Amyotrophic lateral sclerosis (ALS)3 is a progressive neurodegenerative disorder leading to the selective death of motor neurons (1, 2). The majority of cases are sporadic, but about 10% of all cases are familial (fALS). In ~20% of familial cases, a mutant allele of the copper–zinc superoxide dismutase (SOD1) enzyme has been identified (3–5). As of today, over 100 different mutations of SOD1 causing ALS have been reported, the majority of which are point mutations and act in a dominant fashion. Several ALS risk factors have been identified, but the etiology of the disease is largely unclear. A hallmark of the disease is the appearance of intracellular inclusions in degenerating motor neurons (6), both in the familial cases caused by mutations in SOD1 and in the more obscure sporadic cases (6–11). The formation of such protein aggregates precedes neuronal death (12). The inclusions are typically SOD1 and ubiquitin immunoreactive in the mutant SOD1-mediated fALS cases (2, 7–9, 11–13). However, it is unclear how the SOD1 mutants form aggregates and whether other proteins play any role in the aggregation process or the aggregate induced toxicity.

p62 (also called sequestosome 1) was first identified as a phosphotyrosine-independent ligand for the Lck SH2 domain (14). It was later found to be a polyubiquitin-binding protein (15–17). The expression of p62 is up-regulated by several stress conditions, e.g., oxidative stress (18, 19), proteasome inhibition (19–21), or prostaglandin J2-induced accumulation of polyubiquitinated proteins (22). The p62 protein has been proposed to act as a shuttling factor of polyubiquitinated proteins to the proteasome (16, 23). p62 has also been found in protein aggregates in neurodegenerative disorders such as Alzheimer disease (23, 24), Huntington disease (25, 26), Parkinson disease (21, 24) and more recently in ALS. It was found in ALS with dementia (27), in ALS caused by CHMP2B mutations (28) and in other ALS cases (29). However, the role of p62 in mutant SOD1-mediated fALS is currently undocumented. In addition, the mechanistic details of p62 aggregate formation or the timeframe of the accumulation of p62 in relation to ALS progression are barely known.

In the present work, we investigated the p62 protein levels in the G93A SOD1 transgenic mouse model of fALS (30) and studied the role of p62 in fALS mutant SOD1 aggregate formation in NSC34 cells (a mouse motor neuron-like cell line) (31, 32). We found that the p62 level was elevated in the spinal cord of early pre-symptomatic G93A SOD1 transgenic mice and then progressively accumulated during the life of the animals. In addition, p62 was found in the protein aggregates in the G93A mice. In contrast, the level of p62 was generally lower and p62-positive aggregates were extremely rare in mice overexpressing wild-type (WT) SOD1. The p62 protein selectively interacted with familial ALS mutants, but not WT SOD1, suggesting a direct role for p62 in the discrimination between wild-
type and mutant SOD1. Co-transfection of DsRed-tagged p62 with GFP-tagged SOD1 showed that aggregate formation of the fALS-linked SOD1 mutants A4V and G93A was significantly enhanced by p62 overexpression, whereas WT SOD1 did not form aggregates. Deletion of the ubiquitin-association (UBA) domain of p62 decreased its ability of enhancing mutant SOD1 aggregation, but did not completely inhibit it. Further protein interaction data showed that the truncated p62 without the UBA domain could interact with mutant SOD1 as the full-length p62, suggesting that the UBA domain is not essential to the interaction. Based on the above data, p62 plays an important, yet undocumented role in the formation of protein aggregates in familial ALS.

MATERIALS AND METHODS

Animals—Transgenic mice strains overexpressing WT and G93A mutant SOD1 were generously provided by Dr. Zuoshang Xu (University of Massachusetts Medical School). The mice were generated from B6.Cg-Tg(SOD1)2Gur/J and B6.Cg-Tg(SOD1-G93A)1Gur/J stains (30) and bred and maintained as hemizygotes at the University of Kentucky animal facility. Transgenic mice were identified using PCR according to Gurney (33). G93A SOD1 transgenic mice were sacrificed at ages 35, 60, 90, and 125 ± 5 days. Age-matched WT SOD1 transgenic mice were used as controls. Mice were anesthetized with an intraperitoneal injection of 0.1 ml pentobarbital (50 mg/ml, Abbott Laboratories) and transcardially perfused with 0.1M phosphate-buffered saline (PBS), pH 7.5, before spinal cords and other tissues were dissected. All animal procedures were approved by the university IACUC committee.

