Gp78, an ER associated E3, promotes SOD1 and ataxin-3 degradation

Zheng Ying1, Hongfeng Wang1, Huadong Fan1, Xiaodong Zhu2, Jiawei Zhou2, Erkang Fei1 and Guanghui Wang1,*

1Laboratory of Molecular Neuropathology, Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science & Technology of China, Hefei, Anhui 230027, People’s Republic of China and 2Institute of Neuroscience, Chinese Academy of Sciences, Shanghai 200031, People’s Republic of China

Received June 23, 2009; Revised and Accepted August 4, 2009

Superoxide dismutase-1 (SOD1) and ataxin-3 are two neurodegenerative disease proteins in association with familial amyotrophic lateral sclerosis and Machado–Joseph disease/spinocerebellar ataxia type 3. Both normal and mutant types of SOD1 and ataxin-3 are degraded by the proteasome. It was recently reported that these two proteins are associated with the endoplasmic reticulum (ER). Mammalian gp78 is an E3 ubiquitin ligase involved in ER-associated degradation (ERAD). Here, we show that gp78 interacts with both SOD1 and ataxin-3. Overexpression of gp78 promotes the ubiquitination and degradation of these two proteins, whereas knockdown of gp78 stabilizes them. Moreover, gp78 represses aggregate formation of mutant SOD1 and protect cells against mutant SOD1-induced cell death. Furthermore, gp78 is increased in cells transfected with these two mutant proteins as well as in ALS mice. Thus, our results suggest that gp78 functions in the regulation of SOD1 and ataxin-3 to target them for ERAD.

INTRODUCTION

The hallmark of many neurodegenerative disorders, such as Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis (ALS), polyglutamine diseases and prion diseases, is the intracellular aggregates formed by the accumulation of aberrant misfolded disease proteins (1,2). Mutations of these proteins may alter the conformation and result in their misfolding. Furthermore, these misfolded proteins can form aggregates which are highly ubiquitinated, along with molecular chaperones and proteasome subunits (3,4).

Copper–zinc superoxide dismutase-1(SOD1) is an antioxidant enzyme which was originally identified to be localized in cytosol and mitochondria (5–7). Mutations of SOD1 are associated with ALS (8). Ataxin-3 is another neurodegenerative disease protein in association with Machado–Joseph disease (also known as spinocerebellar ataxia type 3) (9). These two disease proteins are misfolded, aggregated and neurotoxic in diseased brains (10,11). It was recently reported that these two proteins are associated with endoplasmic reticulum (ER) and both of them are involved in ER-associated degradation (ERAD) (12–14). Abnormal accumulation of them in the ER induces ER stress (14,15).

ERAD is a protein quality control system in eukaryotes to degrade misfolded proteins in the ER (16). In ERAD process, misfolded or unassembled target proteins are selected as substrates and finally degraded by the cytoplasmic 26S proteasome through ubiquitin–proteasome system (UPS) (17,18). Many unassembled or misfolded proteins are accumulated in the ER and degraded through ERAD (19–22). The disorder of ERAD may result in abnormal accumulation of substrates to cause disease, for example, cystic fibrosis transmembrane conductance regulator, which is tightly associated with cystic fibrosis, is accumulated in the ER if there are impairments of ERAD (23–26).

Ubiquitination of proteins is an important process for protein degradation by ERAD system. Up to date, five E3s (ubiquitin ligases), including gp78, Hrd1, TEB4, Rma1 and RFP2, have been identified to be involved in mammalian ERAD (27–31). Similar to other ERAD E3s, mammalian gp78 is an ER membrane-spanning protein with the RING finger-type ubiquitin ligase activity site that is localized on the ER surface towards the cytoplasmic side (27,32). It was reported that gp78 is involved in ERAD of several substrates, including normal HMG CoA reductase and AAT-deficiency disease protein Z variant of alpha-1-antitrypsin (33,34).
Here we show that ER membrane-anchored E3 gp78 can enhance the ubiquitination of SOD1 and ataxin-3 and promote their degradation by the proteasome, thereby protecting cells against SOD1 and ataxin-3-induced ER stress and cell death.

RESULTS

Gp78 specifically stimulates the degradation of SOD1 and ataxin-3

As both SOD1 and ataxin-3 are involved in ERAD, we wonder whether ERAD associated E3 could promote their degradation. First, we examined their subcellular localization and their degradation. We co-transfected Human Embryonic Kidney 293 (HEK 293) cells with RFP-tagged wild-type (SOD1WT-RFP) or G85R mutant (SOD1 G85R-RFP) SOD1 and EGFP-tagged ER or mitochondria marker, which label ER or mitochondria, respectively. Both wild-type and mutant SOD1s were diffusively distributed in cytoplasm (Fig. 1A). The aggregate formation was observed in cells transfected with G85R mutant SOD1 at a ratio ~10% of positive cells (Fig. 1A). The aggregates formed by mutant SOD1 were localized in the ER, but not mitochondria (Fig. 1A). When transfected cells were treated with MG132, a proteasome inhibitor, the aggregates were significantly increased to be presented in ~40% of positive transfected cells, and the size of aggregates became larger (Fig. 1A). However, tunicamycin, an ER stress inducer, did not significantly increase the aggregate formation (Fig. 1A). These data suggest that inhibition of proteasome results in an abnormal accumulation of mutant SOD1 in the ER. The aggregates formed by mutant ataxin-3 were also observed to be localized in the ER (Fig. 1B).

