Interaction between Familial Amyotrophic Lateral Sclerosis (ALS)-linked SOD1 Mutants and the Dynein Complex*§

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Amyotrophic lateral sclerosis (ALS)3 is an age-dependent neurodegenerative disorder characterized by progressive motor neuron death. More than 90 mutations in the copper-zinc superoxide dismutase (SOD1) gene cause a subset of familial ALS. Toxic properties have been proposed for the ALS-linked SOD1 mutants, but the nature of the toxicity has not been clearly specified. Cytoplasmic inclusion bodies containing mutant SOD1 and a number of other proteins are a pathological hallmark of mutant SOD1-mediated familial ALS, but whether such aggregates are toxic to motor neurons remains unclear. In this study, we identified a dynein subunit as a component of the mutant SOD1-containing high molecular weight complexes using proteomic techniques. We further demonstrated interaction and colocalization between dynein and mutant SOD1, but not normal SOD1, in cultured cells and also in G93A and G85R transgenic rodent tissues. Moreover, the interaction occurred early, prior to the onset of symptoms in the ALS animal models and increased over the disease progression. Motor neurons with long axons are particularly susceptible to defects in axonal transport. Our results demonstrate a direct gain-of-interaction between mutant SOD1 and dynein, which may provide insights into the mechanism by which mutant SOD1 could contribute to a defect in retrograde axonal transport or other dynein functions. The aberrant interaction is potentially critical to the formation of mutant SOD1 aggregates as well as the toxic cascades leading to motor neuron degeneration in ALS.

Amyotrophic lateral sclerosis (ALS)3 is an age-dependent neurodegenerative disorder characterized by the progressive death of motor neurons. Mutations in the gene encoding copper-zinc superoxide dismutase (SOD1) have been linked to ~25% of familial ALS (1, 2). More than 90 mutations, which scatter throughout the protein, are known to date (3). It has been established that SOD1 mutants acquire toxic properties, but the nature of the toxicity remains debatable. It is also unclear how the toxicity causes preferential motor neuron death.

Cytoplasmic inclusion bodies containing mutant SOD1, ubiquitin, and neurofilaments are a pathological hallmark of mutant SOD1-mediated familial ALS (4–10). The soluble high molecular weight (HMW) complexes of SOD1 mutants, which exist prior to large protein aggregates, have also been reported to cause motor neuron death (6, 8). Such HMW complexes or aggregates may disrupt essential cellular functions, such as disturbing mitochondria (11–15) and inhibiting proteasome function (16), thus being toxic to neuron. A comprehensive study of all protein components in the SOD1-containing aggregates would provide valuable insights into the toxicity of mutant SOD1. It is particularly interesting to identify proteins interacting with mutant SOD1 in the soluble HMW complexes in the early stage of protein aggregation. We recently demonstrated that fALS-linked SOD1 mutants A4V, G85R, and G93A can form soluble HMW complexes prior to forming insoluble aggregates in cultured cells (17). In this study, the soluble SOD1-containing HMW complexes were purified and a dynein subunit was identified using mass spectrometry.

The dynein-mediated retrograde transport system is required for many essential cellular functions (18, 19), particularly in motor neurons because of their long axons (20). Dynein-mediated retrograde transport is responsible for returning membrane-bounded organelles from the axon terminal to the cell body and for providing neurotrophic growth factors for neuron survival, injury, and regeneration signaling. Mice with mutations in the dynein heavy chain (DHC), Legs at odd angle (Loa), or Cramping 1 (Cra1), showed defects in retrograde transport and motor neuron degeneration (21). Mice overexpressing the motor protein dynamitin, which inhibited dynein-mediated axonal transport, developed a late-onset progressive motor neuron degenerative disease (22). Studies in ALS patients have revealed decreased speed of retrograde transport of organelles such as mitochondria (23, 24). Reduced transport of dynein-dependent cargoes as well as slow transport of major structural components such as tubulin and neurofilaments have also been found in several ALS transgenic models and can be observed well before onset of symptoms (25–30). Furthermore, mutations in the p150Glued subunit of dynactin, a dynein-
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binding protein involved in retrograde transport, have been reported in familial ALS patients (31, 32). Recent studies crossing the Loa (33) and Cra1 (34) mice with G93A SOD1 transgenic mice showed surprising findings that both DHC mutants can rescue the axonal transport defects and extend the life spans of ALS mice. Although the results were counterintuitive and the mechanisms remain unclear, these two studies strongly suggest that dynein-mediated retrograde transport may play an important role in the mutant SOD1-mediated familial ALS etiology. However, it has remained largely unknown how mutant SOD1 may cause the retrograde axonal transport defect.

