Mahogunin ring finger 1 confers cytoprotection against mutant SOD1 
aggresomes and is defective in an ALS mouse model

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ABSTRACT

Proteotoxicity of misfolded, disease-causing proteins is deeply implicated in the pathomechanisms for neurodegenerative diseases including copper–zinc superoxide dismutase (SOD1)–linked amyotrophic lateral sclerosis (ALS). However, the precise cellular quality control (QC) mechanisms against aggregation of misfolded mutant SOD1 proteins remain elusive. Here, we found that the Mahogunin ring finger-1 (MGRN1) E3 ubiquitin ligase, which catalyzes mono-ubiquitination to the substrate, was dysregulated in the cellular and mouse models of ALS and that it preferentially interacted with various mutant forms of SOD1. Intriguingly, the motor neurons of presymptomatic ALS mice have diminished MGRN1 cytoplasmic distribution. MGRN1 was partially recruited to mutant SOD1 inclusions where they were positive for p62 and Lamp2. Moreover, overexpression of MGRN1 reduced mutant SOD1 aggregation and alleviated its proteotoxic effects on cells. Taken together, our findings suggest that MGRN1 contributes to the clearance of toxic mutant SOD1 inclusions likely through autophagic pathway, and, most likely, the sequestration of MGRN1 sensitizes motor neurons to degeneration in the ALS mouse model. Furthermore, the present study identifies the MGRN1-mediated protein QC mechanism as a novel therapeutic target in neurodegenerative diseases.

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset progressive neurodegenerative disorder that is characterized by the selective death of motor neurons in the spinal cord and cerebral cortex (Bruijn et al., 2004). Dominant mutations in the copper–zinc superoxide dismutase 1 (SOD1) gene encoding the SOD1 enzyme are one of the frequent causes of familial ALS (Bruijn et al., 2004; Raoul et al., 2002; Wong et al., 1995). The overexpression of the human SOD1 gene carrying ALS-linked mutations in mice reproduces the major pathological hallmark of ALS (Gurney et al., 1994). Mutant SOD1 proteins, which are highly ubiquitinated, form insoluble abnormal inclusions with components of the ubiquitin proteasome system (UPS) and autophagy pathway in motor neurons (Banerjee et al., 2010; Cheroni et al., 2009).

The key mechanism of motor neuron death that is linked to mutant SOD1 aggregates remains poorly understood. Recently, it has been shown that supersaturation of the cellular protein is linked to neurodegeneration and aging (Ciryam et al., 2013). Because the UPS facilitates the degradation of mutant SOD1 proteins, the overexpression of mutant SOD1 proteins decreases the elimination capacity of the proteasome system (Allen et al., 2003; Cheroni et al., 2009; Hoffman et al., 1996; Urushitani et al., 2002). Numerous studies have suggested that mutant SOD1 aggregates lead to several critical cellular dysfunctions, such as UPS alterations, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and oxidative stress (Cheroni et al., 2009; Kikuchi et al., 2006; Lin and Beal, 2006).

Despite knowing that abnormal SOD1 protein aggregation results in a toxic gain of function and contributes to ALS disease progression, it is still not clear how neuronal cells survive under such devastating dysfunctions. One possible cellular response is autophagy. Several lines of evidence have suggested that autophagy activation alleviates mutant SOD1–linked toxic insults (Wong and Cuervo, 2010). SOD1G93A transgenic mice show numerous microtubule-associated protein 1A/1B-light chain 3 (LC-3)-labeled autophagic vacuoles in the spinal motor neurons (Li et al., 2008). In addition, it has been shown that p62/SQSTM1 recognizes mutant SOD1 proteins through an autophagy–linked lysosomal pathway and promotes their clearance (Gal et al., 2009; Zhang et al., 2007).