Next, we examined whether the ER-resident E3s could target these two proteins for ubiquitin–proteasome degradation. We co-transfected HEK 293 cells with EGFP-tagged SOD1G85R and FLAG tag alone or FLAG-tagged gp78. The level of SOD1 G85R-EGFP was markedly decreased in cells co-transfected with FLAG-gp78, but not in cells co-transfected with FLAG tag alone (Fig. 2A). However, in the presence of MG132, no changes were observed (Fig. 2A). Both wild-type and mutant SOD1s were found to be lower in cells co-transfected with gp78, although more decreases of mutant SOD1 in the detergent-insoluble fractions than in the soluble fractions were observed (Fig. 2B). However, gp78 did not change the protein level of FLAG-BAP, a bacterial protein that served as a control in these assays (Fig. 2B).

Similar experiments as Figure 2A were performed to determine the effects of overexpression of gp78 on the clearance of ataxin-3. FLAG-gp78 also promoted ataxin-3 degradation that could be blocked by MG132 (Fig. 2C and Supplementary Material, Fig. S1B). As degradation of SOD1 and ataxin-3 promoted by gp78 could be inhibited by MG132, our data suggested that gp78-mediated degradation of SOD1 and ataxin-3 is proteasome-dependent.

To further identify the effect of gp78 on ataxin-3 degradation, we transfected HEK 293 cells with a fixed amount of
wild-type or mutant ataxin-3 and different amounts of FLAG-gp78. Increased amounts of FLAG-gp78 resulted in more decrease of ataxin-3 levels, suggesting that gp78 promotes ataxin-3 degradation in a dose-dependent manner (Fig. 2D). Chase-time assays demonstrated that gp78 increased the elimination rates of both wild-type and mutant ataxin-3s (Fig. 2E).

We next examined whether Hrd1, another ERAD E3, could promote the degradation of ataxin-3. The ataxin-3-EGFP level was decreased in cells co-transfected with FLAG-gp78, but not in those co-transfected with FLAG-Hrd1 (Supplementary Material, Figs S1A and B). In a cell line stably expressing ataxin-3-20Q-EGFP (35), the fluorescence of ataxin-3-20Q-EGFP was abolished in cells overexpressing gp78, but not in cells overexpressing Hrd1 (Supplementary Material, Fig. S2). These data further suggested that the ERAD-mediated degradation of ataxin-3 is specifically driven by gp78.

To further identify the specificity of SOD1 and ataxin-3 degradation driven by gp78, we co-transfected HEK 293 cells with FLAG-gp78 or FLAG-Hrd1 along with α-synuclein or DJ-1 or SOD1. Only SOD1, but not α-synuclein or DJ-1, was decreased in cells transfected with FLAG-gp78 (Fig. 2F). Meanwhile, in cells co-transfected with FLAG-gp78 and ataxin-3 or the truncated N-terminus of huntingtin (Nhrt) containing different length of polyQ tract (Nhrt-16Q or/and 60Q), no changes of Nhrt levels were observed, although ataxin-3-20Q and -80Q were significantly decreased (Fig. 2G). These data suggested that gp78 specifically promotes SOD1 and ataxin-3 degradation and has no effects on other neurodegenerative disease protein degradation.

To investigate whether gp78 promotes endogenous SOD1 and ataxin-3 degradation, we transfected FLAG-Hrd1 or FLAG-gp78 into HEK 293 cells. Transfection of FLAG-gp78 significantly decreased endogenous protein levels of SOD1 and ataxin-3, but not endogenous DJ-1 (Fig. 2H), further suggesting that gp78 specifically facilitates the clearance of SOD1 and ataxin-3. Another ERAD E3, Hrd1, slightly decreased the level of endogenous SOD1 (Fig. 2H) as well as overexpressed SOD1 (Fig. 2F), however, it has no effect on ataxin-3 (Fig. 2H).

Gp78 colocalizes with SOD1 and ataxin-3 and promotes the ubiquitination of these two proteins

