Degradation of Amyotrophic Lateral Sclerosis-linked Mutant Cu,Zn-Superoxide Dismutase Proteins by Macroautophagy and the Proteasome

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Mutations in the Cu,Zn-superoxide dismutase (SOD1) gene cause ~20% of familial cases of amyotrophic lateral sclerosis (fALS). Accumulating evidence indicates that a gain of toxic function of mutant SOD1 proteins is the cause of the disease. It has also been shown that the ubiquitin-proteasome pathway plays a role in the clearance and toxicity of mutant SOD1. In this study, we investigated the degradation pathways of wild-type and mutant SOD1 in neuronal and nonneuronal cells. We provide here the first evidence that wild-type and mutant SOD1 are degraded by macroautophagy as well as by the proteasome. Based on experiments with inhibitors of these degradation pathways, the contribution of macroautophagy to mutant SOD1 clearance is comparable with that of the proteasome pathway. Using assays that measure cell viability and cell death, we observed that under conditions where expression of mutant SOD1 alone does not induce toxicity, macroautophagy inhibition induced mutant SOD1-mediated cell death, indicating that macroautophagy reduces the toxicity of mutant SOD1 proteins. We therefore propose that both macroautophagy and the proteasome are important for the reduction of mutant SOD1-mediated neurotoxicity in fALS. Inhibition of macroautophagy also increased SOD1 levels in detergent-soluble and -insoluble fractions, suggesting that both detergent-soluble and -insoluble SOD1 are degraded by macroautophagy. These findings may provide further insights into the mechanisms of pathogenesis of fALS.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease caused by selective loss of motor neurons (1, 2).

Although most cases of ALS are sporadic, ~10% of ALS cases run in families. Dominant missense mutations in the gene that encodes the Cu,Zn-superoxide dismutase (SOD1) are responsible for 20% of familial ALS (fALS) cases (3). Mice overexpressing mutant SOD1 develop an ALS-like phenotype comparable with human ALS, whereas mice lacking SOD1 do not (4, 5). These findings have led to the conclusion that SOD1 mutants cause motor neuron degeneration by a toxic gain of function. Thus, studies of the degradation process of mutant SOD1 proteins could provide important insights into understanding the mechanisms that underlie the pathology of fALS, and possibly sporadic ALS, and into developing novel therapies for fALS by removing toxic species of mutant SOD1.

Cytoplasmic proteins are mainly degraded by two pathways, the ubiquitin-26 S proteasome pathway (6) and autophagy (7). Previous studies have shown that mutant SOD1 proteins are turned over more rapidly than wild-type SOD1, and a proteasome inhibitor increases the level of mutant SOD1 proteins (8, 9). Dorfin and NEDL1, two distinct ubiquitin ligases, ubiquitinate mutant but not wild-type SOD1 (10, 11). These observations suggest that mutant SOD1 is degraded by the ubiquitin-26 S proteasome pathway and that the increased turnover of mutant SOD1 is mediated in part by this pathway. On the other hand, the 20 S proteasome, a component of the 26 S proteasome, can degrade proteins without a requirement for ubiquitination (12, 13). A recent study has found that metal-free forms of wild-type and mutant SOD1 are degraded by the 20 S proteasome in vitro (14).

Autophagy is an intracellular process that results in the degradation of cytoplasmic components inside lysosomes. At least three forms of autophagy have been described in mammalian cells: macroautophagy, microautophagy, and chaperone-mediated autophagy (7). Macroautophagy is the major and the most well studied form of autophagy; this process begins with a sequestration step, in which cytosolic components are engulfed by a membrane sac called the isolation membrane. This membrane results in a double membrane structure called the autophagosome, which fuses with the lysosome. The inner membrane of the autophagosome and its protein and organelle contents are degraded by lysosomal hydrolases. Recent reports have demonstrated that macroautophagy plays an important role in preventing neurodegeneration in mice (15, 16). Although macroautophagy can be induced by starvation, this

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*The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.