Ablant function of Mahogunin ring finger-1 (MGRN1), a RING domain-containing E3 ubiquitin ligase, causes late-onset spongiform neurodegeneration in mice (He et al., 2003). MGRN1, a unique E3 ligase which catalyzes multi-monoubiquitination to the substrate, and is likely to be involved in the cellular quality control machinery through...
proteasome-independent pathway (Chhangani and Mishra, 2013; Chhangani et al., 2014b; Kim et al., 2007). Mice lacking MGRN1 function exhibit dysregulation of the mitochondrial pathway and neurodegeneration (Sun et al., 2007). Downregulation of MGRN1 disturbs endo-lysosome molecular trafficking of epidermal growth factor and null mice of MGRN1 indicate that MGRN1-deficient neurons are more prone for high vulnerable risk in comparison of other cells due to abnormal endosomal trafficking (Kim et al., 2007). Recently it has also been demonstrated that MGRN1 interacts with transmembrane \( \text{C}^{\text{PrP}} \) and toxic (cyPrP) prion disease proteins; depletion of MGRN1 affects lysosomal morphology and leads to neurodegeneration likely due to such improper sequestration (Chakrabarti and Hegde, 2009). MGRN1 interacts with several proteins such as expanded polyglutamine proteins (Chhangani et al., 2014b), molecular chaperone (Chhangani and Mishra, 2013), \( \alpha \)-tubulin (Srivastava and Chakrabarti, 2014), MC1R/MC2R (Cooray et al., 2011; Perez-Oliva et al., 2009), cyPrP/\( \text{C}^{\text{PrP}} \) (Chakrabarti and Hegde, 2009), TSG 101 (Kim et al., 2007), and NEDD4 (Gunn et al., 2013). Under such crucial biological interactions it is plausible that a loss of function or depletion of MGRN1 can cause multifactorial defects in cells and generate various pathological states in important physiological events. As recently we reviewed (Upadhyay et al., 2015) and previous studies also indicated strong implications of MGRN1 in neuro-pathobiological mechanisms. How misfolded or aggregated proteins affect physiological function of MGRN1 and disturb normal localization at the site of proper recruitment is a crucial question. However, the role of MGRN1 in neurodegenerative diseases has not been clarified. Although abnormal protein accumulation is a prominent feature of neurodegenerative diseases, how the sequestration or loss of function of MGRN1 contributes to neurodegeneration is not known.

In our present study, we found MGRN1 dysregulation in cellular and mouse models of ALS. MGRN1 interacts with normal and mutant SOD1 proteins in cells. The motor neurons showed diminished MGRN1 cytoplasmic labeling in presymptomatic mutant SOD1 mice. MGRN1 was partially recruited to mutant SOD1 inclusions, Fig. 1. Mahogunin ring finger-1 (MGRN1) E3 ubiquitin ligase is dysregulated in the cells and mice expressing ALS-linked mutant superoxide dismutase (SOD1) protein. (A) Lysates from COS-7 cells transfected with green fluorescent protein (GFP)-tagged wild-type (WT) and mutant (SOD1G37R and SOD1G85R) SOD1 were analyzed by immunoblotting with anti-MGRN1 and anti-actin antibodies. (B–C) Immunoblotting of MGRN1 in the brain and spinal cord samples of WT (Control: B6) and ALS mice (SOD1G85R and SOD1G37R). (D) As shown in Fig. 1A six well plate cultured cells were used from three independent experiments for quantification. Bar diagram showing the quantification of the band intensities of the wild-type and mutant SOD1-expressing cells and the blots were quantified from three different experiments with NIH Image analysis software. (E–F) Quantification of the band intensities in the immunoblots using brain and spinal cord (SC) lysates of ALS mice (SOD1G85R and SOD1G37R; n = 3 animals) and similar groups (n = 3 animals) of Control (B6) mice. The actin protein levels in each were used for the normalization of each sample. (G) The dot blots that were prepared with the extracts from the brain and SC of WT and early symptomatic ALS mice were detected by immunosassays with anti-SOD1, anti-MGRN1, anti-p62, and anti-ubiquitin antibodies. (H–K) Quantification of the dot-blot results shown in G. The inclusions of various proteins were quantified by a densitometric analysis with NIH image software in WT (control; n = 3 animals) and ALS mice (n = 3 animals) in brain (H, J) and SC (I, K) samples.
which were positive for p62 and Lamp2 in the spinal motor neurons of ALS mice. Finally, MGRN1 alleviates the cytotoxicity mediated by abnormal inclusions of mutant SOD1 and thus, exerts a cytoprotective effect. Overall, these findings suggest that an MGRN1-mediated protein quality control mechanism alleviates the mutant SOD1-linked proteotoxicity likely through autophagic pathway.

2. Materials and methods

2.1. Materials

3-((4,5-Dimethylthiazol-2-yl)-2,5-diphenytlentetrazolium bromide (MTT), Bafilomycin A1 (Baf), chloroquine, cycloheximide, MGRN1–specific siRNA oligonucleotides, and all cell culture reagents were obtained from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). The protein G–agarose beads were obtained from Roche Applied Science (Indianapolis, IN, USA). The TRizol reagent, Lipofectamine®2000, OptiMEM, and reverse transcription-polymerase chain reaction (RT-PCR) kits were purchased from Life Technologies Corporation (Grand Island, NY, USA). The pcDNA™ 3.1 plasmid was purchased from Life Technologies Corporation. The pEGFP-Hsp70 (Addgene 15215), pcDNA3-EGFP (Addgene 13031), pcDNA3-cmv (Addgene 16011), pFI148 pSOD1G37R AcGFP1 (Addgene 26409), and pFI150 pSOD1G93AAcGFP1 (Addgene 26411) plasmids were purchased from Addgene (Cambridge, MA, USA). The wild-type and mutant SOD1 plasmids have been described elsewhere (Mishra et al., 2013).