Plasmid Construction—The construction of GFP-tagged SOD1 (SOD1-GFP) plasmids was previously reported (34). The C-terminally 3xHA-tagging vector p3xHA-CMV14 was made by replacing the 3xFLAG tag of p3XFLAG-CMV10 (Sigma) with the GST sequence of pGEX-2T (Amersham Biosciences). The p62 lacking the UBA domain (p62-UBA) with the GST sequence of pGEX-2T (Amersham Biosciences) was obtained from Sigma with the GST sequence of pGEX-2T (Amersham Biosciences). The p62-UBA vector unaltered. The 3xHA-tagged SOD1 plasmids were constructed by PCR amplifying the p62 coding sequence that was subsequently inserted into the EcoRI and BamHI sites of the pDsRed-monomer C1 vector (Clontech). The p62 lacking the UBA domain (p62-UBAD lacking Glu353, Leu403) was also tagged with DsRed in a similar fashion (DsRed-p62-UBAD). A GST-tagging vector for mammalian cells was made by replacing the 3xFLAG tag of p3XFLAG-CMV10 (Sigma) with the GST sequence of pGEX-2T (Amersham Biosciences). The p62 and p62-UBAD fragments were subcloned from the respective DsRed-tagged constructs into the GST-tagging vector. The sequence fidelity of all constructs was verified by sequencing.

Cell Culture, Transfection, and Cell Viability—NSC34 cells were cultured at 37 °C under 5% CO2, 95% air in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (31, 32). 80% confluent cells were transfected with the indicated plasmids using Lipofectamine (Invitrogen) following the manufacturer’s instruction. Cells were treated, harvested, and lysed as described in individual experiments.

NSC34 cells were transfected with SOD1-GFP constructs and untagged p62 or its vector control in 6-well plates. Forty-eight hours after transfection, cells were stained with 6.25 μg/ml propidium iodide for 10 min. Cells with SOD1-GFP aggregates and dead cells were counted using a Zeiss Axiosvert 100 epifluorescent microscope in ten random viewfields. Three independent experiments were carried out, and the p values were calculated using the Student’s t test.

Immunoprecipitation and GST Pull-down Assays—Dissected mouse spinal cords were lysed in radioimmuneprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with protease inhibitor mixture (P8340, Sigma, 1:1,000 dilution), 0.2 mM phenylmethylsulfonyl fluoride, and 0.625 mg/ml N-ethylmaleimide (E3876, Sigma). Lysates were cleared by centrifugation at 1,000 × g for 10 min at 4 °C, and the protein concentration determined by Bradford assay (Bio-Rad). 1000 μg of each extract was precleared for 1 h at 4 °C with 50 μl of 50% protein G-Sepharose (Amersham Biosciences) in a total volume of 500 μl of RIPA buffer. Extracts were then incubated with 2 μg of ubiquitin monoclonal antibody (sc-8017, Santa Cruz Biotechnology) overnight at 4 °C, followed by incubation with 50 μl of 50% protein G-Sepharose (Amersham Biosciences) for 2 h. Protein G beads were collected by centrifugation for 5 min at 500 × g and washed three times with ice-cold RIPA buffer. Proteins were eluted by boiling in 2× SDS sample buffer and subjected to SDS-PAGE followed by Western blotting analysis.

NSC34 cells were co-transfected with 0.5 μg of p62 construct and 0.5 μg of 3xHA-SOD1 construct. 28 h post-transfection, the medium were changed to fresh medium containing 20 μM MG-132 (Calbiochem) or vehicle control (Me2SO). Cells were cultured for additional 20 h, harvested, and lysed in 1× RIPA buffer containing protease inhibitor mixture (P8340, Sigma, 1:1,000 dilution), 10 μM MG-132 and 0.625 mg/ml N-ethylmaleimide. After incubation on ice for 20 min, the lysates were cleared by centrifugation at 1,000 × g for 10 min at 4 °C and the protein concentrations were determined using Bradford assay (Bio-Rad). The immunoprecipitation was carried out as described above using anti-HA antibodies (sc-7392 mouse monoclonal or sc-805 rabbit polyclonal, Santa Cruz Biotechnology). Proteins were eluted by boiling in 2× SDS sample buffer and subjected to SDS-PAGE followed by Western blotting analysis. The GST pull-down experiments were carried out similarly as above using glutathione-Sepharose (Amersham Biosciences) and the manufacturer’s protocol.