As gp78 is an E3 ubiquitin ligase and it could target SOD1 and ataxin-3 for proteasomal degradation, we examined whether
gp78 could increase their ubiquitination. We co-transfected HEK 293 cells with EGFP-tagged wild-type SOD1 and gp78-myc and performed immunoprecipitation assays using anti-GFP antibodies. In the absence of MG132, transfection of gp78 slightly increased the ubiquitination of wild-type SOD1, although in the presence of MG132, the ubiquitination of SOD1 was significantly increased in gp78 transfected cells (Fig. 3A). Meanwhile, gp78-myc could be co-immunoprecipitated when SOD1-EGFP was immunoprecipitated (Fig. 3A). Similar results were obtained in cells co-transfected with gp78 and EGFP-tagged (Fig. 3B) or FLAG-tagged (Supplementary Material, Fig. S3) mutant SOD1, but more ubiquitinated mutant SOD1 than wild-type SOD1 was observed. Ubc7/UBE2G2 has been recently reported to be a major E2 for gp78 and for ERAD (36–38). Using an in vitro ubiquitination assay, we found that SOD1 was ubiquitinated by gp78 in the presence of Ubc7/UBE2G2 (Fig. 3C). These data suggest that gp78 ubiquitates SOD1 and that Ubc7/UBE2G2 is an E2 for SOD1 ubiquitination. In the presence of other E2s, UbcH5a and UbcH6, ubiquitination of SOD1 by gp78 in vitro was also observed (data not shown). To further examine the interactions between SOD1 and gp78, we transfected HEK 293 cells with FLAG-tagged wild-type SOD1 or mutant SOD1 and performed immunoprecipitation assays. When SOD1s were immunoprecipitated, endogenous gp78 was co-immunoprecipitated (Supplementary Material, Fig. S4). Interestingly, the amounts of gp78 co-immunoprecipitated were higher in cells co-transfected with mutant SOD1s than in that co-transfected with wild-type SOD1 (Supplementary Material, Fig. S4), suggesting stronger interactions between endogenous gp78 and mutant SOD1. The interactions of endogenous SOD1 or ataxin-3 and gp78 in mouse brain homogenates were also confirmed using immunoprecipitation assays (Fig. 3D). Experiments similar to Figure 3A were performed to investigate the effects of gp78 on the ubiquitination and degradation of ataxin-3. Gp78 increased the ubiquitination of ataxin-3 (Fig. 3E). Degradation of ataxin-3 enhanced by gp78 could be blocked by MG132 (Fig. 3E). Stronger interaction between gp78 and mutant ataxin-3 was also observed in immunoprecipitation assays (Fig. 3F).

To further examine whether gp78 is colocalized onto ER-associated aggregates formed by SOD1 and ataxin-3, we co-transfected HEK 293 cells with RFP-tagged SOD1s, ER marker and gp78-myc; or RFP-tagged SOD1s, gp78-EGFP and FLAG-Ub; or EGFP-tagged ataxin-3, gp78-myc and FLAG-Ub, respectively. Under MG132 treatment, gp78 was colocalized onto the aggregates formed by SOD1 that were labeled by ER-marker (Supplementary Material, Fig. S5). Meanwhile, both gp78 and ubiquitin were recruited onto the aggregates formed by SOD1 and ataxin-3 (Fig. 4A and B, Supplementary Material, Fig. S6). In spinal cords of SOD1G93A transgenic mice, gp78 was colocalized onto the SOD1 aggregates (Fig. 4C) and the ubiquitinated aggregates (Fig. 4D).

Functional domains in gp78 involved in interaction with SOD1 and ataxin-3

To investigate if there is a direct interaction between gp78 and SOD1 or ataxin-3, we evaluated the binding of gp78 with these two proteins using in vitro GST pull-down assays. Our data showed that the C-terminus of gp78 (gp78 C, amino acids 309–643) interacted with both wild-type and mutant SOD1 (Supplementary Material, Fig. S7), but not with ataxin-3 (data not shown), indicating that gp78 directly interacts with SOD1. To precisely define the domains required for binding to these two proteins, we created different mutants of gp78 (Fig. 5A). We transfected HEK 293 cells with FLAG-tagged gp78 deletion mutants and EGFP-tagged mutant SOD1 and performed immunoprecipitation assays. Our data showed that the loop (amino acids 379–455) between RING and Cue domains of gp78 interacted with SOD1 (Fig. 5B). Similar experiments were performed to identify the domain in gp78 interaction with ataxin-3, showing that the N-terminus of gp78 (amino acids 1–340) interacted with ataxin-3 (Fig. 5C).

It was reported that both an E3 activity RING finger domain and a ubiquitin-binding Cue domain in the C-terminus of gp78 are involved in gp78-mediated ERAD (32). To explore which domain is responsible for gp78 to mediate ubiquitination and degradation of SOD1 and ataxin-3, we co-transfected cells with EGFP-tagged ataxin-3-20Q or ataxin-3-80Q, along with FLAG-tagged wild-type gp78, or gp78ΔC (Δ309–643). Gp78 ΔC failed to promote the degradation of both wild-type and mutant ataxin-3 (Supplementary Material, Fig. S8A). Meanwhile, gp78 ΔC increased the stability of ataxin-3 (Supplementary Material, Fig. S8B). Decreased ubiquitination of ataxin-3 (Supplementary Material, Fig. S8C) and SOD1 (Supplementary Material, Fig. S8D) in cells co-transfected with gp78 ΔC as compared with those co-transfected with wild-type gp78 were observed. These data suggested that the C-terminus of gp78 is necessary for promoting ubiquitination and degradation of SOD1 and ataxin-3.

We next examined that the different deletion and point mutants of gp78 on SOD1 degradation. The deletion of RING finger (ΔRING) and the point mutant of RING finger (Rm) that result in a loss of E3 ligase activity failed to down-regulate SOD1G93A, but the deletion mutant (ΔCue) or the three-point mutant (Cm) of cue domain did not affect SOD1G93A degradation (Fig. 5D, left panel), suggesting that RING finger and the E3 ligase activity of gp78 are important for driving the degradation of SOD1. The quantitative data are shown in Figure 5D (right panel). Similar to Figure 3A, gp78 increased SOD1G93A ubiquitination even without MG132 treatment, however, RING finger mutant of gp78 (FLAG-gp78 Rm) failed to ubiquitinate SOD1G93A (Fig. 5E), suggesting that E3 ligase activity of gp78 is necessary for SOD1 ubiquitination and degradation.