We hereby provide a direct connection between retrograde axonal transport and mutant SOD1. We demonstrate in this study that the ALS-linked SOD1 mutants A4V, G85R, and G93A interact with the dynein complex, whereas WT SOD1 does not. We further show the aberrant interactions between the dynein complex and ALS-linked SOD1 mutants in spinal cord and sciatic nerve of G93A and G85R transgenic mice and G93A transgenic rats. We also show that mutant SOD1 and the dynein complex are largely colocalized in the protein aggregates in the spinal cord motor neurons of the G93A and G85R SOD1 transgenic ALS mice. The significance of this novel interaction between mutant SOD1 and dynein and its potential relationship to ALS pathophysiology is discussed.

MATERIALS AND METHODS

Plasmids Construction—Mouse dynein intermediate chain (DIC) was amplified from the pEGFP-DIC construct (generously provided by Dr. Trina A. Schroer, Johns Hopkins University) by PCR and was inserted into the BamHI and NotI sites of the mammalian expression vector pEBG that expresses glutathione S-transferase fusion proteins. Mouse DIC was also amplified and inserted into the EcoRI and BamHI sites of the pDsRed2 vector (Clontech) to construct DIC-DsRed plasmid. The SOD1-FLAG and SOD1-GFP constructs were described in the previous study (17). The fidelity of all the constructs was confirmed by DNA sequencing.

Chemical Cross-linking and FLAG Immunoprecipitation—NSC34 and HEK293T cells were cultured at 37 °C under 5% CO2, 95% air in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum and antibiotics as described (35). Cells were transfected with the indicated plasmids using Lipofectamine (Invitrogen) and were harvested and lysed 48 h after transfection unless indicated otherwise. NSC34 cells transfected with SOD1-FLAG were harvested and lysed in 1 ml of 1M tris-buffer (50 mM HEPES, pH 7.5). After preclearing, the cell lysate was incubated with 2 ml disuccinimidyl suberate on ice for 2 h and the reaction was quenched by adding 20 μl of 1 M Tris. The cross-linking reaction mixture was immunoprecipitated with anti-FLAG M2 affinity gel (A2220, Sigma) at 4 °C overnight. The agarose beads were washed three times with RIPA buffer and boiled with 2× SDS loading buffer. The supernatant was loaded onto 10% SDS-PAGE for protein separation.

Mass Spectrometry Analysis and Protein Identification—The immunoprecipitated SOD1 containing cross-linked products were resolved by SDS-PAGE and visualized with Sypro Ruby staining. Protein bands were sliced from the gel and subjected to in-gel trypsin digestion and LC-MS/MS analysis as in our previous studies (35, 36). Alternatively, the immunoprecipitated samples were digested by trypsin and the resulting mixtures of peptides were subjected to LC-MS/MS shotgun proteomic analysis without running SDS-PAGE as described in Ref. 36. Both MS and MS/MS data were acquired by Qstar XL Q-TOF mass spectrometer (Applied Biosystems, Foster City, CA) under information-dependent acquisition mode. The LC-MS/MS data were subjected to data base search for protein identification using a local MASCOT search engine. Multiple databases (NCBI, Swiss-Prot, and MSDB) were searched using the parameters as previously described (35, 36), yielding identification of proteins in each sliced gel band.