Western Blotting Analysis—After SDS-PAGE, proteins were transferred onto nitrocellulose membranes in 25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol. Membranes were blocked in 5% milk in TBS with 0.1% Tween-20 (TBST) at room temperature followed by incubation with the indicated primary antibodies in TBST. Anti-SOD1 (sc-11407, Santa Cruz Biotechnology), anti-p62 (goat polyclonal,


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sc-10117, Santa Cruz Biotechnology), anti-HA (sc-7392 and sc-805, Santa Cruz Biotechnology), and anti-GST (sc-459, Santa Cruz Biotechnology) were used at 1:1,000 dilution. Anti-p62 (mouse monoclonal, 610832, BD Bioscience), ant ubiquitin (sc-8017, Santa Cruz Biotechnology), anti-DesRed (632496, Clontech), anti-GFP (sc-8334, Santa Cruz Biotechnology) were used at 1:500 dilution. After primary antibody incubation, membranes were washed with TBST and incubated with the indicated secondary antibodies in 5% milk in TBST at room temperature. Membranes were then washed with TBST before the protein of interest was visualized using Supersignal West Pico or Dura Enhanced Chemiluminescent (ECL) substrate (Pierce).

**Fluorescence Microscopy**—Immunohistological analyses were performed on spinal cords of G93A and WT SOD1 transgenic mice at ages 35, 60, 90, and 125 ± 5 days. Spinal cords were dissected out, post-fixed overnight in 4% paraformaldehyde in 0.1 M PBS, dehydrated, and embedded in Paraplast X-tra (Tyco Healthcare). Sections (6 μm) were deparaffinized, rehydrated, and boiled in 0.01 M citrate buffer (pH 6.0) in a microwave oven at a high power setting for 15 min to retrieve antigens. Sections were then blocked in 10% heat-inactivated fetal bovine serum (FBS) in 0.1 M PBS with 0.1% Triton X-100 (PBST) for 30 min and incubated with primary antibodies diluted in 2% FBS-PBST overnight at room temperature. The rabbit SOD1 antibody (sc-11407, Santa Cruz Biotechnology), the mouse ubiquitin antibody (sc-8017, Santa Cruz Biotechnology) and the goat p62 antibody (sc-10117, Santa Cruz Biotechnology) were used in Fig. 1, and double-positive aggregates were counted in ten randomly chosen view fields. Statistical analysis was carried out using Student’s t test to calculate p values.

**RESULTS**

**Accumulation of p62 and Polyubiquitinated Proteins in G93A SOD1 Transgenic Mice**—We followed the accumulation of p62 and polyubiquitinated proteins in the G93A SOD1 transgenic mice and the control WT SOD1 transgenic mice. G93A transgenic mice were sacrificed at the presymptomatic 35 and 60 days of age, at 90 days when the disease onset is expected, and at 125 days when the mice reach the end stage of the disease. Age-matched WT SOD1 transgenic mice were also sacrificed. Protein extracts were prepared from the spinal cord and sciatic nerve of the G93A and WT mice. The Western blotting experiments were performed using a mouse monoclonal p62 antibody (610832, BD Bioscience), and the results are shown in Fig. 1. The level of p62 was detectably higher in the G93A spinal cords as early as 35 days of age and clearly showed continued elevation during later stages in Western blotting experiments (Fig. 1A). In comparison, the level of p62 in WT mouse spinal cord was much lower at all time points. In addition, compared with WT, elevated levels of p62 were also observed in G93A transgenic mice sciatic nerves containing motor neuron axons (Fig. 1B). This elevation was detectable at 90 days and even greater at 125 days.