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2 The abbreviations used are: ALS, amyotrophic lateral sclerosis; fALS, familial ALS; SOD1, Cu,Zn-superoxide dismutase(s); 3-MA, 3-methyladenine; siRNA, short interfering RNA; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
pathway may take place constitutively in mammals (17). In cultured cells, inhibition of macroautophagy does not alter enhanced green fluorescent protein (EGFP) levels (18) or glycéraldehyde-3-phosphate dehydrogenase protein levels, suggesting that not all cytosolic proteins are degraded by macroautophagy. To date, however, there have been no reports of macroautophagy in mutant SOD1 clearance.

In this study, we investigated the pathway by which human wild-type SOD1 and the A4V, G85R, and G93A SOD1 mutants are degraded in neuronal and nonneuronal cells. We show that wild-type and mutant SOD1 proteins are degraded by both the proteasomal pathway and macroautophagy. The experiments with inhibitors of these degradation pathways suggested that mutant SOD1 are degraded more rapidly than wild-type SOD1 in part by macroautophagy and that the contribution of macroautophagy to mutant SOD1 clearance is approximately equal to that of the proteasome pathway. Macroautophagy decreases mutant SOD1 protein levels in both nonionic detergent-soluble and -insoluble fractions. In addition, we provide data indicating that macroautophagy has a role in mutant SOD1-mediated cell death.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The expression plasmids pcDNA3-hSOD1 containing wild-type, A4V, G85R, and G93A mutant SOD1 were kindly donated by Ryosuke Takahashi (Kyoto University, Kyoto, Japan) and Makoto Urushitani (Laval University, Quebec, Canada) (19). To construct a plasmid expressing human wild-type SOD1 with the HA tag at the carboxyl terminus of SOD1, HA-tagged SOD1 fragments were amplified by PCR using wild-type SOD1 cDNA (Open Biosystems, Huntsville, AL) as the template. The PCR products were digested with XhoI and NotI and cloned into an XhoI-NotI-digested plasmid pCI-neo vector (Promega, Madison, WI). The primers used were 5′-AAAACTCGAGCCGCCAAGATGGCGACGAAGGC-CCGTGTGCG-3′ and 5′-CTCTGGAACATCGTATGGGTATTGGGCGATCCCAATT-

**Cell Culture and Transfection**—The mouse neuroblastoma cell line Neuro2a, the human neuroblastoma cell line SH-SY5Y, and the monkey kidney-derived cell line COS-7 were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum and 20 μM retinoic acid (12 h after transfection). Approximately 90% of cells in dishes (wells) were transfected in our experimental conditions (data not shown), and there was no notable differences in the transfection efficiency among the wells (supplemental Fig. S1).

**Treatment of Cells with Epoxomicin, 3-Methyladenine, Cycloheximide, Rapamycin, or NH₄Cl**—Cells grown in 12- or 6-well plates to 50–80% confluence were transfected with expression plasmids containing wild-type, A4V, G85R, or G93A mutant SOD1. 24 h after transfection, cells were incubated with epoxomicin (10 nM, 1 μM, 5 μM, or 10 μM; Sigma), 3-methyladenine (3-MA) (10, 20, or 30 mM; Sigma), rapamycin (100 or 200 nM; Sigma), 20 mM NH₄Cl, and/or carrier (Me₂SO or water) as a control. In some experiments, 10 μg/ml cycloheximide (Sigma) was added to the cells to avoid the confounding effects of ongoing protein synthesis. Epoxomicin, cycloheximide, and rapamycin were dissolved in Me₂SO, NH₄Cl in water. 3-MA was freshly dissolved in culture medium 30 min before use.