Animals—Transgenic mice strains overexpressing WT and G93A mutant SOD1 (37) were generously provided by Dr. Zuoshang Xu (University of Massachusetts Medical School) and maintained as hemizygotes at the University of Kentucky animal facility. Transgenic mice overexpressing G85R mutant SOD1 (4) and transgenic rats overexpressing G93A mutant (38) (purchased from Taconic, Germantown, NY) were bred and maintained at the University of Massachusetts Medical School animal facility. Transgenic positives were identified using PCR according to Gurney et al. (37). G93A SOD1 transgenic mice were sacrificed at ages 60, 90, and 125 ± 5 days. Age-matched WT SOD1 transgenic mice as well as non-transgenic littermates were used as controls. Pre-symptomatic G85R transgenic mice were sacrificed at the age of 167 days. Mice were anesthetized with an intraperitoneal injection of 0.3 ml of pentobarbital (50 mg/ml, Abbott Laboratories) and transcardially perfused with 0.1 M PBS, pH 7.5. Spinal cords and other tissues were dissected. All animal procedures were approved by each university’s IACUC committee.

GST Pulldown and Immunoprecipitation of DIC—HEK293 cells transfected with both SOD1-FLAG and DIC-GST constructs were harvested and lysed in 1 ml of RIPA buffer. The protein concentration was determined by Bradford assay (BioRad). Four hundred micrograms of lysate was incubated with 20 μl of a 50% slurry of glutathione-Sepharose 4B (Amersham Biosciences) in a total volume of 1 ml for 1 to 2 h at 4 °C to allow isolation of the GST fusion proteins. The glutathione beads were then collected, washed, and boiled in 2× SDS sample loading buffer. The proteins released from the beads were subjected to 12% SDS-PAGE followed by Western blotting.

DIC immunoprecipitations were carried out for the dissected mice spinal cords and sciatic nerves using DIC mouse monoclonal antibody (sc-13524, Santa Cruz) and protein G-Sepharose (Amersham Biosciences). The immunoprecipitated proteins were subjected to 12% SDS-PAGE followed by Western blotting. Antibodies used in the Western blotting are rabbit anti-SOD1 (sc-11407, Santa Cruz), mouse anti-DIC, rabbit anti-DHC (sc-9115, Santa Cruz), and mouse anti-p150Gluad (catalog number 610473, BD Pharmingen). Intensities of Western blotting bands were quantified using the Kodak 1D software (version 3.6.1).

Fluorescence Microscopy—Immunohistological analyses were performed on lumbar spinal cords and sciatic nerves of ALS animals at various ages as indicated. Spinal cords were dis-
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Identification of Dynein in the SOD1-containing High Molecular Weight Complexes—We recently reported that the ALS-linked SOD1 mutants have different conformations in live cells and form soluble HMW complexes prior to the formation of large aggregates, whereas such complexes were not observed for WT SOD1 (17). A recent study demonstrated retarded post-translational folding kinetics as a common feature of multiple ALS-linked SOD1 mutants (39). The impaired folding abilities will likely increase the population of misfolded intermediates of mutant SOD1 and lead to formation of HMW complexes, supporting our results.

In this study, the HMW complexes of the A4V mutant were captured in the presence of the chemical cross-linker disuccinimidyl suberate and visualized by Western blotting as shown in Fig. 1A. In contrast, WT SOD1 migrated only as monomers and dimers in SDS-PAGE after being cross-linked. The cross-linked A4V SOD1-containing HMW complexes were effectively immunoprecipitated as shown in Fig. 1B. Fig. 1C shows the Sypro Ruby staining of the SDS-PAGE resolved immunoprecipitation product. The protein bands were subsequently identified by proteomic and mass spectrometric analyses and are shown in the figure. SOD1 monomer, cross-linked SOD1 dimer, and IgG light chain were three major protein species. Copper chaperone for SOD1 and Hsp70 were identified and they have been reported to be associated with SOD1. Copper chaperone for SOD1 plays a critical role in facilitating copper binding to SOD1 (40, 41) and heat shock proteins are molecular chaperones for misfolded SOD1 mutants (7, 42). The proteomic analysis also identified DHC and SOD1 as a cross-linked species from the top of the SDS-PAGE. In addition, the alternative shotgun proteomic approach also identified DHC in the immunoprecipitation sample of A4V SOD1, but not in the sample of WT SOD1. This novel finding, combined with the reported involvement of dynein and axonal transport in ALS, suggests that the dynein complex including the heavy chain and mutant SOD1 may interact with each other in vivo. The above hypothesis will be tested in this study.