The accumulation of polyubiquitinated proteins closely paralleled that of p62. At each age, a greater amount of high molecular weight ubiquitin-positive signal was detected in the G93A mouse spinal cord compared with WT (Fig. 1A). In addition, the amount of polyubiquitinated proteins increased more rapidly in G93A mice than in WT mice as the animals aged. A similar pattern of accumulation of polyubiquitinated proteins was observed in the sciatic nerves of G93A mice as well (Fig. 1B).

Polyubiquitinated proteins were immunoprecipitated from WT and G93A mouse spinal cord extracts using an ubiquitin antibody and immunoblotted for p62 using a goat polyclonal antibody (sc-10117, Santa Cruz Biotechnology) (Fig. 1C). All samples contained detectable amounts of p62, even in the case of the WT samples, probably because of the fact that p62 was enriched by the ubiquitin immunoprecipitation. The levels of p62 co-precipitated by the polyubiquitinated proteins were always higher in the G93A mouse spinal cords than in the age-matched WT mouse spinal cords. The elevation became more significant in the 90 and the 125 days samples. The same results were obtained using the mouse monoclonal antibody that was used in Fig. 1, A and B. Control experiments were carried out using IgG in immunoprecipitation, and p62 was not detected in the controls.

The accumulation of p62 in G93A mice was also demonstrated using immunofluorescence staining studies, and the results are shown in Fig. 2. p62 was hardly detectable in the WT mouse at various ages except for a minute amount in a small subset of cells in 125-day-old animals. In contrast, p62-positive staining emerged in the motor neurons of 35-day-old G93A
mouse spinal cord and increased with age. Moreover, the p62 staining closely resembled protein aggregates in the motor neurons of G93A mice and was observed long prior to the disease onset.

Co-localization of p62, SOD1, and Ubiquitin in Protein Aggregates in Spinal Cord Motor Neurons of the G93A SOD1 Transgenic Mice—The fALS-linked SOD1 mutant G93A was co-localized with p62 in the protein aggregates in the spinal cord motor neurons of G93A SOD1 transgenic mice as demonstrated by co-immunostaining studies (Fig. 3A). Images from WT SOD1 mice are not shown because p62 staining was barely observed (see Fig. 2).

Co-immunostaining images of SOD1 and ubiquitin are shown in Fig. 3B. In WT SOD1 mice, ubiquitin staining was strong in both the cytoplasm and the nucleus. In the G93A SOD1 mice, loss of nuclear ubiquitin signal accompanied by an increase in cytoplasmic ubiquitin staining was observed as early as 35 days of age (data not shown) but was more profound at 60 days (Fig. 3B). At 60 days of age, ubiquitin-positive aggregates appeared in the neurites and generally in the white matter of the G93A spinal cord samples. By 90 days, the ubiquitin-positive aggregates appeared in the cell bodies as well, along with the progression of the accumulation of the ubiquitin aggregates in the white matter. At 125 days, multiple ubiquitin aggregates were seen in the white matter and in the few remaining motor neurons. It is noted that the accumulation of p62 (Fig. 2), the co-aggregates of p62 and SOD1 (Fig. 3A), and the co-aggregates of SOD1 and ubiquitin (Fig. 3B) emerged in concert during the disease onset and progression. Moreover, Fig. 3C is the triple staining of SOD1, p62, and ubiquitin, demonstrating the co-localization of the three proteins in aggregates.

Co-precipitation of fALS-linked SOD1 Mutants and p62 from NSC34 Cells—To test whether p62 specifically interacted with mutant SOD1, we co-transfected DsRed-p62 and HA-tagged WT, A4V, or G93A mutant SOD1 into NSC34 cells. The HA tag was selected to be fused at the C terminus of SOD1 because it lacks lysine residues, thus avoiding potential fortuitous ubiquitination sites. A set of transfected cells was treated with MG-132 (a proteasome inhibitor), whereas another set was treated with Me2SO vehicle control only. Cell extracts were prepared and subjected to immunoprecipitation using an anti-HA antibody. The immunoprecipitated samples were blotted with anti-DsRed to determine the presence of p62, and the results are shown in Fig. 4. Without MG-132, p62 was co-precipitated with the fALS-linked SOD1 mutants A4V and G93A, but not with WT SOD1. Treatment of the transfected cells with MG-132 (a proteasome inhibitor), whereas another set was treated with Me2SO vehicle control only. Cell extracts were prepared and subjected to immunoprecipitation using an anti-HA antibody. The immunoprecipitated samples were blotted with anti-DsRed to determine the presence of p62, and the results are shown in Fig. 4. Without MG-132, p62 was co-precipitated with the fALS-linked SOD1 mutants A4V and G93A, but not with WT SOD1. Treatment of the transfected cells with MG-132 greatly enhanced the co-precipitation of p62 with the fALS SOD1 mutants, but p62 was hardly detectable in the WT SOD1 immunoprecipitation sample. In contrast, Western blotting of cell lysates showed that p62 was expressed in all cells. A DsRed vector control was also used in all experiments, and DsRed was never co-precipitated with SOD1-HA under all experimental conditions (data not shown).