**Cell Fractionation**—For preparation of nonionic detergent-soluble and -insoluble fractions, adherent cells were harvested and lysed on ice for 15 min in 1% Triton X-100 lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and protease inhibitors (Complete, EDTA-free; Roche Applied Science). Lysates were centrifuged at 20,000 × g for 10 min at 4 °C, and the supernatants were pooled and designated as the detergent-soluble fractions. After the pellets were washed with 1% Triton X-100 lysis buffer, they were solubilized with SDS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 3% SDS, 1% Triton X-100, and protease inhibitors) and sonicated. The resulting solution was used as the detergent-insoluble fraction. For preparation of total cell lysates containing both detergent-soluble and -insoluble fractions, cells were lysed in SDS buffer and sonicated. Protein concentrations were determined with the protein assay kit (Bio-Rad) or the DC protein assay kit (Bio-Rad).

**Western Blot Analysis**—Western blotting was performed using standard procedures as described previously (20). The primary antibodies used were as follows: anti-SOD1 rabbit polyclonal antibody (1:4000; Stressgen Bioreagents, Victoria, Canada), anti-α-tubulin mouse monoclonal antibody (1:4000; Sigma), anti-β-actin mouse monoclonal antibody (1:5000; Sigma), anti-HA mouse monoclonal antibody (1:4000; Sigma), anti-Beclin 1 mouse monoclonal antibody (1:500; BD Transduction Laboratories, San Diego, CA), anti-Agg7/Atg7 rabbit polyclonal antibody (1:500; Rockland, Gilbertsville, PA). After overnight incubation with primary antibodies at 4 °C, each blot was probed with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:20,000; Pierce). Immunoreactive signals were visualized with SuperSignal West Dura extended duration substrate (Pierce) or SuperSignal West Femto maximum sensitivity substrate (Pierce) and detected with a chemiluminescence imaging system (FluorChem; Alpha Innotech, San Leandro, CA). The signal intensity was quantified by densitometry using FluorChem software (Alpha Innotech).

**Short Interfering RNA (siRNA) Preparation and Transfection**—Double-stranded siRNA targeting mouse Beclin 1, mouse Atg7 and EGFP were purchased from RNAi Co., Ltd.

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3 T. Kabuta, Y. Suzuki, and K. Wada, unpublished data.

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(Roche Applied Science). For experiments with differentiated Neuro2a cells, the medium was changed to differentiation medium (Dulbecco’s modified Eagle’s medium supplemented with 1% fetal calf serum and 20 μM retinoic acid) 24 h after transfection. Approximately 90% of cells in dishes (wells) were transfected in our experimental conditions (data not shown), and there was no notable differences in the transfection efficiency among the wells (supplemental Fig. S1).
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Tokyo, Japan. Sequences targeted by siRNA were selected using siDirect (RNAi Co., Ltd.): mouse Beclin 1 siRNA, sense (5' /H11032 -GUC-UACAGAAAGUGCUAAUAG-3') and antisense (5' /H11032 -AUUAGC-ACUUUCUGUAGACAU-3'); mouse Atg7 siRNA, sense (5' /H11032 -GAGCGGCGCGCUAGAACA-3') and antisense (5' /H11032 -UUC-UACCAGCGCGCUAA-3'); EGFP siRNA, sense (5' /H11032 -GCC-ACACGCUAUAUGCUGG-3') and antisense (5' /H11032 -AUGAUA-UAGACGUGUGGCUCA-3'). EGFP siRNA was used as a control. Cells (3 x 10^5) were cotransfected with 1 μg of DNA and 3 μg of siRNA using Lipofectamine PLUS reagent (Invitrogen).