2.2. Primary and secondary antibodies

The monoclonal green fluorescent protein (GFP-11814460001) antibody was purchased from Roche Applied Science. The monoclonal anti-Hsp70 (sc-32239), polyclonal anti-SOD1 (sc-11407), polyclonal anti-GFP (sc-8334), polyclonal anti-MGRN1 (sc-160518), monoclonal anti-ubiquitin (sc-58448), and siRNA-MGRN1 oligonucleotides were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-FLAG (F7425), polyclonal anti-MGRN1 (HPA007653), monoclonal anti-p62/SQSTM1 (WH0008878M1), anti-c-Myc (M4439), anti-actin (A2066), and anti-Lamp2 (SA1402250) were obtained from Sigma-Aldrich Co. LLC. Polyclonal anti-ubiquitin (Z0458) was purchased from Dako (Glostrup, Denmark). Multi ubiquitin antibody was purchased from MEDICAL & BIOLOGICAL LABORATORIES (MBL: Nagoya, Japan). The anti-human SOD1 antibody (M062-3) was obtained from MBL International (Woburn, MA, USA). Monoclonal misfold SOD1–specific antibody (clone C4F6) was obtained from MediMabs (Montreal, Canada). Anti-mouse IgG–fluorescein isothiocyanate (FITC) was obtained from Vector Laboratories, Inc. (Burlingame, CA, USA). The Zenon® Secondary Detection-Based Zenon® Alexa Fluor® 350 Mouse IgG labeling Kit (Z-25,000); was obtained from Life Technologies Corporation.

2.3. Cell culture, transfection, and cell viability assay

COS-7 cells and 293T cells were maintained at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich Co. LLC) with 100 U/mL penicillin and 100 μg/mL streptomycin and 10% heat-

Fig. 2. MGRN1 interacts with wild-type and mutant SOD1 proteins. (A) MGRN1 and SOD1 co-immunoprecipitation (CO-IP). Lysates were prepared from COS-7 cells transfected with Myc-MGRN1 and FLAG-tagged wild-type (SOD1) and mutant (SOD1G37R, SOD1G93A) SOD1 plasmids. Transfected cells were processed for CO-IP with an anti-Myc antibody. After the CO-IP, the blots were probed with anti-FLAG, anti-SOD1, anti-Myc, or anti-MGRN1 antibodies. (B) As described above, the same cell lysates were used for CO-IP with an anti-actin antibody, and immunoblotting was performed with anti-Myc, anti-MGRN1, anti-FLAG, or anti-SOD1 antibodies. The arrowheads represent exogenous (upper) and endogenous (lower) SOD1. (C) As an immunoprecipitation control, COS-7 cells were expressed with the above-described constructs, and, after 48 h, the cell lysates were prepared and immunoprecipitated with an anti-actin antibody or Protein G–agarose (control). The pulled-down products and cell lysates were used for immunoblotting and sequentially probed with anti-actin and anti-FLAG antibodies.
inactivated fetal bovine serum. Cells were transiently transfected with various constructs as described in the figures, and the Lipofectamine®2000 transfection reagent was used according to the manufacturer’s protocol for transfections. The cells were treated with reagents at 37 °C, at a subconfluent density; the reagents were added drop-wise into the 6-well tissue culture plates. The cells were processed for different immunofluorescence stainings and immunoblotting experiments after 48 h when the transfection efficiency was about 70%. Cell viability was assessed with MTT, as described previously (Mishra et al., 2013; Mulherkar et al., 2009).

2.4. Immunocytochemistry technique, counting of aggregates, and statistical analysis

COS-7 cells were plated into 2-well chamber slides and transiently transfected as described above. Transfected cells were processed for double immunolabeling as follows. Cells were rinsed 4 times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and blocked with 2% goat serum for 1 h. The cells were then incubated with primary antibodies (1:500) overnight at 4 °C. The next day, the cells were washed three times with Tris-buffered saline with Tween (TBST), and a fluorochrome-conjugated secondary antibody was used for 2 h. The cells were then washed 4 times with PBS, which was followed by 4′,6-diamidino-2-phenylindole (DAPI) staining. After mounting with Vectashield mount medium (Vector Laboratories, Burlingame, CA), these cells were visualized with a fluorescence microscope (Leica DM 6000B, Leica Microsystems GmbH, Wetzlar, Germany). For counting aggregates, about 500 cells were observed, and aggregate formation was counted manually under a fluorescence microscope. Cells retaining more than one aggregate were counted as having a single inclusion or aggregate. The statistical significance for comparisons of the means of more than two groups, one-way ANOVA was used. In some experiments the statistical analyses between the groups and intergroup comparisons were determined with Student’s t-tests and Microsoft Excel software.