The SOD1-HA immunoprecipitation samples were also blotted with an ubiquitin antibody (Fig. 4). The levels of polyubiquitinated SOD1 were significantly higher in the presence of MG-132. In addition, the amounts of polyubiquitinated SOD1 were profoundly higher for the fALS-linked SOD1 mutants than WT in the presence of MG-132.

The above results clearly demonstrate the specific interaction between p62 and the fALS-linked SOD1 mutants A4V and G93A. In addition, proteasome inhibition elevated the amount of polyubiquitinated SOD1.
of mutant SOD1 that interacted with p62. Moreover, the amount of p62 co-precipitated with mutant SOD1 increased in parallel to the levels of the polyubiquitinated SOD1. These data suggest that the interaction between p62 and mutant SOD1 can be partially mediated through polyubiquitinated mutant SOD1.

**Enhancement of Aggregate Formation of Mutant SOD1 but Not WT SOD1 by p62 Expression**—We tested whether p62 played a role in the aggregation of mutant SOD1 by co-expressing GFP-tagged SOD1 and DsRed-tagged p62 in NSC34 cells. The aggregate formation was measured by simultaneously monitoring the GFP-positive SOD1 aggregates and DsRed-positive p62 aggregates under fluorescence microscope. The fluorescence emitted by GFP and DsRed fluorophores can be confidently distinguished from each other. NSC34 cells were selected because of their low levels of endogenous p62 (data not shown). When SOD1-GFP and DsRed alone were co-expressed in NSC34 cells, aggregates of the GFP-tagged SOD1 mutants A4V and G93A were observed in ~2–3% of the transfected cells 24 h post-transfection. In the same time frame, GFP-tagged WT SOD1 did not form any aggregates.

When DsRed-tagged p62 and SOD1-GFP were co-transfected into NSC34 cells, the proportion of cells with GFP-positive aggregates was increased from ~3% to 37% for A4V mutant \((p = 9.4 \times 10^{-7})\) and to 25% for G93A mutant \((p = 9.3 \times 10^{-6})\) 24 h post transfection (Fig. 5A). In contrast, WT SOD1 still did not form aggregates when p62 was co-expressed. The expression levels of SOD1 and DsRed constructs were assessed by Western blotting (Fig. 5B), showing that they were approximately equal for each set of SOD1 constructs. Thus, the aggregate formation differences were truly influenced by p62. The results demonstrate that p62 was able to facilitate the formation of mutant SOD1 aggregates but WT SOD1 did not form aggregates regardless of whether p62 was co-expressed.

![Immunostaining of p62 in spinal cord motor neurons.](image)