(FIGURE 1. Both mutant and wild-type SOD1 are degraded by the proteasome. A, i, Neuro2a cells were transiently transfected with wild-type or mutant A4V human SOD1. 24 h after transfection, cells were treated with 10 μg/ml cycloheximide for the indicated time and lysed. Total cell lysates were analyzed by immunoblotting using anti-SOD1 or anti-α-tubulin antibody. ii, Neuro2a cells transfected with G93A SOD1 were incubated with or without 10 nM epoxomicin in the presence of 10 μg/ml cycloheximide for the indicated time and lysed. Total cell lysates were analyzed by immunoblotting using anti-SOD1 or anti-α-tubulin antibody. iii, the relative levels of wild-type or G93A SOD1 (percentage of 0-h control) were quantified by densitometry. Mean values are shown with S.E. (n = 3). B and C, Neuro2a cells were transiently transfected with wild-type or mutant A4V, G85R, or G93A human SOD1. 24 h after transfection, cells were incubated with or without 10 nM epoxomicin in the presence of 10 μg/ml cycloheximide for 24 h. Total cell lysates were analyzed by immunoblotting using anti-SOD1 antibody. The electrophoretic mobility of G85R SOD1 was greater than that of wild-type SOD1. α-Tubulin was used as a loading control. Asterisks indicate endogenous mouse SOD1 (B). The relative level of wild-type or mutant SOD1 was quantified by densitometry. Mean values are shown with S.E. (n = 3). *, p < 0.05; **, p < 0.01 (C). D and E, human SH-SY5Y cells were incubated with or without 10 nM epoxomicin in the presence of cycloheximide for 24 h. Total cell lysates were analyzed by immunoblotting with anti-SOD1 antibody (D). The relative level of human endogenous SOD1 was quantified by densitometry. Data are expressed as the means ± S.E. (n = 3). *, p < 0.05 (E).)
Quantitative Assessment of Cell Viability and Cell Death—
One day before transfection, Neuro2a cells were seeded at 5 \times 10^4 cells/well in 24-well plates. 24 h after transfection with 0.4 \mu g of DNA/well, cells were cultured in differentiation medium with or without 10 \mu M 3-MA for 24 h. Cell death was assessed by a lactate dehydrogenase release assay using the CytoTox-ONE homogenous membrane integrity assay (Promega) according to the manufacturer’s protocol. The percentage of cytotoxicity (Fig. 7G) was calculated according to this protocol. For assessment of cell viability, we used the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy- methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and the ATP assay with the CellTiter 96 AQueous One Solution cell proliferation assay (Promega) and CellTiter-Glo luminescent cell viability assay (Promega), respectively, according to the manufacturer’s protocols. Measurements with a multiplate reader were performed after samples were transferred to 96-well assay plates.

Statistical Analysis—For comparison of two groups, the statistical difference was determined by Student’s t test. For comparison of more than two groups, analysis of variance was used. If the analysis of variance was significant, Dunnett’s multiple comparison test was used as a post hoc test.

RESULTS

Wild-type and Mutant SOD1 Are Degraded by the Proteasome—To determine whether SOD1 is degraded by the proteasome pathway, we assessed the effect of proteasome inhibitors on SOD1 protein clearance. Peptide aldehydes, such as
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MG132 or ALLN, and lactacystin are widely used proteasome inhibitors. However, peptide aldehydes also inhibit cathepsins and calpains, and lactacystin inhibits cathepsin A (21, 22). Because these inhibitors are not proteasome-specific and may interfere with lysosomal function, we used epoxomicin as a selective proteasome inhibitor (23, 24). We observed protein clearance of human SOD1 in Neuro2a cells transfected with mutant or wild-type SOD1 in the presence of the translation inhibitor cycloheximide (Fig. 1A, i and ii). Consistent with previous reports (9, 11), wild-type SOD1 exhibited a relatively long half-life (half-life of more than 24 h) compared with mutant SOD1 (~10 h; G93A) (Fig. 1A, iii). The degradation of wild-type and mutant SOD1 was suppressed by epoxomicin treatment (Fig. 1, B and C) (~14-h increase in half-life; G93A; Fig. 1A, ii). Our finding that mutant SOD1 is degraded by the proteasome is in agreement with previous reports (8, 9). To determine whether endogenous human wild-type SOD1 is also degraded by the proteasome, SOD1 clearance was examined using the human neuroblastoma SH-SY5Y cell line. The proteasome inhibitor treatment promoted the accumulation of human SOD1 proteins (Fig. 1, D and E). These results indicate that endogenous wild-type SOD1 is degraded by the proteasome, also consistent with a previous report (14).