2.5. Coimmunoprecipitation and immunoblotting experiment

COS-7 cells were transiently transfected with different constructs. After 48 h of transfection, the cells were rinsed with PBS, and placed on ice for 45 min with Nonident P-40 lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonident P-40, and complete protease inhibitor mixture), to prepare the lysate. Cell lysates were briefly sonicated twice and centrifuged for 10 min at 10,000 × g at 4 °C, and the supernatants (total protein lysates) were used for immunoprecipitation, as described previously (Mishra et al., 2009). In each immunoprecipitation experiment, 200 μg of protein in 0.2 mL of Nonident P-40 lysis buffer was used for a pull-down experiment and incubated for 4 h with the desired antibody. After the coimmunoprecipitation experiment, the samples were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were transferred onto nitrocellulose membranes and incubated under blocking buffer [5% skim milk in TBST (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween)] for 1 h. Homogenate extracts from the brain and spinal cord of wild-type and ALS mice were used for a dot-blot analysis on membrane-filter assays, as previously described (Wanker et al., 1999). After blocking, the membranes were incubated with different primary antibodies in TBST and then processed for secondary antibody incubation. All of the blots were developed with ECL substrate. All primary antibodies were used for immunoblotting at a 1:500 dilution.

2.6. Degradation assay, RT-PCR analysis, and RNA interference experiments

COS-7 cells were plated in 6-well plates and transiently cotransfected with wild-type and mutant SOD1 constructs along with MGRN1 plasmids. Forty-eight h after transfection, the cells were incubated for 4 h with a cycloheximide solution (15 μg/mL). The proteins in the cell lysate were separated by SDS-PAGE and processed for the immunoblot analysis. The blots were incubated with the appropriate primary antibodies. Quantification of the band intensities of the immunoblots was performed with NIH Image analysis software. Actin was used to normalize the data. For the RT-PCR analysis, total RNA was isolated from the transiently transfected cells using TRIzol reagent, and, then, semi-quantitative RT-PCR was performed with MGRN1-specific and β-actin-specific primers. The details of the RT-PCR conditions were previously described (Chhangani and Mishra, 2013). To deplete the endogenous MGRN1 from the cells, the cells were plated in 6-well tissue culture plates. RNA interference experiments were conducted with transiently transfected MGRN1-siRNA or Control-siRNA oligonucleotides. Transfected cells were collected for RNA isolation, and a RT-PCR analysis was performed, as explained above. Some cells were collected, and the lysate was processed for the immunoblot analysis with a MGRN1 antibody.

2.7. Transgenic mice and fluorescent immunohistochemical staining of spinal cord sections

SOD1-transgenic mice [LoxSOD1G37R (Boillee et al., 2006), SOD1G85S (Bruijn et al., 1997), and SOD1G93A (Gurney et al., 1994)] have been described previously. All animal experiments were conducted with the approval of the Animal Care Committee of Nagoya University and were in accordance with their requirements. Presymptomatic and symptomatic ALS-transgenic mice (n = 3) and similar groups (n = 3) of Control (B6) mice were perfused with sterile PBS, which was followed by 4% paraformaldehyde in PBS. For the double-immunofluorescence labeling, lumbar spinal cords were dissected and fixed with 4% paraformaldehyde in phosphate buffer, treated (for cryoprotection) with 30% sucrose for at least 18 h at 4 °C, and frozen in mounting medium (Yamanaka et al., 2008). Cryosections (20 μm) were directly mounted on the slides, probed with primary antibodies, and later incubated with a fluorescent-conjugated secondary antibodies. Lumbar spinal motor sections were mounted with Prolong antifade reagent and observed on a confocal microscope (LSM700, Carl Zeiss AG, Oberkochen, Germany), and nuclear staining was detected by DAPI. For quantification of MGRN1-immunoreactivity in the lumbar ventral large neurons, mean fluorescence intensities (MFI) within cell bodies of large neurons (major axis >20 μm) in the anterior horn area were measured using ZEN software (Carl Zeiss, Germany). The neuronal MFI was normalized to that of the ipsilateral posterior horn area in the same section.