Knockdown of gp78 decreases sod1 and ataxin-3 ubiquitination and increases their stability

To further confirm the effects of gp78 on SOD1 and ataxin-3 degradation, we synthesized oligonucleotides against gp78 (si-gp78) or control RNA oligonucleotides and co-transfected them with SOD1G93A-EGFP into HEK 293 cells. In gp78 knockdown cells, the levels of SOD1G93A-EGFP were increased (Fig. 6A). Meanwhile, ubiquitination of SOD1G85R-RFP was decreased (Fig. 6B) and the stability of SOD1G85R-RFP was increased (Fig. 6C) in gp78 knockdown cells.
Mutant SOD1 and ataxin-3 upregulate endogenous gp78 by triggering ER stress

It was reported that accumulation of misfolded proteins in the ER can induce ER stress and upregulate ERAD E3s, like Hrd1 and gp78 (39,40). As mutant SOD1 and expanded polyQ proteins could also induce ER stress and cell death (15,41,42), we therefore examined if mutant SOD1 and ataxin-3 could increase gp78 levels via ER stress. In spinal cords of SOD1G93A transgenic mice, we found that gp78 was significantly increased (Figs 7A and B). Bip and caspase-12 are two ER stress marker proteins (15). Increased levels of Bip, gp78 and cleaved caspase-12 were observed in cells treated with tunicamycin (Fig. 7C), suggesting that ER stress induces Bip and gp78 expressions. In cells expressing SOD1G85R and the C-terminal fragments of ataxin-3 containing expanded polyQ tracts (ataxin-3-80QF-EGFP and ataxin-3-130QF-EGFP), increases of Bip and gp78 were observed (Fig. 7D). The quantitative data are shown in Figure 7E and F. These data suggest that mutant SOD1 and ataxin-3 induce ER stress, leading to an increase of gp78.

Gp78 inhibits aggregate formation and cell death mediated by mutant SOD1

As we have shown that gp78 enhances SOD1 degradation, we wonder if gp78 could affect aggregate formation of SOD1 in
the ER. We therefore co-transfected HEK 293 cells with SOD1\textsuperscript{G85R}-EGFP and gp78-myc or gp78 siRNA. The aggregate formation was decreased by overexpression of gp78 (Fig. 8A). However, in cells transfected with si-gp78, the number and size of aggregates were increased, similar to those treated with MG132 (Fig. 8A). The quantitative data showed the effects of overexpression and knockdown of gp78 on SOD1 aggregate formation (Fig. 8B). In N2a cells expressing SOD1\textsuperscript{G93A}-EGFP, knockdown of gp78 greatly increased cell death (Supplementary Material, Fig. S9). Furthermore, knockdown of gp78 could increase aggregate formation of SOD1 (Fig. 8C) and cell death (Fig. 8D) in primary cultured neurons of spinal cords from SOD1\textsuperscript{G93A} transgenic mice.

Figure 5. Gp78 stimulates the degradation and ubiquitination of SOD1 and ataxin-3 in a RING finger dependent manner. (A) Schematic diagram of gp78 constructs used in this study. (B and C) Different constructs of FLAG-gp78 and SOD1\textsuperscript{G93A}-EGFP or ataxin-3-80Q-EGFP were co-transfected into HEK 293 cells. Twelve hours after transfection, MG132 (10 \mu M) was added and incubated for 12 h. Cell lysates were processed for immunoprecipitation analysis and then subject to immunoblot analysis with indicated antibodies. The asterisk indicates IgG heavy chains. (D) HEK 293 cells were co-transfected with SOD1\textsuperscript{G93A}-EGFP and different constructs of FLAG-gp78 as indicated. The cell lysates were subjected to immunoblot analysis with indicated antibodies (left panel). Quantitative data from three independent experiments were shown (right panel). The results were indicated mean ± SE. \( * \), \( P < 0.05 \) one-way ANOVA. (E) SOD1\textsuperscript{G93A}-EGFP was co-transfected with FLAG-gp78 or FLAG-gp78 Rm, cell lysates were processed for immunoprecipitation analysis and then subject to immunoblot analysis with indicated antibodies.
We next examined the effects of knockdown of gp78 on mutant SOD1-induced ER stress and cell death. Knockdown of gp78 increased the level of Bip, whereas overexpression of gp78 decreased the level of Bip in HEK 293 cells (Fig. 8E). Taken together, our data suggest that gp78 inhibits aggregate formation of mutant SOD1 and protects cells from death mediated by ER stress induced by mutant SOD1.

DISCUSSION

The UPS is an important system to degrade misfolded proteins and several RING finger-type E3s, such as dorfin, chip and parkin, are involved in the ubiquitination and degradation of SOD1 and ataxin-3 to repress their aggregate formation and toxicity (43–47). In our present study, we show that ER-associated E3 gp78 interacts with SOD1 and ataxin-3 and increases their ubiquitination to drive ERAD-mediated clearance of these two proteins. Overexpression of gp78 promotes SOD1 and ataxin-3 degradation, although knockdown of gp78 increases their stability and aggregate formation. Our observation that gp78 is an E3 to promote their ubiquitination and degradation suggests that gp78 may function as a quality control E3 to facilitate ER-associated SOD1 and ataxin-3 degradation through ERAD.