Wild-type and Mutant SOD1 Are Also Degraded by Macroautophagy—To date, there have been no reports of macroautophagy participating in human SOD1 clearance. We therefore investigated whether wild-type or mutant SOD1 was degraded by macroautophagy using 3-MA, an inhibitor of macroautophagy (18, 25, 26), and ammonium chloride, an inhibitor of lysosomal proteolysis (26). We initially confirmed that 3-MA inhibits the formation of autophagosomes in Neuro2a cells using green fluorescent protein-LC3, a marker of autophagosomes (27) (supplemental Fig. S2). Moreover, we also showed that the clearance of α-synuclein, an established substrate for macroautophagy (28), was inhibited by 3-MA or ammonium chloride treatment (supplemental Fig. S3). Treatment of Neuro2a cells with 3-MA promoted the accumulation of G93A mutant SOD1 proteins (Fig. 2A). In the presence of cycloheximide, the degradation of wild-type and mutant SOD1 was suppressed by treatment with 3-MA (Fig. 2, B and C) (a more than 14-h increase in half-life; G93A, Fig. 2B), indicating that wild-type and mutant SOD1 are degraded by macroautophagy in these cells and that the accumulation of SOD1 proteins by 3-MA is not due to increased protein synthesis. These results, together with Fig. 1, suggest that mutant SOD1 are degraded more rapidly than wild-type SOD1 by macroautophagy (it is estimated that 15–20% of wild-type SOD1 and 25–30% of mutant SOD1 were degraded by macroautophagy during the 24-h incubation). The clearance of mutant G93A SOD1 was also decreased by treatment with ammonium chloride (Fig. 2D). As shown in Supplemental Fig. S4 and Fig. 2D, the protein level of endogenous mouse SOD1 was increased by 3-MA or ammonium chloride treatment. The result shown in Fig. 2D further supports the role of the lysosomes in SOD1 degradation. To test the role of macroautophagy on SOD1 degradation in differentiated neuronal cells or neurons, we also used differentiated Neuro2a cells. In differentiated Neuro2a cells, 3-MA increased both wild-type and mutant SOD1 protein levels in the presence or absence of cycloheximide (data not shown). To determine whether endogenous human SOD1 is degraded by macroautophagy, the clearance of endogenous SOD1 was examined in SH-SY5Y cells. As shown in Fig. 2, E and F, the degradation of endogenous SOD1 proteins was inhibited by 3-MA.

For further confirmation of the clearance of SOD1 by macroautophagy, we used rapamycin to induce macroautophagy (29, 30), and gene silencing with siRNA to inhibit macroautophagy. Treating Neuro2a cells with rapamycin decreased HA-tagged G93A SOD1 levels (Fig. 3, A and B). In differentiated Neuro2a cells, SOD1 protein levels were also decreased by rapamycin (Fig. 3C). Beclin 1 is a component of a class III phosphatidylinositol 3-kinase complex that is crucial for macroautophagy (31). Silencing of the Beclin 1 gene by siRNA inhibits the generation of autophagosomes, thus preventing macroautophagy (32). Atg7 protein is also essential for macroautophagy (17). We initially confirmed that Beclin 1 or Atg7 expression was knocked down by Beclin 1 or Atg7 siRNA, respectively (Fig. 4, A and B). We also showed that α-synuclein level was increased by Beclin 1 or Atg7 siRNA (supplemental Fig. S3). We observed inhibited degradation of wild-type and mutant SOD1 in cells with Beclin 1 siRNA (Fig. 4, A and C) or Atg7 siRNA (Fig. 4, B and D) compared with cells with control siRNA (~14-h increase in half-life; G93A; Fig. 4E). The results shown in Figs. 2–4 demonstrate that wild-type and mutant SOD1 are also...
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In neuronal cells, ammonium chloride or 3-MA treatment stimulates the accumulation of HA-tagged wild-type SOD1 and G93A SOD1 (Fig. 5A) or mutant G93A SOD1 (Fig. 5B), respectively. Treatment of the cells with epoxomicin also increased wild-type and mutant SOD1 levels (Fig. 5C and supplemental Fig. S5). These results indicate that wild-type and mutant SOD1 are degraded by both macroautophagy and the proteasome.