**FIGURE 2. Immunostaining of p62 in spinal cord motor neurons.** G93A and WT SOD1 transgenic mice were sacrificed at 35, 60, 90, and 125 days. Spinal cords were collected, fixed in 4% paraformaldehyde and embedded. Sections (6 \(\mu\)m) were prepared and incubated with p62 and SOD1 antibodies and DAPI. p62 and SOD1 were visualized with Alexa Fluor 488 (green) and Alexa Fluor 594 (red) labeled secondary antibodies, respectively. The images were obtained using a Leica DM IRBE laser scanning confocal microscope with a \(\times 100\) objective. All scale bars are 10 \(\mu\)m. The results shown are representative of immunostaining of three sets of animals.
We further evaluated the role of the UBA domain of p62 in aggregate formation. The UBA domain has been attributed to the ability of p62 to bind polyubiquitin chains (15, 17). When DsRed-p62-UBA\textsubscript{H9004} instead of the full-length p62, was co-expressed with SOD1-GFP, the percentage of cells forming GFP-positive A4V aggregates decreased from 37\% to 10\% ($p < 10^{-5}$) (Fig. 5A). For G93A SOD1, the percentage of cells forming aggregates decreased from 25 to 5\% ($p = 2.5 \times 10^{-5}$). The results showed that the UBA truncation significantly impaired p62's ability to facilitate the aggregation of mutant SOD1, indicating that the UBA domain likely plays a role in promoting mutant SOD1 aggregation. However, compared with the DsRed alone control, co-expression of DsRed-p62-UBA\textsubscript{H9004} marginally increased the percentage of cells forming A4V aggregates ($p = 0.01$). This trend was also observed in G93A but the difference was statistically insignificant.

The Role of UBA Domain in Mutant SOD1-p62 Interaction—To further evaluate the role of the UBA domain of p62 in aggregate formation. The UBA domain has been attributed to the ability of p62 to bind polyubiquitin chains (15, 17). When DsRed-p62-UBA\textsubscript{Δ} instead of the full-length p62, was co-expressed with SOD1-GFP, the percentage of cells forming GFP-positive A4V aggregates decreased from 37\% to 10\% ($p = 7.8 \times 10^{-5}$) (Fig. 5A). For G93A SOD1, the percentage of cells forming aggregates decreased from 25 to 5\% ($p = 2.5 \times 10^{-5}$). The results showed that the UBA truncation significantly impaired p62's ability to facilitate the aggregation of mutant SOD1, indicating that the UBA domain likely plays a role in promoting mutant SOD1 aggregation. However, compared with the DsRed alone control, co-expression of DsRed-p62-UBA\textsubscript{Δ} marginally increased the percentage of cells forming A4V aggregates ($p = 0.01$). This trend was also observed in G93A but the difference was statistically insignificant.

The Role of UBA Domain in Mutant SOD1-p62 Interaction—To further evaluate the role of the UBA domain in mutant SOD1-p62 interaction, we performed co-precipitation experiments using GST-p62 and GST-p62-UBA\textsubscript{Δ} constructs and 3XHA-tagged SOD1. The expression levels of the GST-tagged p62 proteins were rather low in the absence of MG-132 (data not shown), thus the experiments were carried out in the presence of 20 \mu m MG-132. We carried out two sets of experiments: GST pull-down followed by HA Western blot (Fig. 6A), and HA immunoprecipitation followed by GST Western blot (Fig. 6B). Consistent with earlier results in Fig. 4, the specific interaction between p62 and the fALS-linked SOD1 mutants (A4V and G93A), but not WT SOD1, were demonstrated in both experiments. Surprisingly, GST-p62-UBA\textsubscript{Δ} also co-precipitated with A4V and G93A mutants in both experiments. The GST only control was included in all experiments and no interaction between SOD1 and GST was observed (data not shown). The expression levels of GST-p62, GST-p62-UBA\textsubscript{Δ}, and SOD1-HA were comparable in the experiments as shown...
in Fig. 6C. The data suggest that the UBA domain is not essential for the interaction between mutant SOD1 and p62. The role of the UBA domain in SOD1 interaction and aggregation is discussed in detail under “Discussion.”

The Effect of p62 Overexpression on Cell Viability—To test whether the enhanced formation of large aggregates in the presence of overexpressed p62 would directly increase cell death, we measured viability of NSC34 cells co-transfected with GFP-tagged WT, A4V, or G93A SOD1 and p62 (Fig. 7). 48 h post-transfection, the percentage of cells containing A4V aggregates increased from 8% in the absence of p62 to 22% in the presence of overexpressed p62 ($p = 2.5 \times 10^{-4}$). Despite the increased aggregation, the percentage of dead cells out of the transfected cells remained
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DISCUSSION

The appearance and accumulation of intracellular protein inclusions in affected motor neurons is a hallmark of ALS. The p62 protein has been found in intraneuronal or intraglial protein inclusions in a number of neurodegenerative diseases such as Alzheimer (23), Parkinson (21), and Huntington diseases (25, 26). More recently, p62 was detected in intraneuronal protein aggregates in ALS with dementia patients (27), in oligodendroglial inclusions in the motor cortex of patients with ALS phenotypes caused by mutations in CHMP2B (28) and in other ALS cases (29). This study is to determine whether and how p62 may contribute to mutant SOD1 aggregation and subsequently ALS etiology.