3. Results

3.1. Dysregulation of MGRN1 in ALS-linked transgenic mice prior to neurodegeneration

To test whether the endogenous MGRN1 E3 ubiquitin ligase was dysregulated by mutant SOD1 proteins, we first examined the expression levels of MGRN1 in cultured cells expressing mutant forms of SOD1 proteins. We observed that the endogenous levels of MGRN1 were depleted in the cells expressing mutant SOD1G37R and SOD1G85S proteins and not in the cells expressing wild-type SOD1.
protein (Fig. 1A). Mutant SOD1-overexpressing cells showed about a 0.4-fold decrease in the endogenous levels of MGRN1 (Fig. 1D). To further examine alteration of MGRN1 levels in vivo, we investigated the endogenous levels of MGRN1 in ALS-linked SOD1G37R (12 month old) - and SOD1G85R (10 month old)-transgenic mice. We observed that the protein levels of MGRN1 were reduced by about half in both the spinal cord and brain samples of SOD1G37R- and SOD1G85R-transgenic mice in comparison to wild-type control mice of the same age (Fig. 1B, C, E and F). Previous work showed that ubiquitin, proteasomes, and Hsc-70 are positively stained with the neuronal SOD1 inclusions of three different ALS mouse models (G37R, G85R, and G93A SOD1) (Watanabe et al., 2001). Therefore, we hypothesized that the aggregation of misfolded SOD1-aggregated species in ALS-transgenic mice might sequester and coaggregate MGRN1 and deplete its endogenous levels compared to normal mice. To address these questions, we performed a dot-blot analysis with homogenate extracts from the brain and spinal cord of wild-type (control) and ALS mice. MGRN1 and other proteins such as SOD1, ubiquitin, and p62 were found to form aggregates in the brains and spinal cords of ALS-transgenic mice (Fig. 1G). Quantification of these dot-blot analyses suggested that MGRN1 strongly aggregated with ubiquitin, p62, and mutant SOD1 proteins in the brains and spinal cords of ALS-transgenic mice (Fig. 1H–K).

3.2. MGRN1 interacts with wild-type and mutant SOD1 proteins

Our initial in vitro and in vivo observations suggested that MGRN1 was sequestered with mutant SOD1 inclusions presumably through their mutual interactions. To determine if MGRN1 interacted with normal and mutant SOD1 proteins, we first conducted a detailed co-immunoprecipitation experiment. FLAG-tagged SOD1, SOD1G37R, SOD1G85R, or SOD1G93A, and Myc-tagged MGRN1 constructs were separately co-transfected into COS-7 cells, and immunoprecipitation was then performed with an anti-Myc antibody. Immunoblots were obtained from the co-immunoprecipitated species and probed with anti-FLAG, anti-SOD1, anti-Myc, and anti-MGRN1 antibodies. As shown in Fig. 2A, MGRN1 preferentially interacted with exogenous mutant SOD1 proteins compared to wild-type endogenous SOD1. To further confirm this interaction, we performed a reverse co-immunoprecipitation analysis. The same cell lysates were used to pull down SOD1 with an anti-SOD1 antibody. Detection of proteins in the precipitates was then performed sequentially with anti-Myc, anti-MGRN1, anti-FLAG, and anti-SOD1 antibodies. Both the wild-type and mutant forms of SOD1 proteins interacted with endogenous as well as exogenous MGRN1 (Fig. 2B). Lastly, we performed various control immunoprecipitation experiments; the cell lysates that were used earlier were separately pulled down by anti-actin or protein G agarose only, and the blots were developed with anti-actin and anti-FLAG antibodies (Fig. 2C). These observations suggested the possible molecular pathological relevance and interaction of MGRN1 with wild-type and mutant SOD1 proteins, and they supported our initial observation of MGRN1 dysregulation in the ALS-transgenic mouse model.