Interaction between Mutant SOD1 and the Dynein Complex in Cultured Cells—GST pulldown experiments were carried out to test whether mutant SOD1 interacts with the dynein complex. DHC (~500 kDa) is difficult for genetic manipulation, thus DIC, a scaffold between dynein heavy chains and light chains, was selected in this study. WT and mutant SOD1 was tagged with FLAG, and DIC was tagged with GST as shown in Fig. 2A. SOD1-FLAG and GST-DIC were co-transfected in HEK293 cells, the cell lysate was subjected to GST pulldown and the pulldown product was subjected to SDS-PAGE and Western blotting analysis (Fig. 2B). When GST-DIC and WT-SOD1 were co-expressed in HEK293 cells, GST-DIC was pulled

RESULTS

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down by glutathione beads, but WT-SOD1 was not pulled down together with GST-DIC. When GST-DIC and A4V SOD1 were co-expressed, A4V was pulled down together with GST-DIC. As a control, GST and A4V SOD1 were co-expressed, A4V was not pulled down with GST. Thus, A4V SOD1 was pulled down because it interacted with DIC and the GST domain did not contribute to the interaction.

We further tested whether other ALS-linked SOD1 mutants interact with dynein. The same experiment was carried out with G85R SOD1 and G93A SOD1. As shown in Fig. 2C, A4V, G85R, and G93A were all pulled down with GST-DIC, but WT SOD1 was not. Negative controls co-transfecting GST and SOD1 were performed and showed that none of the mutants was pulled down with GST alone (identical to the last lane of Fig. 2B, data not shown), thus the interactions were between mutant SOD1 and DIC, but not an artifact of the GST domain.

To further verify the interaction, reverse co-precipitation experiments were performed. WT or mutant SOD1-FLAG was pulled down using anti-FLAG M2 affinity gel and co-immuno-precipitation of GST-DIC was analyzed by SDS-PAGE and anti-GST Western blotting. As shown in Fig. 2D, DIC was co-precipitated with all three SOD1 mutants A4V, G85R, and G93A, but not with WT SOD1. As a control GST alone was not pulled down with any of the SOD1 mutants (right panel of Fig. 2D), supporting again that the interactions were not caused by the GST domain. The co-precipitation experiments in both directions (Fig. 2C and 2D) strongly support the interaction between mutant SOD1 and DIC.

Furthermore, we demonstrated that the overexpression of GST-DIC did not disrupt the dynein complex and other associated motor proteins. As shown in the supplementary Fig. S1, DHC, DIC, light intermediate chain (DLIC), and light chain (DLC) were all identified by mass spectrometry in the GST-DIC pulldown sample. In addition, p150Glued (dynactin heavy chain, the protein linking dynein and the transported cargo) was pulled down with GST-DIC as shown in the Western blot in Fig. 2C. The results suggest that the dynein complex as well as dynactin complex and other associated proteins involved in retrograde transport remained largely in place when GST-DIC was overexpressed.

**Interactions between Mutant SOD1 and the Dynein Complex in Transgenic Animals**—We further tested whether mutant SOD1 interacted with dynein in three different ALS animal models: G93A SOD1 and G85R SOD1 transgenic mice and G93A SOD1 transgenic rats. Spinal cord and sciatic nerve homogenates were subjected to immunoprecipitation using DIC monoclonal antibody and subsequent Western blotting analyses. In 60-, 90-, and 125-day-old G93A SOD1 transgenic mice spinal cords, DIC was ubiquitously detected as it was
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In addition, similar DIC immunoprecipitation was carried out using spinal cord homogenates of G85R SOD1 transgenic mice and G93A SOD1 transgenic rats. The results in Fig. 3, C and D, demonstrate the interaction between dynein and two different SOD1 mutants (G85R and G93A) in both additional ALS animal models. In all animals examined, neither WT human SOD1 nor endogenous mouse SOD1 was co-precipitated with dynein.

Moreover, we examined whether the interaction between mutant SOD1 and dynein existed outside the motor neuron cell bodies. As shown in Fig. 3E, G93A mutant SOD1 was co-immunoprecipitated by DIC in sciatic nerve homogenate, whereas WT SOD1 in sciatic nerve was hardly co-immunoprecipitated. The data demonstrate that mutant SOD1 can interact with dynein in axons where axonal retrograde transport occurs.