E3s play important roles in regulating misfolded protein degradation by the proteasome. Many of E3s are presented in the cytoplasm and nucleus where they facilitate the degradation of proteins, including some neurodegenerative disease related proteins such as α-synuclein and poly-Q-expanded proteins (46,48). Mammalian gp78 has been reported to exist in the ER and to function as an E3 for regulating misfolded or unfolded ER-resident proteins. It was reported that SOD1 binds to derlin-1 (12) and ataxin-3 binds to VCP/p97 (13,14,49). As derlin-1 and VCP/p97 are two key components of ERAD machinery, these interactions provide a basis for ER-localization of SOD1 and ataxin-3, and therefore link SOD1 and ataxin-3 to ERAD. Our study also showed that SOD1 and ataxin-3 are accumulated in the ER under MG132 treatment (Fig. 1 and Supplementary Material, Fig. S5). It is much likely that SOD1 and ataxin-3 are similar to ER-resident proteins to be subject to ERAD, when they are accumulated in the ER. In comparison with SOD1 and ataxin-3, non-ER-colocalized neurodegenerative disease proteins, such as α-synuclein, N htt and DJ-1, are not affected by gp78 (Fig. 2F and G), suggesting that gp78 specifically functions in the ubiquitination and degradation of SOD1 and ataxin-3. Interestingly, it was reported that another ER-resident E3, Hrd1, suppresses A53T mutant α-synuclein-mediated neurodegeneration (50), suggesting that α-synuclein may be closely associated with ERAD. E3 ligases have high specificity for their own substrates, it is therefore possible that ER-associated E3s differentially regulate their substrates. It is also possible that mutant α-synuclein, but not WT α-synuclein, is accumulated in the ER to induce ER stress and to be associated with ERAD.

Previous studies have shown that ERAD E3 can eliminate the misfolded proteins within both ER lumen and cytosol (51,52). Interestingly, Doa10, yeast homologous to mammalian ERAD E3 TEB4, could promote the ubiquitination and degradation of non-ER localization substrates (52). It was recently reported that another mammalian ERAD E3 Hrd1 is also involved in the degradation of misfolded cytoplasmic N htt containing expanded polyglutamine tracts (53). A possible explanation is that their RING finger catalytic center

Figure 6. Knockdown of gp78 stabilizes SOD1 and ataxin-3 and inhibits their ubiquitination. (A) HEK 293 cells were transfected with SOD1G93A-EGFP, along with siRNAs targeting two regions of gp78 or negative control siRNA. The indicated proteins were detected using immunoblot analysis 24 h after transfection. (B) HEK 293 cells were co-transfected with SOD1G85R-RFP and siRNAs. Twenty-four hours after transfection, MG132 was added and incubated for 12 h. The cells were subjected to immunoprecipitation analysis using anti-SOD1 antibodies, and the inputs and immunoprecipitants were subject to immunoblot analysis with indicated antibodies. (C) SOD1G85R-RFP was co-transfected with control siRNA, myc-gp78 or siRNA targeting gp78 into HEK 293 cells. Sixteen hours after transfection, the cells were treated with CHX and the turnover of SOD1G85R-RFP was examined over an 8 h period.
Figure 7. Mutant SOD1 and ataxin-3 induce ER stress and upregulate endogenous gp78. (A) Expression of gp78 in motor neurons in spinal cord anterior horn of 4-month-old SOD1<sup>G93A</sup> transgenic mice and age-matched non-transgenic mice were detected using immunohistochemical staining with anti-gp78 antibodies. The arrows indicate positive-stained motor neurons. Scale bar, 20 μm. (B) Spinal cord homogenates from 2- or 4-month-old SOD1<sup>G93A</sup> transgenic mice were processed for immunoblot analysis. (C) HEK 293 cells were treated with increasing amounts of tunicamycin for 8 h. The levels of Bip, caspase-12 and gp78 were detected using immunoblot analysis. (D) EGFP-tagged C-terminus of ataxin-3 containing 20Q, 80Q, 130Q or full length of WT or mutant SOD1 was transfected into HEK 293 cells for 20 h. Cell lysates were subjected to immunoblot analysis with indicated antibodies. Lipofectamine-treated (NT) cells and cells treated with tunicamycin (8 h) were used as a negative and a positive control, respectively. (E and F) Quantitative data from three independent experiments of (D) were shown. The results were indicated mean ± SE. *, P < 0.05; **, P < 0.01, one-way ANOVA.
Figure 8. Gp78 protects cells against ER stress induced cell death caused by mutant SOD1 and ataxin-3. (A) HEK 293 cells were co-transfected with SOD1G85R-EGFP, along with si-control RNA (control), gp78-myc or siRNA of gp78 (si-gp78) and visualized 36 h after transfection. The cells treated with 10 μM MG132 (lower panel, right) or without MG132 (upper panel and the left picture at lower panel) were indicated. (B) SOD1WT or SOD1G85R-RFP was co-transfected with si-control RNA (control), gp78-myc or siRNA targeting gp78 into N2a cells for 24 or 48 h with or without 2 μM MG132 treatment. The aggregates were counted. The quantitative data represent the means of percentage of SOD1-RFP aggregates in 100 positive cells from three independent transfections. The results were indicated mean ± SE. *, P < 0.05; **, P < 0.01, one-way ANOVA. (C) si-control RNA (control) or si-gp78 RNA was transfected into neurons of spinal cord cultures of SOD1 G93A transgenic mice (P0). The cultures were subjected to immunofluorescence analysis using rabbit polyclonal anti-SOD1 antibodies (red) and a neuronal marker antibody, anti-MAP-2 (green). Scale bars, 10 μm. Quantitative data of left panel from three independent experiments were shown (right panel). The results were indicated mean ± SE. *, P < 0.05 one-way ANOVA. (D) The quantitative data represent the means of percentage of PI-positive cells in 100 primary cultured neurons of spinal cord from SOD1G93A transgenic mice (E14) from three independent si-control and si-gp78 RNA transfections. The results were indicated mean ± SE. *, P < 0.05 one-way ANOVA. (E) EGFP-tagged SOD1G85R was co-transfected with si-control RNA (control), si-gp78 RNA or gp78-myc into HEK 293 cells. Cells were collected 20 h after transfection and subjected to immunoblot analysis. Cells treated with tunicamycin for 8 h served as a positive control.
towards the cytoplasmic side of the ER allows its interaction with misfolded cytoplasmic proteins to perform its E3 activity. In gp78, there are two important domains, RING finger and Cue domains which are associated with its activity (32). In our study, we show that RING finger, but not Cue domain, is necessary for SOD1 and ataxin-3 degradation (Fig. 5D) and ubiquitination (Fig. 5E), suggesting that gp78 E3 ligase activity is critical for regulation of SOD1 and ataxin-3 degradation.