3.3. MGRN1 is sequestered in the spinal motor neurons of presymptomatic and symptomatic mutant SOD1 transgenic mice

Abnormal SOD1 accumulation and dysregulation of oxidative phosphorylation have been observed in the mitochondria of symptomatic ALS mice (Liu et al., 2004; Mattiazzi et al., 2002; Vijayvergiya et al., 2005). On the other hand, the loss of MGRN1 function has been shown to result in mitochondrial dysfunction and neurodegeneration in MGRN1 null-mutant mice (Sun et al., 2007). The findings of these studies and our current observations of dysregulated endogenous levels of MGRN1 in mutant SOD1-expressing cells and mice may represent a triggering factor in the onset of the disease. This prompted us to further investigate whether the sequestration of MGRN1 was observed in the spinal motor neurons of presymptomatic ALS-transgenic mice. In order to determine the localization of MGRN1, we performed a detailed immunohistochemical profile of MGRN1, and other critical proteins, such as SOD1, Lamp2, ubiquitin, and p62, in the lumbar spinal cord of both wild-type and presymptomatic ALS-transgenic mice. MGRN1 immunoreactivities were predominantly observed in the cytoplasm of motor neurons in the spinal cord of the control (non-transgenic) mice. The spinal cord sections of ALS SOD1G85R (Fig. 3) and SOD1G93A-transgenic (Fig. 4) mice demonstrated cytoplasmic inclusions with MGRN1 immunostaining, which was greatly diminished and colocalized with mutant SOD1 (Fig. 3A & Fig. 4A). As shown in Figs. 3 and 4, mutant SOD1 aggregates showed positive double immunofluorescence staining for MGRN1 and various critical components of proteolytic pathways, such as ubiquitin, Lamp2, and p62 inclusion bodies, in the same set of spinal cord samples. To further examine whether misfolded SOD1 species are involved in dysregulated MGRN1 expression in spinal motor neurons, we performed immunostaining using misfolded SOD1-specific antibody, C4F6. MGRN1 was partially colocalized with misfolded SOD1G93A proteins at onset of disease (Fig. 5A). Abnormal accumulation of MGRN1 was observed both in axons and somata of the neurons. Despite of MGRN1 aggregation occasionally observed, the overall signal intensities of MGRN1 in the somata of anterior horn neurons were significantly diminished before the onset of the disease in SOD1G85R mice (Fig. 5B, C), suggesting that depletion of functional MGRN1 in the neurons of mutant SOD1 mice. Altogether, these immunohistochemical observations suggested that MGRN1 levels were lower in presymptomatic and onset ALS-transgenic mice and that it was partially mislocalized with aberrant SOD1 macroautophagic aggregates.

3.4. MGRN1 overexpression reduces the steady-state levels of mutant SOD1 proteins and mitigates their aggregation in cells

In cells, most abnormal proteins are selectively targeted by both proteasome and autophagy pathways (Chhangani et al., 2014a). Moreover, these aberrant proteins are ubiquitinated and positive for various components of the cellular quality control pathway (Chhangani et al., 2013). In our current study, we demonstrated the partial recruitment of MGRN1 with mutant SOD1 proteins in presymptomatic and symptomatic ALS-transgenic mice. Therefore, we speculated that such an association could promote the degradation of mutant SOD1 proteins in cells. Because MGRN1 targets TSG101 for multiple mono-ubiquitination through a proteosomal-independent pathway (Kim et al., 2007), we next performed an immunocytochemical analysis of the cells and observed that chloroquine-mediated autophagy inhibition induced the colocalization of endogenous MGRN1 with inclusions of SOD1G37R-YFP and SOD1G85R-YFP abnormal proteins (Fig. 6A–D). In order to determine whether MGRN1 influences mutant SOD1 protein levels in mammalian cells, we transiently cotransfected cells with control and MGRN1 plasmids along with wild-type and mutant SOD1G93A constructs and treated them with cycloheximide to inhibit protein synthesis, and the levels of wild-type and mutant SOD1 protein over time were analyzed with an immunoblot analysis. The overexpression of MGRN1 reduced the steady-state levels of mutant SOD1G93A protein compared to wild-type SOD1 protein in cells, and, moreover, degradation of SOD1 proteins was retarded in the presence of Bafilomycin, an inhibitor for autophagy (Fig. 6E – H).

To examine whether MGRN1 preferentially targets mutant SOD1 protein in MGRN1 activity dependent manner, we coexpressed wild-type SOD1 and SOD1G93A mutant constructs along with MGRN1 or the catalytically inactive form MGRN1H130A plasmids in the cells; some cells were treated with 50 nM Baf (Fig. 7A, B). We found that the overexpression of MGRN1 significantly reduced the levels of mutant SOD1G93A protein compared to wild-type SOD1 (Fig. 7A, B). In contrast, no significant decrease in the mutant SOD1G93A contents was detected in the catalytically inactive form of the MGRN1-transfected cells.  

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Treatment with Baf further stabilized the degradation of abnormal SOD1G93A protein, suggesting that MGRN1 facilitated SOD1G93A clearance through the autophagy pathway. The current observations suggested that there were multiple routes of mutant SOD1 protein degradation specifically when the accumulation of abnormal SOD1 proteins impaired the proteasomal functions of cells. To confirm the degradation of other mutant SOD1 species by MGRN1, COS-7 cells were transiently transfected with SOD1G37R and MGRN1-GFP or MGRN1AVVA-GFP and processed for immunofluorescence. Compared to MGRN1AVVA-GFP-inactive mutants, MGRN1-GFP-positive cells retained very few diffuse inclusions (Fig. 7C – F). Next, we tested the effects of the overexpression (Fig. 7G) and knockdown (Fig. 7H) of MGRN1 on SOD1 aggregate formation. In the cells expressing SOD1G37R-YFP, the overexpression of MGRN1 decreased the number of aggregates, whereas the knockdown of MGRN1 increased aggregate formation in the cells. Taken together, these findings indicate that...
MGRN1 overexpression accelerated the clearance of misfolded SOD1 inclusions partly through autophagy pathway and suppressed the intracellular aggregate formation of mutant SOD1.