Colocalization between Mutant SOD1 and the Dynein Complex—We examined whether mutant SOD1 and the dynein complex is colocalized in spinal cord motor neurons in transgenic ALS animal models. Fig. 4A shows the fluorescence staining of SOD1 and DHC in the spinal cord motor neurons from WT SOD1 and G93A SOD1 transgenic mice at the ages of 60, 90, and 125 days. Motor neurons in the lumbar spinal cord are distinguished by their large sizes and their localization in the ventral horn in the 4,6-diamidino-2-phenylindole-stained slide as well as the hematoxylin and eosin-stained adjacent section. At all three different ages, the morphology of the motor neurons appears to be healthy in the WT SOD1 transgenic mice. WT SOD1 expression in motor neurons was relatively low, but the distribution of WT SOD1 was rather ubiquitous in the cell bodies without appearance of protein aggregates. There was little colocalization between WT SOD1 and DHC in the spinal cord motor neurons except some random overlap (see first, third, and fifth rows of Fig. 4A). In G93A SOD1 transgenic mice, protein aggregates immunopositive for SOD1 were commonly observed at the boundaries of vacuoles in the motor neurons. Moreover, strong colocalization of G93A SOD1 and DHC was observed in those vacuole-associated protein aggregates (indicated by arrows in the second, fourth, and sixth rows of Fig. 4A). The protein aggregates in motor neurons and the colocalization of G93A SOD1 and DHC all increased over the progression of the disease in the G93A SOD1 transgenic mice.

The colocalization of SOD1 and DIC was also characterized and the results are shown in Fig. 4B. There was little colocalization between WT SOD1 and DIC in the spinal cord motor neurons of WT SOD1 transgenic mice (see the first row in Fig. 4B). The colocalization of G93A SOD1 and DIC in the vacuole-associated proteins aggregates were also evident in the G93A transgenic mouse spinal cord motor neurons (indicated by the arrow in the second and third rows in Fig. 4B).

Furthermore, colocalization of SOD1 and dynein was also investigated in the sciatic nerves of SOD1 transgenic mice. As shown in Fig. 4C, colocalization of strong DHC staining and G93A SOD1 aggregates could be observed at both 60 and 90 days in the sciatic nerves of the G93A SOD1 mice. In contrast, even DHC and SOD1 staining was observed in the sciatic nerves of the WT SOD1 mice. The above results obtained from co-immunostaining of SOD1 and two different dynein subunits (heavy chain and intermediate chain) in both spinal cords and sciatic nerves of the G93A SOD1 transgenic mice demonstrate

Immunoprecipitated by this antibody (Fig. 3A, second row). At all three different ages, G93A SOD1 was co-immunoprecipitated with DIC, whereas WT SOD1 was not (Fig. 3A, top row). Moreover, quantitative results (Fig. 3B) showed that the amount of co-precipitated G93A SOD1 increased during disease progression. Compared with the 60-day-old pre-symptomatic mice, higher levels of co-precipitated G93A SOD1 were observed in the 90-day-old and 125-day-old end-stage mice (* indicated p < 0.02 compared with SOD1 intensity in G93A, 60 days). C, interactions between dynein and G85R SOD1, but not WT SOD1 in G85R transgenic mice. D, interactions between dynein and G93A SOD1 in transgenic rat. E, co-immunoprecipitation of mutant SOD1 and dynein in sciatic nerve from G93A transgenic mice. G93A mutant SOD1 was co-precipitated by DIC in sciatic nerve homogenate, whereas WT SOD1 was hardly co-precipitated. The experiments in C–E were carried out as in A. All experiments were performed three times independently and the representative images are shown.