It was reported that mutant SOD1 and ataxin-3 inhibit mammalian ERAD (12–14). They impair the retrotranslocation of misfolded ERAD substrates in the ER lumen, leading to accumulation of proteins in the ER, thereby inducing ER stress (12–14). ER stress-induced cell death caused by abnormal accumulation of disease proteins are involved in pathogenesis of several neurodegenerative disorders including ALS and polyglutamine diseases (42,54–59). In ALS mice (Fig. 7A and B) and in cells overexpressing mutant SOD1 or the fragments of ataxin-3 with expanded polyQ tracts (Fig. 7D), gp78 is up-regulated. As overexpression of gp78 could down-regulate SOD1 and ataxin-3 (Fig. 2), the up-regulation of gp78 may be a cellular protective response to ER stress induced by these mutant proteins. It was reported that ER-associated E3s, such as Hrd1, act as a protector of ER stress to facilitate the clearance of toxic misfolded ER-resident proteins (39). Therefore, gp78 here functions as a comprehensive ‘sweeper’ in clearance of both misfolded ER membrane-associated cytoplasmic proteins and ER luminal proteins thereby mitigating ER stress. Increases of cell death and aggregate formation of SOD1 in primary cultured neurons from ALS mice by knockdown of gp78 (Fig. 8C) and colocalization of gp78 and ubiquitinated SOD1 aggregates (Fig. 7D), gp78 is up-regulated. As overexpression of gp78 in animal model and transfected cells were observed in our study, it is possible that mutant proteins are more resistant to degradation, leading to abnormal accumulation of disease proteins and ER stress. These phenomena are similar to that mutant huntingtin is resistant to its degradation by the proteasome and results in impairments of proteasome activity, although it is a substrate degraded by the proteasome (60). Colocalization of gp78 with ubiquitinated SOD1 aggregates in transfected cells (Fig. 4A) and colocalization of gp78 and SOD1 inclusions in ALS mice (Fig. 4C and D) support that ALS shares a common mechanism with some other neurodegenerative diseases in dysfunction of the UPS.

In conclusion, our study provides evidence that gp78 facilitates the degradation of two ER-associated proteins SOD1 and ataxin-3 by ERAD pathway and suppresses their aggregation in the ER. Disorders of ERAD may affect these disease protein degradation, thus contributing to disease pathogenesis.