3.5. MGRN1 confers cytoprotection against mutant SOD1 toxic species and improves cell viability

The synchronization of motor neuron pathology and abnormal protein accumulation in ALS has been previously explored (Ferraiuolo et al., 2011). However, the mechanism underlying the interplay of E3 ubiquitin ligases and the cytoprotection against proteotoxicity that is mediated by mutant SOD1 proteins in ALS largely remains unknown. Based on these intriguing observations, such as the diminished levels and aggregates of MGRN1 in motor neurons of SOD1-mutant mice and the preferential elimination of mutant SOD1 proteins by MGRN1 overexpression, we further investigated whether MGRN1 provided cytoprotection against the proteotoxicity of mutant SOD1 proteins. When the endogenous MGRN1 were depleted through small interfering RNA (siRNA), we observed the accumulation of mutant SOD1 proteins (Fig. 8A–D). In the cell viability assay, we noticed that the partial depletion of MGRN1 generated additive vulnerable effects in the cells expressing aberrant SOD1 proteins (Fig. 8E and F). To further validate this observation, we performed another set of cell viability assays in which we separately overexpressed MGRN1 or its catalytically inactive form MGRN1 AVVA in SOD1G93A- (Fig. 8I) and SOD1G37R-expressing (Fig. 8J) cells with an Hsp70 chaperone construct. The cell viability analyses indicated that MGRN1 provided a defense against mutant SOD1 aggregation and that this effect was slightly more prominent in the cells coexpressing MGRN1 and Hsp70 chaperone. Overall, these results indicated that MGRN1 alleviated mutant SOD1-mediated cytotoxicity and possibly elicited a cascade of events, which included the functional recruitment of MGRN1 with multifactorial toxic inclusions of abnormal SOD1 proteins.

4. Discussion

Our present study shows that the MGRN1 E3 ubiquitin ligase interacted with both wild-type and mutant SOD1 proteins in cells. The overexpression of MGRN1 facilitated the degradation of mutant SOD1 proteins. Moreover, mutant SOD1 proteins dysregulated endogenous MGRN1 levels and overexpression of MGRN1 alleviated their proteotoxic insults. Taken together, the findings of all of these studies support our current conclusion that MGRN1 is a novel cytoprotective factor to alleviate mutant SOD1-mediated proteotoxicity.

Previous reports showed that mutations in MGRN1 cause spongiform neurodegeneration in mice (He et al., 2003). MGRN1-mutant mice also exhibit mitochondrial dysfunction at an early age (Sun et al., 2007). Subsequently, it has been observed that MGRN1 promotes monoubiquitination of tumor susceptibility gene 101 (TSG101). This observation elaborates a novel function of MGRN1 via proteasome-independent degradation pathway (Kim et al., 2007). Mutant MGRN1 causes spongiform neurodegeneration in mice and it also
Fig. 7. MGRN1 suppresses mutant SOD1 aggregation in cells. (A) COS-7 cells were transfected with MGRN1-GFP or MGRN1*AVVA*-GFP mutant constructs, and cell lysates were prepared after 48 h of transfection. All of the blots were sequentially developed with anti-GFP, anti-SOD1, and anti-actin antibodies. (B) COS-7 cells were cotransfected with MGRN1-GFP or MGRN1*AVVA*-GFP in the presence of FLAG-tagged SOD1G93A, and some cells were treated with 50 nM of bafilomycin (Baf) for 12 h prior to collection. Immunoblots were probed with anti-FLAG and anti-actin antibodies. (C–F) COS-7 cells were cotransfected with FLAG-tagged SOD1 (C, E) or FLAG-tagged SOD1G37R (D, F) plasmids with MGRN1-GFP (C, D) or MGRN1*AVVA*-GFP mutant (E, F) plasmids, and, after 48 h, the cells were incubated with an anti-FLAG antibody. A rhodamine-conjugated secondary antibody was used to detect FLAG-tagged SOD1. DAPI was used to stain nuclei. Scale bar, 20 μm. (G–H) As described in sections C–F, some sets of YFP-tagged SOD1G37R-expressing COS-7 cells were cotransfected in a concentration-dependent manner with an empty vector (Control) and Myc-MGRN1 (G) or MGRN1 mutant, Scrambled siRNA and MGRN1-siRNA (H). The quantitative immunofluorescence analysis demonstrates SOD1G37R-YFP aggregates in 100 cells. The values are presented as the mean ± SD of three independent experiments and each performed in triplicate. *, p < 0.05 compared with (pcDNA or Scrambled siRNA) expressed cells.
interacts with cytosolic prion proteins (PrPs) (Chakrabarti and Hegde, 2009; He et al., 2003). Melanocortin receptors (MCRs) are also ubiquitinated as substrates of MGRN1 and are regulated by MGRN1 (Cooray et al., 2011). In contrast, our present study together with our recent works revealed a novel role of MGRN1 in recognizing misfolded proteins and in reducing proteotoxicity (Chhangani and Mishra, 2013; Chhangani et al., 2014b). In this work, mutant SOD1 proteins are identified as new target proteins recognized by MGRN1 in addition to mutant huntingtin and ataxin-3 proteins with expanded polyglutamine (Chhangani et al., 2014b). To date, several E3 ubiquitin–protein ligases, such as gp78 (Ying et al., 2009), MITOL (MARCH-V) (Yonashiro et al., 2009), Dorfin (Niwa et al., 2002), and E6-AP (Mishra et al., 2013), have been known to recognize and target misfolded SOD1 proteins for degradation through ubiquitin proteasome system (UPS). In comparison to those E3 ligases, MGRN1 is a unique E3 ligase catalyzing monoubiquitination, which eliminates misfolded proteins through UPS-independent pathway. Possibilities of MGRN1 to recognize other misfolded proteins linked to neurodegenerative diseases and the mechanisms of MGRN1-mediated protein degradation should be explored further.