The Contributions of the Proteasome Pathway and Macroautophagy to Mutant SOD1 Degradation Are Comparable—We then assessed the relative contributions of proteasomal degradation and macroautophagy to the clearance of mutant SOD1. As shown in Fig. 6A, 10 μM 3-MA entirely suppresses the (3-MA-sensitive) macroautophagy-mediated degradation of mutant SOD1. 1 μM epoxomicin also entirely suppresses the (epoxomicin-sensitive) proteasome-mediated degradation of mutant SOD1 (Fig. 6B and supplemental Fig. S6). Therefore, we compared mutant G93A SOD1 levels in 1 μM epoxomicin-treated cells with that of 10 μM 3-MA-treated cells. The SOD1 protein level in 3-MA-treated cells was comparable with that of epoxomicin-treated cells (Fig. 6C–F). An increased accumulation of mutant SOD1 was detected in cells cotreated with both inhibitors compared with that of 3-MA-treated cells or epoxomicin-treated cells (Fig. 6E and F). These data further support the idea that mutant SOD1 proteins are degraded by both macroautophagy and the proteasome and indicate that, in these cells, the contribution of macroautophagy to mutant SOD1 clearance is approximately equal to that of the proteasome pathway.

Macroautophagy Reduces the Toxicity of Mutant SOD1—Previous studies have shown that mutant SOD1-expressing cells are more susceptible to cell death induced by proteasome inhibition (33). We examined whether inhibiting the macroautophagy-mediated degradation of mutant SOD1 could also induce cell death in Neuro2a cells using three different assays.

FIGURE 4. Silencing of macroautophagy genes promote the accumulation of SOD1 proteins. A and C, Neuro2a cells were cotransfected with SOD1 (wild-type, A4V, G85R, or G93A) and siRNA (Beclin 1 siRNA or control EGFP siRNA). 24 h after transfection, total cell lysates were prepared and analyzed by immunoblotting using anti-SOD1 or anti-Beclin 1 antibody. α-Tubulin was used as a control (A). Levels of SOD1 were quantified by densitometry, and the levels are expressed as -fold level of SOD1 in cells with Beclin 1 siRNA over cells with control siRNA. Data are presented as the means ± S.E. (n = 3). *, p < 0.05; **, p < 0.01 (C). B and D, Neuro2a cells were cotransfected with SOD1 (wild-type, A4V, G85R, or G93A) and siRNA (Atg7 siRNA or control siRNA). 24 h after transfection, total cell lysates were prepared and analyzed by immunoblotting using anti-SOD1, anti-Atg7, or anti-α-tubulin antibody (B). Levels of SOD1 were quantified by densitometry, and the levels are expressed as -fold level of SOD1 in cells with Atg7 siRNA over cells with control siRNA. Data are presented as the means ± S.E. (n = 3). *, p < 0.05; **, p < 0.01 (D). E, Neuro2a cells cotransfected with G93A SOD1 and siRNA (control, Atg7, or Beclin 1 siRNA) were treated with 10 μg/ml cycloheximide for the indicated time and lysed. Total cell lysates were analyzed by immunoblotting using anti-SOD1 or anti-α-tubulin antibody.
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**FIGURE 5.** Mutant and wild-type SOD1 are degraded by both macroautophagy and the proteasome in COS-7 cells. **A,** COS-7 cells were transiently transfected with HA-tagged human wild-type SOD1 or G93A SOD1. 24 h after transfection, cells were incubated with or without 20 mM NH4Cl for 24 h. Total cell lysates were analyzed by immunoblotting using anti-HA antibody or anti-SOD1 antibody. β-Actin and α-tubulin were used as loading controls. **B** (i), COS-7 cells transfected with G93A mutant SOD1 were incubated with or without 10 mM 3-MA in the presence of cycloheximide for 24 h. Total cell lysates were analyzed by immunoblotting using anti-SOD1 antibody (i). Levels of SOD1 were quantified by densitometry, and the levels are expressed as -fold level of SOD1 in cells with 3-MA over control. Data are presented as the means ± S.E. (n = 3). **,** p < 0.01 (ii). **C,** COS-7 cells were transfected with wild-type or mutant A4V or G93A SOD1. 24 h after transfection, cells were incubated with or without 10 nM epoxomicin for 24 h. Total cell lysates were analyzed by immunoblotting.