**MATERIAL AND METHODS**

**Plasmid constructs**

SOD1, ataxin-3, α-synuclein, DJ-1, truncated N-terminal fragments of huntingtin (Nhtt) and FLAG-UB constructs were described previously (35,61–64). pCIneo-gp78 and pCIneo-FLAG-Hrd1 were kindly provided by Dr Shengyun Fang (University of Maryland Biotechnology Institute). pET-28-His-Ubc7 was kindly provided by Dr Baoliang Song (Institute of Biochemistry and Cell Biology, SIBS, CAS). The following constructs were generated by standard PCR methods: Full-length gp78 cDNA was first amplified by PCR using primers 5'-cccaagctatgctgctgctc-3' and 5'-gtctagatggtgggcaacagg-3' from pCIneo-gp78 and inserted into p3xFLAG-myc-CMV-24 (Sigma) at HindIII/XbaI sites. FLAG-Hrd1 was generated by subcloning the PCR products amplified with primers 5'-cccaagctatgctgctgctc-3' and 5'-gtctagatggtgggcaacagg-3' into p3xFLAG-myc-CMV-24 vector at HindIII/XbaI sites. For the construct of gp78-myc, full length gp78 cDNA was excised from FLAG-gp78 with HindIII/BamHI sites and subcloned into pCS2-MT vector. The deletion mutants and point mutations of FLAG-tagged gp78 were generated by the Site Directed Mutagenesis using Mutanbest kit (Takara) with following primers: 5'-gggtgca tctgtggtggac-3' and 5'-gtctagatggtgggcaacagg-3' for FLAG-gp78 Δ341–463; 5'-gtctagatggtgggcaacagg-3' and 5'-attc aagtctcttgaatctgatg-3' for FLAG-gp78 Δ341–497; 5'-gggtgtgtgtgtgtgtgtgtgtgtgt-3' and 5'-attc aagtctcttgaatctgatg-3' and 5'-tgctagagatgtagagcggcttgctg-3' for FLAG-gp78 Δ456–464; 5'-ccctctctcttttgatgtagagcggcttgctg-3' and 5'-tgctagagatgtagagcggcttgctg-3' and 5'-attc aagtctcttgaatctgatg-3' for FLAG-gp78 Δ341–378 (ΔRING); 5'-gggtgtgtgtgtgtgtgtgtgtgtgt-3' and 5'-attc aagtctcttgaatctgatg-3' for FLAG-gp78 Δ456–497 (ΔCue); and 5'-gggtgtgtgtgtgtgtgtgtgtgtgt-3' and 5'-ccctctctcttttgatgtagagcggcttgctg-3' for FLAG-gp78 Cm in which three amino acids from 467 to 469 (MFP) were converted to GGR, respectively. The C-terminal deletion mutant (Δ309–463) of gp78 (FLAG-gp78 ΔC) was constructed by subcloning a deletion fragment excised from p3xFLAG-myc-CMV-24-gp78 into FLAG vector at HindIII/EcoRI sites. For myc-ataxin-3 constructs, ataxin-3 cDNA containing 20 or 80 CAG repeats was digested from pAS2-1-ataxin-3-20Q or pAS2-1-ataxin-3-80Q and cloned into pAC2-MT vector. For FLAG-myc construct, the fragments of ataxin-3 with expanded polyQ tracts was digested from pAS2-1-ataxin-3-20Q or pAS2-1-ataxin-3-80Q and cloned into pAC2-MT vector. For the construction of gp78-myc, full length gp78 cDNA was excised from FLAG-gp78 with HindIII/BamHI sites and subcloned into pCS2-MT vector. pEGFP-Mito marker was cloned from pECFP-Mito (Clontech) by changing ECFP tag to EGFP tag through BamHI/NcoI sites. ER marker, pEGFP-ER, was kindly provided by Dr Cuiqing Zhu (Fudan University, China).

All constructs were confirmed by sequencing.

**In vitro binding assay**

An aliquot containing 20 µg of protein from the soluble fraction of *Escherichia coli* cell lysates expressing GST or GST-gp78 C (309–643) was incubated with 20 µl of glutathione agarose beads (Pharmacia) for 20 min at room temperature. After washing three times with 1×PBS, beads bound with GST or GST-gp78 C were incubated with 50 µg of protein from the supernatants of *E. coli* crude extract containing recombinant His-SOD1 WT or His-SOD1 G85R expressed by pET-15b-SOD1 WT, or pET-15b-SOD1 G85R in 0.25 ml HNTG-buffer [20 mM HEPES-KOH (pH 7.5), 100 mM NaCl, 0.1% Triton X-100 and 10% glycerol] for 1 h at 4°C. After incubation, the beads were washed four times with 1 ml HNTG buffer to remove unbound proteins. Bound proteins were collected by washes and eluted with 6×SDS-PAGE loading buffer [50 mM HEPES-KOH (pH 7.5), 50 mM DTT, 2% SDS, 5% glycerol, 0.05% bromophenol blue, and 0.5% Bio-Rad protein assay dye reagent (Bio-Rad)] and analyzed on 10% SDS-PAGE.
were eluted from the beads by boiling in SDS sample buffer and detected by immunoblot analysis.

**In vitro ubiquitination assay**

Ten nanogram of E1 (Sigma), 200 ng of His-Ubc7, 2 μg of GST-gp78 C, 2 μg of FLAG-SOD1G85R-His and 5 μg of GST-Ub were mixed and incubated at 37°C for 2 h in 50 μl of reaction buffer (4 mM ATP in 50 mM Tris–HCl, pH 7.5, and 4 mM MgCl2). The reactions were stopped by adding SDS sample buffer and detected by immunoblot analysis.

**Cell culture and transfection**

HEK 293 cells or Mouse Neuroblastoma (N2a) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO) containing 10% newborn calf serum (NCS) (GIBCO). A stable HEK 293 cell line expressing EGFP-fused ataxin-3-20Q (35) was cultured in DMEM with 10% NCS and 200 μg/ml G418 (GIBCO). Cells were transfected with expressing vectors using LipofectAMINE™ 2000 reagent (Invitrogen) at ~40% confluence in DMEM without serum. Cycloheximide (CHX) and tunicamycin were purchased from Sigma, and MG132, from Calbiochem. Primary neuron cultures from mouse spinal cords (P0 or E14) were prepared with standard neuronal culture procedures. Briefly, the dissociated neurons were plated in DMEM containing 10% fetal bovine serum (FBS) (Hyclone). After culturing for 12 h, media were changed to neurobasal media (GIBCO) containing 3 μg/ml glutamate (Sigma) and 1×B27 (GIBCO). Cultures were assayed for RNAi studies 8 days after plating.