Western blotting and immunostaining results (Figs. 1 and 2) clearly demonstrated that p62 accumulated in parallel with the elevation of polyubiquitinated proteins in the spinal cords and sciatic nerves of G93A SOD1 transgenic mice starting at the early pre-symptomatic stage. Immunostaining data showed the co-localization of p62 with the SOD1-and ubiquitin-positive protein aggregates in affected cells in G93A transgenic mice (Fig. 3). The results show that p62 accumulated in protein aggregates in G93A transgenic mice long before the disease onset, suggesting that p62 may be involved in the mutant SOD1-mediated ALS etiology.

The p62 mRNA levels in the spinal cords of WT and G93A transgenic mice were measured by quantitative RT-PCR. No statistically significant differences in p62 mRNA levels were found between WT and G93A mice except in the end stage animals (data not shown). The moderate increase of p62 mRNA levels (10–20%) in the end stage G93A mice probably do not fully account for the observed accumulation of p62 at the protein level at all ages. It is likely that the p62 turnover rate decreased because of the oxidative stress and proteasome impairment in ALS. More interestingly, the binding of p62 to misfolded and polyubiquitinated proteins could be a more probable reason for the observed accumulation of p62 localized in protein aggregates.

Co-precipitation experiments from cultured NSC34 cells showed that p62 interacted selectively with fALS-linked SOD1 mutants, but not with WT SOD1. Treatment of the cells with the proteasome inhibitor MG-132 caused an increase in the amount of p62 associated with SOD1 mutants, suggesting that p62 selectively interacted with a subset of mutant SOD1 molecules otherwise targeted for proteasomal destruction (Fig. 4). The MG-132 treatment also caused the accumulation of polyubiquitinated SOD1 mutants. In transgenic mice, the levels of the polyubiquitinated proteins in G93A SOD1 mouse spinal cords were significantly higher than those in the age-matched WT SOD1 mice (Fig. 1). In parallel, greater amount of p62 was co-precipitated from the G93A SOD1 mice using an ubiquitin antibody (Fig. 1C). It has also been reported that p62 binds to polyubiquitinated proteins (15, 17). Thus, polyubiquitination may partially contribute to the interaction between mutant SOD1 and p62. However, deletion of the UBA domain of p62 did not abolish the interaction between p62 and mutant SOD1 (Fig. 6), suggesting that the association between the UBA domain and the polyubiquitin chain is not essential to the p62-mutant SOD1 interaction. It is likely that p62 can recognize the misfolded mutant SOD1 via a polyubiquitination-independent mechanism. Thus, the interaction between p62 and mutant SOD1 is possibly mediated by two distinct mechanisms. Fig. 8 shows a model illustrating p62 interacting with mutant SOD1 via polyubiquitination-dependent and -independent mechanisms. The UBA-independent interaction between p62 and misfolded proteins is a novel finding regarding p62 interaction and remains to be elucidated.

To determine whether p62 directly contributes to the formation of mutant SOD1-containing protein aggregates, we have studied the effect of p62 overexpression on the aggregate formation by WT SOD1 and the fALS-linked mutants A4V and G93A in NSC34 cells that have low levels of endogenous p62. The aggregate formation was followed in live cells by simultaneously monitoring the aggregation of GFP-tagged SOD1 and DsRed-tagged p62 under a fluorescence microscope. Co-expression of p62 greatly enhanced the aggregate formation of the fALS-linked SOD1 mutants, but not WT SOD1 (Fig. 5). The data show that p62 can differentiate between WT and mutant SOD1, and specifically facilitate the aggregation of mutant SOD1. Deletion of the ubiquitin-binding domain of p62 significantly impaired its ability to facilitate the formation of mutant SOD1 aggregates (Fig. 5). The result shows that the UBA domain of p62 makes a significant contribution to the forma-
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tion of mutant SOD1 aggregates. The interaction results (Figs. 4 and 6) and the protein aggregation data (Fig. 5) together suggest that the UBA domain plays a critical role in facilitating the aggregation of mutant SOD1 although it is not essential to the interaction between p62 and mutant SOD1. The molecular mechanisms by which p62 interacts with mutant SOD1 and promotes protein aggregation remain to be better defined in future studies.