For assessment of cell viability, we used the MTS assay and ATP assay, and for assessment of cell death, we used the lactate dehydrogenase release assay. In untreated differentiated Neuro2a cells, there was no statistically significant difference in cell viability or cell death among control cells, wild-type SOD1-expressing cells, and mutant SOD1-expressing cells (Fig. 7, A–C). However, when cells were treated with 3-MA, mutant SOD1-expressing cells showed significantly increased cell death and significantly decreased cell viability compared with control cells or wild-type SOD1-expressing cells (Fig. 7, D–F). When compared with cell death of 3-MA-untreated cells, cell death of 3-MA-treated cells was increased in mutant SOD1-expressed cells but not in cells with wild-type SOD1 (Fig. 7G). From these results, we conclude that macroautophagy reduces mutant SOD1-mediated toxicity in this cell model.

**DISCUSSION**

Using inhibitors of macroautophagy and proteasomal degradation, we have shown that both wild-type and mutant SOD1 proteins are degraded by both pathways. Accumulating evidence has shown that mutant SOD1 is degraded by the ubiquitin-proteasome pathway (8, 9, 19). However, most of these studies have used lactacystin or a peptide aldehyde, both of which are not proteasome-specific inhibitors. Our data on the effect of the selective proteasome inhibitor epoxomicin also indicate that mutant SOD1 is degraded by the proteasome. Because wild-type SOD1 is not ubiquitinated by the ubiquitin ligases (10, 11), it has been proposed that wild-type SOD1 is not a substrate of the proteasome. However, a recent report has suggested that wild-type SOD1 can be degraded by the 20 S proteasome without ubiquitination (14). Moreover, we show here that epoxomicin treatment increases both overexpressed and endogenous wild-type SOD1 levels. Our data together with the previous reports support the idea that wild-type SOD1 is degraded by the 20 S proteasome in mammalian cells.

In this study, we demonstrated for the first time that macro-
autophagy is another pathway for degradation of wild-type and mutant SOD1. Our findings are consistent with a previous report that rat wild-type SOD1 is present in autophagosomes and lysosomes in rat hepatocytes (although they did not examine whether rat SOD1 was degraded by macroautophagy in those cells) (38). We propose that the contribution of macroautophagy to mutant SOD1 degradation is comparable with that of the proteasome pathway in the cell types we tested. Recent studies have demonstrated that transgenic mice with neuron-specific expression of mutant SOD1 do not exhibit an ALS-like phenotype (39, 40) and that neurodegeneration is delayed when motor neurons expressing mutant SOD1 are surrounded by healthy nonneuronal wild-type cells (41). In addition, Urushitani et al. (42) have shown that chromogranins promote secretion of mutant SOD1 from cells expressing the mutant protein, and they proposed that secreted mutant SOD1 can be toxic to neighboring cells. These studies strongly suggest that the expression of mutant SOD1 in nonneuronal cells may be involved in mutant SOD1-mediated neurotoxicity. In nonneuronal COS-7 cells, mutant SOD1 is also degraded by both the proteasome and macroautophagy (Fig. 3). Thus, not only the proteasome but also macroautophagy may play an important role in the degradation of mutant SOD1.

