevented neurotoxicity by mutant SOD1 with intact Cys\textsuperscript{6} and Cys\textsuperscript{111} residues. Cell viability was measured by the WST-1-based assay. Data are mean ± SD values of three independent experiments. Statistical analyses were carried out by unpaired t-test. *, p < 0.01. nr, SOD1 without replacement in cysteine residue; 4×CS, all four cysteines replaced by serines.
Figure 1 (Niwa et al.)

SOD1-MycHis

| MG132 (μM) | 0 | 0.5 | 1 | 2 | 4 | 8 |
|------------|---|-----|---|---|---|---|
| **A**      |   |     |   |   |   |   |
| Soluble, 2-ME(-) |
| 250        |   |     |   |   |   |   |
| 200        |   |     |   |   |   |   |
| 150        |   |     |   |   |   |   |
| 100        |   |     |   |   |   |   |
| 50         |   |     |   |   |   |   |
| 37         |   |     |   |   |   |   |
| 25         |   |     |   |   |   |   |
| 20         |   |     |   |   |   |   |
| **B**      |   |     |   |   |   |   |
| Soluble, 2-ME(+) |
| 250        |   |     |   |   |   |   |
| 200        |   |     |   |   |   |   |
| 150        |   |     |   |   |   |   |
| 100        |   |     |   |   |   |   |
| 50         |   |     |   |   |   |   |
| 37         |   |     |   |   |   |   |
| 25         |   |     |   |   |   |   |
| 20         |   |     |   |   |   |   |
| **C**      |   |     |   |   |   |   |
| Insoluble  |
| 25         |   |     |   |   |   |   |
| 20         |   |     |   |   |   |   |

**D**

WB: anti-Myc

WB: anti-αTub
Figure 3 (Niwa et al.)

A

| SOD1-MycHis | WT | G85R | G93A |
|-------------|----|------|------|
| nr          | C6.111S | C57.146S | 4xCS |
| Crude       | Cellulose Acetate | Crude | Cellulose Acetate |
| Soluble     | anti-Myc | Soluble | anti-Myc |
| Insoluble   | Insoluble | Insoluble | Insoluble |

B

| SOD1-MycHis | WT | G85R | G93A |
|-------------|----|------|------|
| nr          | C6.111S | C57.146S | 4xCS |
| Crude       | Cellulose Acetate | Crude | Cellulose Acetate |
| Soluble     | anti-Myc | Soluble | anti-Myc |
| Insoluble   | Insoluble | Insoluble | Insoluble |

(kDa)

IP: anti-Myc, WB: anti-Ub

IP: anti-Myc, WB: anti-Myc
Figure 4 (Niwa et al.)

A

\[
\begin{align*}
\text{nr} & \quad \text{C6,111S} & \quad \text{C57,146S} & \quad 4xCS \\
\% \text{ of cells with inclusions in GFP(+) cells} & \quad & \quad & \\
\hline
\text{SOD1-GFP} & \quad 30 & \quad 20 & \quad 10 & \quad 0 \\
\text{WT} & \quad 20 & \quad 15 & \quad 10 & \quad 5 \\
\text{G85R} & \quad 15 & \quad 10 & \quad 5 & \quad 0 \\
\text{G93A} & \quad 0 & \quad 0 & \quad 0 & \quad 0 \\
\end{align*}
\]

B

G93A SOD1-GFP

nr       C6,111S   C57,146S   4xCS

C

\[
\begin{align*}
\text{nr} & \quad \text{C6,111S} & \quad \text{C57,146S} & \quad 4xCS \\
\text{Absorbance} [A_{\text{450nm}}] & \quad & \quad & \\
\hline
\text{SOD1-GFP} & \quad 2.5 & \quad 2.0 & \quad 1.5 & \quad 1.0 \\
\text{WT} & \quad 2.0 & \quad 1.5 & \quad 1.0 & \quad 0.5 \\
\text{G85R} & \quad 1.5 & \quad 1.0 & \quad 0.5 & \quad 0.0 \\
\text{G93A} & \quad 0.5 & \quad 0.0 & \quad 0.0 & \quad 0.0 \\
\end{align*}
\]

D

\[
\begin{align*}
\text{nr} & \quad \text{C6,111S} & \quad \text{C57,146S} & \quad 4xCS \\
\text{SOD1-GFP/GAPDH} & \quad & \quad & \\
\hline
\text{SOD1-GFP} & \quad 1.2 & \quad 1.0 & \quad 0.8 & \quad 0.6 \\
\text{WT} & \quad 1.0 & \quad 0.8 & \quad 0.6 & \quad 0.4 \\
\text{G85R} & \quad 0.8 & \quad 0.6 & \quad 0.4 & \quad 0.2 \\
\text{G93A} & \quad 0.6 & \quad 0.4 & \quad 0.2 & \quad 0.0 \\
\end{align*}
\]
Figure 5 (Niwa et al.)

2-ME (-)

(kDa)

2-ME (+)

WB: anti-SOD1
Figure 6 (Niwa et al.)

(A) SOD1<sup>WT</sup>-MycHis

Chase Time (h) | nr | C6, 111S | C57, 146S | 4xCS
--- | --- | --- | --- | ---
0 |  |  |  | 
2 |  |  |  | 
6 |  |  |  | 
12 |  |  |  | 

WB: anti-Myc

(B) SOD1<sup>G93A</sup>-MycHis

Chase Time (h) | nr | C6, 111S | C57, 146S | 4xCS
--- | --- | --- | --- | ---
0 |  |  |  | 
2 |  |  |  | 
6 |  |  |  | 
12 |  |  |  | 

WB: anti-Myc

% protein remaining vs. Chase Time (h)

SOD1<sup>WT</sup>-MycHis

% protein remaining vs. Chase Time (h)

SOD1<sup>G93A</sup>-MycHis

% protein remaining vs. Chase Time (h)
Disulfide bond mediates aggregation, toxicity and ubiquitylation of familial amyotrophic lateral sclerosis-linked mutant SOD1
Jun-ichi Niwa, Shin-ichi Yamada, Shinsuke Ishigaki, Jun Sone, Miho Takahashi, Masahisa Katsuno, Fumiaki Tanaka, Manabu Doyu and Gen Sobue

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