It has been reported that p62 plays a role in protein aggregation. It was reported that p62 expression was increased in cells expressing expanded polyglutamine and was associated with the polyglutamine inclusions (26). Moreover, inhibition of p62 expression interfered with the formation of ubiquitin positive inclusions (22). It has been further shown that p62 serves as a shuttling factor for polyubiquitinated proteins to the proteasome (23) and that p62 actually interacts with the proteasome (16). It is likely that p62 initially targets misfolded and/or oxidatively damaged SOD1 for proteasomal degradation. However, the proteasome activities have been reported to be impaired in ALS (36, 37), thus the accumulation of proteins that are shuttled for destruction may well likely trigger the formation of aggregates (35, 38).

The results of this study also showed that p62 formed aggregates independent of co-expression of either WT or mutant SOD1 (Fig. 5). The results are in line with previous reports that p62 can form fibrillar aggregates itself (39), and that p62 can oligomerize via its PB1 domain (40, 41). The results from this study also show that p62 selectively interacted with the fALS-linked SOD1 mutants. Thus we hypothesize that p62 facilitates mutant SOD1 aggregation by self-aggregation and interacting with mutant SOD1. Fig. 8 shows a model illustrating p62 interacting with mutant SOD1 and promoting mutant SOD1 aggregation. The fALS-linked SOD1 mutants have been characterized as less stable than WT SOD1 with altered conformations and prone to form oligomeric structures in vitro (42, 43) and in vivo (34). Such decreased stabilities and increased oligomerization may occur due to the intrinsic properties of the mutants and may not need involvement of any other cellular factors. However, the formation of large protein aggregates could be greatly facilitated by p62 via linking already existing mutant SOD1 microaggregates of varied polymerization levels together with each other and potentially with other cellular proteins.

It has been debated for quite some time if the formation of protein aggregates is a cytoprotective mechanism or a cause of motor neuron death. Likely, there is truth to both conceptions. The initial aggregation of misfolded and/or oxidatively damaged proteins may promote the survival of the affected neurons by sequestration of potentially harmful proteins into aggregates. On the other hand, large protein aggregates can be detrimental to neurons by interfering with vital cellular processes. For instance, proteasome activities can be overwhelmed by the large protein aggregates. In fact, proteasome impairment has been reported in ALS (36, 37). Other than proteasomes, autophagy is another pathway to degrade unwanted proteins. It has recently been reported that besides the proteasomal mechanisms, autophagy plays an important role in fALS SOD1 degradation (44). It has been suggested that p62 targets polyubiquitylated proteins to the autophagy machinery (25, 45). Thus, the interaction between p62 and mutant SOD1 may not only contribute to the formation of mutant SOD1 aggregates, but also could interfere with degradation of other proteins by the proteasome and/or autophagy.

In addition, p62 has been reported to be involved in multiple signaling pathways (46). The atypical protein kinase Cs (aPKCs) bind to p62 and other proteins to stimulate the activation of the transcription factor NF-κB (47, 48), which plays an important role in regulating genes involved in neuronal development and survival (49). In addition, p62 functions as a scaffold for nerve growth factor receptor p75 (47) as well as intracellular p75 effector p75 (50). It remains unknown whether and how the above signaling pathways, in which p62 participates, change in mutant SOD1-mediated fALS.

The cell viability results in the presence and absence of p62 overexpression suggest that the elevated levels of p62 promoted aggregate formation of the fALS-linked SOD1 mutants, but did not directly cause significant increase of cell death under the experimental conditions in this study. The results of this study provide evidence that p62 accumulates in mutant SOD1-mediated fALS and that p62 selectively interacts with mutant SOD1 and promotes mutant SOD1 aggregation. The mechanisms by which the p62-mutant SOD1 interaction influences motor neuron survival need to be elucidated in future studies.

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