We found that MGRN1 partially co-localizes with misfolded mutant SOD1 and preferentially degrades mutant SOD1. Moreover, degradation of mutant SOD1 is dependent on the activity of MGRN1 and is inhibited by bafilomycin, an inhibitor for autophagy. This is in line with our previous finding that inhibition of autophagy elevates the level of endogenous MGRN1 and recruits ubiquitin-positive aggregates in cells (Chhangani et al., 2013). Together with the current findings that MGRN1 is partially recruited and colocalized with Lamp2 and p62, important components for autophagy-lysosomal pathway, MGRN1 facilitates degradation of misfolded protein through autophagy pathway. Since monoubiquitination of substrate protein determines its endocytosis and membrane trafficking (Polo et al., 2002), MGRN1 may participate in targeting mutant SOD1 to autophagy-lysosomal pathway. Moreover, association of MGRN1 with molecular chaperone, Hsp70 and preferential degradation of mutant protein by MGRN1 suggest that MGRN1 has a capability to target selectively aberrant proteins for their clearances with help of chaperones and components of autophagy-linked protein quality control mechanism (Chhangani and Mishra, 2013).

Previously, it has been observed that macroautophagy and the proteasome pathway alleviate mutant SOD1-mediated neurotoxicity (Kabuta et al., 2006). It has also been shown that macroautophagy promotes the degradation of mutant SOD1 proteins partly through the action of small heat shock protein HspB8, when aberrant SOD1 forms insoluble aggregates and impairs proteasomal activity (Crippa et al., 2010; Kabuta et al., 2006). Recently, we observed that administration of Cystatin C (CysC) provides cytoprotection against mutant SOD1-mediated toxicity partly through induction of autophagy (Watanabe et al., 2014). This study also support our hypothesis that MGRN1-mediated mutant SOD1 clearance via autophagy is effective, because...
UPS is known to be impaired in mutant SOD1 models. Through interaction with mutant SOD1 proteins, MGRN1 may facilitate their delivery into autophagosome or may improve efficient recognition of SOD1 by UPS through assisting the function of other E3 ubiquitin ligases by yet unidentified mechanism. Our results suggested that MGRN1 overexpression dramatically suppressed the aggregation of mutant SOD1 proteins in cells and alleviated their associated cytotoxicity. A partial depletion of MGRN1 induced the cell death that is linked to mutant SOD1 proteins, and the diminished levels of MGRN1 in presymptomatic ALS mice contributed to the molecular mechanism of the disease. Taken together, our findings suggested MGRN1 is a novel cytoprotective factor to eliminate abnormal SOD1 through autophagy-lysosomal pathway. Considering that there are several known candidate cytoprotective factors against SOD1-mediated toxicities, the combination of these neuroprotective factors including MGRN1 may provide an opportunity for ameliorating the toxicities of mutant SOD1 and perhaps other misfolded proteins linked to neurodegenerative diseases.

Disclosure statement

The authors do not have any actual or potential conflicts of interests to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnbd.2015.11.017.

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References