**Immunoblot analysis and antibodies**

Cells were first lysed in TSPI buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 μg/ml of aprotinin, 10 μg/ml of leupeptin, 0.5 μM Pefabloc SC, and 10 μg/ml of pepstatin] containing 1% NP-40, and then the proteins were separated by 10% SDS PAGE and transferred onto polyvinylidene difluoride membrane (Millipore). To prepare the detergent soluble and insoluble fractions, cell lysates were centrifuged at 12 000g for 20 min at 4°C. Mouse spinal cord and brain homogenates were prepared from SOD1G93A transgenic mice or age-matched non-transgenic mice.

The following primary antibodies were used: mouse monoclonal antibodies against FLAG (Sigma), Tubulin (Santa Cruz), GFP (Santa Cruz), GAPDH (Chemicon), SOD1 (Santa Cruz), Ub (Santa Cruz), myc (Santa Cruz), anti-MAP-2 (Lab Vision Corporation) and ataxin-3 (MJ2-5-3) (35); rabbit polyclonal antibodies against DJ-1 (Chemicon), myc (Santa Cruz), Bip (Stressgen Bioreagents) and caspase-12 (Cell signaling Biotech). Monoclonal anti-FLAG antibody conjugated with HRP was purchased from Sigma. Rabbit polyclonal antibodies against gp78, SOD1 or ataxin-3 were generated by immunizing rabbits with GST-gp78 C, His-SOD1WT or His-ataxin-3-20Q and then were purified with affinity column. The secondary antibodies, sheep anti-mouse IgG-HRP antibody and anti-rabbit IgG-HRP antibody (Amersham Pharmacia Biotech) were used. The proteins were visualized using an ECL detection kit (Amersham Pharmacia Biotech).

**Immunoprecipitation**

Crude cell lysates were sonicated in TSPI buffer containing 1% NP-40. Cellular debris was removed by centrifugation at 12 000g for 20 min at 4°C. The supernatants were incubated with the antibodies in 0.01% BSA for 4 h at 4°C. After incubation, protein G Sepharose (Roche) was used for precipitation. The beads were washed with TSPI buffer four times, and proteins were eluted with SDS sample buffer for immunoblot analysis.

**Immunocytochemistry and immunohistochemistry**

HEK 293 cells grown and transfected on cover slides were washed with PBS and fixed with 4% paraformaldehyde for 5 min at room temperature. The cultures were treated with 0.25% Triton X-100 for 15 min and blocked by 4% FBS in PBS and then incubated overnight at 4°C with the primary antibody following an incubation with FITC or Rho-conjugated donkey anti-mouse or -rabbit secondary antibodies (Santa Cruz Biotechnology) or Alexa Fluor-350-labeled goat anti-mouse IgG (Invitrogen). Nucleus was stained with DAPI (Sigma).

Four month old ALS mice and age-matched non-transgenic mice were perfused through the heart with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) under deep urethane anesthesia. The spinal cords were removed, post-fixed overnight in the same fixative at 4°C, and treated with 30% sucrose at 4°C overnight. The sections were stored in 0.1 M PBS containing 0.1% sodium azide at 4°C. Immunohistochemical staining experiments were performed using indicated antibodies and detected with FITC or Rho-conjugated donkey anti-mouse or -rabbit secondary antibodies (Santa Cruz Biotechnology) or Vectastain Elite ABC kit (Vector Laboratories).

**RNA interference**

Double-stranded oligonucleotides designed against the region starting from 496 or 1128 of human gp78 cDNA were synthesized by Shanghai GenePharma (Shanghai, China). The sequences are si-gp78-1, sense 5'-GGAUCGAUUUGAA UAUCUUTT-3' and anti-sense 5'-UUAAGAGACAUUCU UAUAATT-3' and anti-sense 5'-GGAUCGAUUUGAA UAUCUUTT-3'. Meanwhile, an irrelevant oligonucleotide was served as a negative control. The transfection was performed with Oligofectamine (Invitrogen) according to its manufacture.

**Mice**

SOD1G93A transgenic mice [B6SJL-TgN(SOD1-G93A)1Gur] from the Jackson Laboratory or age-matched non-transgenic mice as the controls were used. All of the mice experiments were performed according to the protocols approved by the Animal Welfare Committee of University of Science and Technology of China.
SUPPLEMENTARY MATERIAL

Supplementary material is available at HMG online.

ACKNOWLEDGEMENTS

The authors are grateful to Dr Shengyun Fang (University of Maryland Biotechnology Institute) for kind gifts of pCIneo-gp78 and pCIneo-FLAG-Hrd1 plasmids, Dr Baoliang Song (Institute of Biochemistry and Cell Biology, SIBS, CAS) for pET-28-His-Ubc7 plasmids.

Conflict of Interest statement. None declared.

FUNDING

This work was supported in part by the National Natural Sciences Foundation of China (30770664), the National High-tech Research and Development program of China (2006AA02A408, 863-project (2006AA02A408), the CAS Knowledge Innovation Project (KSCX2-YW-R-138) and the Bureau of Education of Anhui (ZD2008008-2).

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