FIGURE 3. Interactions between the dynein complex and mutant SOD1 in transgenic animals. A, interaction between dynein and G93A SOD1, but not WT SOD1 in the spinal cords of G93A transgenic mice at 60, 90, and 125 days. Mouse spinal cord homogenates were subjected to immunoprecipitation (IP) using a mouse monoclonal DIC antibody. The immunoprecipitation product was resolved in SDS-PAGE and blotted with SOD1, DIC, DHC, and p150Glued antibodies. G93A SOD1 was co-immunoprecipitated with DIC, whereas WT SOD1 was not. The negative control of the left column was hemagglutinin antibody immunoprecipitation of the spinal cord homogenate of a 125-day-old G93A transgenic mouse. B, increased interaction between mutant SOD1 and dynein during disease progression. The amount of SOD1 coprecipitated with DIC at various ages were quantified with Kodak 1D 3.6.1 software. The values are obtained from three independent experiments and are presented as SOD1 intensity (top row) normalized to the immunoprecipitated DIC intensity (second row). The statistics analysis was performed using Student’s t test. * indicated p < 0.02 compared with SOD1 intensity in G93A, 60 days. C, interactions between dynein and G85R SOD1, but not WT SOD1 in G85R transgenic mice. D, interactions between dynein and G93A SOD1 in transgenic rat. E, co-immunoprecipitation of mutant SOD1 and dynein in sciatic nerve from G93A transgenic mice. G93A mutant SOD1 was co-precipitated by DIC in sciatic nerve homogenate, whereas WT SOD1 was hardly co-precipitated. The experiments in C–E were carried out as in A. All experiments were performed three times independently and the representative images are shown.
the colocalization of mutant SOD1 and the dynein complex in protein aggregates in motor neuron cell bodies and axons.

In addition, we tested the colocalization of mutant SOD1 and DIC in the spinal cord of G85R SOD1 transgenic mice. As shown in Fig. 4D, G85R SOD1 and DIC were colocalized in protein aggregates inside motor neurons in the spinal cord of G85R SOD1 transgenic mice. In contrast, WT human SOD1 or endogenous mouse SOD1 was largely not colocalized with DHC or DIC in age-matched WT SOD1 transgenic mice, as shown in Fig. 4, A–C. The colocalization of dynein and an additional ALS-linked mutant A4V was also demonstrated in cultured cells when the GFP-tagged A4V SOD1 and DsRed-tagged DIC were co-expressed in HEK293 cells. The fluorescence microscopic images are shown in supplementary Fig. S2.
DISCUSSION

The results from this study provide evidence that the ALS-linked SOD1 mutants, but not WT SOD1, interact and colocalize with the dynein complex in vitro and in vivo. Our initial cross-linking results identified dynein heavy chain as a component of the mutant SOD1-containing high molecular weight complexes. To eliminate potential ambiguities inherent to the cross-linking methods, we performed independent analyses using co-precipitations in both directions, i.e. GST DIC pull-down followed by SOD1 Western blot (Fig. 2, B and C) and SOD1-FLAG immunoprecipitation followed by DIC Western blot (Fig. 2D) to confirm the interaction between mutant SOD1 and dynein. Moreover, we confirmed the interaction and colocalization of mutant SOD1 and the endogenous mouse dynein complex in multiple transgenic mouse models using immunoprecipitation (Fig. 3) and confocal microscopy (Fig. 4) techniques.

In the co-immunoprecipitation studies, the interaction between mutant SOD1 and dynein was demonstrated in three different transgenic animal models, G93A SOD1 transgenic mouse and rat and G85R SOD1 transgenic mouse (Fig. 3). In the colocalization studies, mutant SOD1 was found to be colocalized with two different dynein subunits, DHC and DIC in protein aggregates in the spinal cord and sciatic nerves of the G93A SOD1 transgenic mice (Fig. 4). The colocalization of mutant SOD1 and DIC in protein aggregates was also demonstrated in G85R SOD1 transgenic mice. In addition, the amount of mutant SOD1 co-immunoprecipitated by DIC and the colocalization of mutant SOD1 and dynein were observed in presymptomatic mice and increased at the disease onset and the later stage of the disease progression (Fig. 3B). The data clearly support the notion that the ALS-linked SOD1 mutants, but not normal SOD1, interact and colocalize with the dynein complex in vitro and in vivo. It remains possible that WT SOD1 may be able to interact with dynein, but with a significantly lower affinity. Thus, no WT SOD1 was clearly detected in the dynein coprecipitation experiments in this study. The significance of the findings in this study is discussed in the context of dynein-mediated retrograde transport, aggregate formation, and motor neuron degeneration in ALS.