FIGURE 6. The contribution of macroautophagy to SOD1 clearance is comparable with that of the proteasome. A, Neuro2a cells transfected with mutant G93A SOD1 were incubated with or without 10, 20, or 30 mM 3-MA for 24 h. Total cell lysates were analyzed by immunoblotting. B, Neuro2a cells transfected with mutant G93A SOD1 were incubated with or without 1, 5, or 10 μM epoxomicin (epox) for 24 h. Total cell lysates were analyzed by immunoblotting. C and D, Neuro2a cells transfected with mutant G93A SOD1 were incubated with or without 10 mM 3-MA or 1 μM epoxomicin for 24 h. Total cell lysates were analyzed by immunoblotting (C). The relative level of mutant G93A SOD1 was quantified by densitometry. Data are presented as the means ± S.E. (n = 3), **, p < 0.01 in comparison with control (analysis of variance with Dunnett’s multiple comparison test) (D).

FIGURE 7. Macroautophagy inhibition causes mutant SOD1-mediated cell death. A–G, Neuro2a cells were transiently transfected with control empty vector (A, B, D, and E) or human SOD1 (wild type, A4V, G85R, or G93A). 24 h after transfection, cells were incubated in differentiation medium with (D–G) or without (A–C and G) 10 mM 3-MA for 24 h, and the lactate dehydrogenase release assay (A, D, and G), MTS assay (B and E), or ATP assay (C and F) were performed. The percentage of nonviable cells in each sample was calculated from the lactate dehydrogenase release assay (G). The experiment in G was performed independently of A and D. Data are expressed as the means ± S.E. (n = 4 in A, C, D, F, and G; n = 3 in B and E), *, p < 0.05; **, p < 0.01 in comparison with control (A, B, D, and E) or with wild-type SOD1 (C and F) (analysis of variance with Dunnett’s multiple comparison test). **, p < 0.01 (G; t test).
role in clearance of mutant SOD1 in fALS in nonneuronal cells as well as in neuronal cells.

It has been well established that mutant SOD1-mediated toxicity is caused by a gain of toxic function rather than the loss of SOD1 activity (1, 2). The appearance of mutant SOD1 aggregates in motor neurons in fALS patients and mouse models of fALS (34, 35) has suggested that aggregation has a role in neurotoxicity. However, conflicting results have been reported on the correlation between aggregate formation and cell death. A recent study has shown that the ability of mutant G85R and G93A SOD1 proteins to form aggregates correlates with neuronal cell death using live cell imaging techniques (36). Another report has concluded that aggregate formation of A4V and V148G SOD1 mutants does not correlate with cell death (37). These controversies also exist in other neurodegenerative diseases (43–46). Our current data suggest that macroautophagy degrades toxic species of mutant SOD1 and that the accumulation of mutant SOD1 proteins leads to greater cell death. However, whether the toxic SOD1 species are monomers, oligomers, or aggregates cannot be determined from our study, because a variety of mutant SOD1 species, including detergent-soluble SOD1 monomers and detergent-insoluble monomers, dimers, and aggregates, were accumulated by macroautophagy inhibition (Fig. 8).

Our data show that macroautophagy reduces mutant SOD1-mediated toxicity and that induction of macroautophagy decreases mutant SOD1 protein levels. Niwa et al. (10) have shown that the ubiquitin ligase Dorfin ubiquitinates mutant SOD1 and prevents the neurotoxicity of mutant SOD1. Taken together, these data imply that macroautophagy inducers, acti-
vators of the ubiquitin-proteasome pathway, or a combination of the two have therapeutic potential for fALS. In conclusion, our results demonstrate that mutant SOD1 is degraded by at least two pathways, macroautophagy and the proteasome pathway, and that the clearance of mutant SOD1 by macroautophagy reduces its cell toxicity. These findings may provide insight into the molecular mechanisms of the pathogenesis of fALS.

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