Axonal transport is particularly critical to motor neurons because they have long axons. Missense point mutations in the dynein heavy chain result in progressive motor neuron degeneration in heterozygous mice, and in homozygotes this is accompanied by the formation of Lewy-like inclusion bodies (21). Axonal transport has been observed to be defective and slowed in ALS transgenic mice (29, 30). It has been suggested that defects in axonal transport are an early feature of toxicity mediated by mutant SOD1, which subsequently can cause motor neuron degeneration. In addition, point mutations in the p150Glued subunit of dynactin, a dynein-binding protein involved in retrograde transport, have been reported in familial ALS patients (31, 32). Although critically important, it has remained largely unknown how mutant SOD1 causes defects in retrograde axonal transport. The results from this study demonstrate a direct connection between mutant SOD1 and the dynein complex that is essential for retrograde transport. Based on these results, we propose that the misfolded SOD1 mutants acquired an aberrant “gain-of-interaction” with dynein, thus overloading the dynein-mediated retrograde transport and impairing axonal transport in motor neurons, which ultimately leads to motor neuron death. The hypothesis is illustrated in Fig. 5 and is particularly supported by the observation that the interaction and colocalization between mutant SOD1 and dynein occur prior to the disease onset and increase over the course of disease progression in transgenic mice.

The gain-of-interaction between mutant SOD1 and dynein can be a mechanism utilized to form large protein aggregates. The appearance of mutant SOD1-containing aggregates resembles that of aggresomes (6, 17), which rely on microtubule-mediated transport for assembly (43, 44). Aggresomes are colocalized with the microtubule organization center, suggesting that the cell body-directed retrograde transport is involved. Because mutant SOD1 interacts with the dynein complex that is essential for retrograde transport, it is hypothesized that the aberrant interaction is exploited to transport the misfolded mutant SOD1 to form aggregates at the microtubule organization center (see Fig. 5). The hypothesis is supported by the increased colocalization of dynein and SOD1 aggregates in G93A transgenic mice over the disease progression (see Fig. 4). Additional studies from our laboratory, which examine aggregate formation of dynein-interacting and non-dynein-interacting SOD1 proteins, also suggest that the interaction between SOD1 and dynein is critical to the formation of large protein aggregates.4

The results that DHC and the dynactin subunit p150Glued were co-immunoprecipitated in cultured cells (Fig. 2 and supplemental Fig. S1) and mouse spinal cords and sciatic nerves (Fig. 3) suggest that the dynein-dynactin complex and the retrograde transport machinery are not completely disrupted by the aberrant interaction between mutant SOD1 and dynein. It is more likely that the efficiency of the retrograde transport may be reduced when the transport system is overloaded with misfolded mutant SOD1. Indeed, a slower retrograde transport has been reported in ALS transgenic mice (30). A unique difference between spinal cord motor neurons and other cell types is the

4 A.-L. Ström, P. Shi, F. Zhang, and H. Zhu, unpublished data.
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large size and the essential role of axonal transport in motor neurons. Thus, a less efficiently functional retrograde transport may contribute to selective motor neuron degeneration, whereas other cell types may be spared in the same time frame.

The downstream pathways causing motor neuron death remain to be determined. It is possible that large mutant SOD1 aggregates, once formed, can disrupt essential cellular functions and lead to cell death. More interestingly, the gain-of-interaction between mutant SOD1 and dynein can competitively impair the efficient transport of neuronal growth factors that are essential for supporting motor neuron survival. Consequently, a chronically reduced supply of trophic factors could cause the age-related progressive neurodegeneration. This hypothesis is partially supported by recent studies that local delivery of growth factors can delay the disease onset and progression in animal models (45–47). Another alternative mechanism is that mutant SOD1, via its interactions with the dynein machinery, is transported to mitochondria where mutant SOD1 forms aggregate and executes toxicity toward motor neurons. In general support of this hypothesis are the data that mutant SOD1 interacts with Bcl-2 and aggregates on the mitochondrial surface in spinal cord, but not in other tissues such as liver (14, 15).

Other interesting questions rise from the findings of this study and remain to be further investigated. First, it is unclear what forms of mutant SOD1 interact with the dynein complex. It is illustrated in Fig. 5 that misfolded forms of mutant SOD1, such as those with disordered loops related to zinc loss, may preferentially interact with the dynein complex. It remains to be clarified whether monomeric, dimeric mutant SOD1, or the high molecular weight complexes interact with the dynein complex. As discussed earlier, normal WT SOD1 may be able to interact with dynein with an extremely low affinity, thus undetected in most of the co-precipitation experiments. The only exception is that a minute amount of WT SOD1 was observed in the immunoprecipitation of the sciatic nerve lysates (Fig. 3E). In addition, it is possible that the heterodimer of WT and mutant SOD1 may contribute to the interaction as such heterodimers have been previously reported to accelerate the disease progression (48). Second, the interaction between mutant SOD1 and the dynein complex is clearly demonstrated by the pull-down and immunoprecipitation experiments (Figs. 2 and 3), but we cannot exclude the possibility that additional adaptor or molecular chaperone protein may mediate the interaction between mutant SOD1 and dynein. Third, it is unclear which subunit(s) of the dynein complex interacts with mutant SOD1. Two recent studies showed that both Loa and Cra1 mutations in DHC can attenuate the axonal transport defects and extend the lifespan of ALS mice (33, 34). We hypothesize that mutant SOD1 directly interacts with DHC and that the Loa and Cra1 DHC mutants do not interact with mutant SOD1 or with significantly reduced affinity. Consequently, the toxicity induced by the mutant SOD1-dynein interaction would be ameliorated. This would explain the rescued axonal transport and the extended life spans of the Loa/SOD1<sup>G93A</sup> and Cra1/SOD1<sup>G93A</sup> mice. Further studies are needed to identify and characterize the dynein component that directly interacts with mutant SOD1, which will provide new insights regarding mutant SOD1 toxicity.

The finding in this study of an interaction between the dynein complex and the ALS-linked SOD1 mutants suggests that dynein function, known to be critical for normal retrograde axonal transport, could be perturbed directly by mutant SOD1. It is hypothesized that the aberrant gain-of-interaction between mutant SOD1 and dynein facilitate protein aggregate formation and cause defective retrograde transport, which ultimately result in motor neuron degeneration. Further studies are ongoing to test the above hypothesis.

Acknowledgments—We are grateful to Dr. Zuoshang Xu for providing WT, G85R, and G93A SOD1 transgenic mice to establish the colonies in our laboratories. Wyeth and ALS Association are acknowledged for making the G93A SOD1 transgenic rats available through Taconic. Renee Kilty is acknowledged for technical assistance in animal studies and critical reading of the manuscript.

REFERENCES

1. Deng, H. X., Hentati, A., Tainer, J. A., Iqbal, Z., Cayabab, A., Hung, W. Y., Getzoff, E. D., Hu, P., Herzfeldt, B., Roos, R. P., Warner, C., Deng, G., Soriano, E., Smyth, C., Parge, H. E., Ahmed, M., Roses, A. D., Hallevell, R. A., Pericak-Vance, M. A., and Siddique, T. (1993) Science 261, 1047–1051

2. Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O’Regan, J. P., Deng, H. X., Rahman, Z., Križus, A., McKenna-Yasek, D., Cayabab, A., Gaston, S. M., Berger, R., Tanzi, R. E., Halperin, J. J., Herzfeldt, B., Vandenbergh, R., Hung, W.-Y., Bird, T., Deng, G., Mulder, D. W., Smyth, C., Laing, N. G., Soriano, E., Pericak-Vance, M. A., Haines, J. L., Rouleau, G. A., Gusella, J. S., Horvitz, H. R., and Brown, R. H. (1993) Nature 362, 59–62

3. Gaudette, M., Hirano, M., and Siddique, T. (2000) Amyotroph Lateral Scler. Other Motor Neuron Disord. 1, 83–89

4. Bruijn, L. I., Becher, M. W., Lee, M. K., Anderson, K. L., Jenkins, N. A., Copeland, N. G., Sisodia, S. S., Rothstein, J. D., Borchelt, D. R., Price, D. L., and Cleveland, D. W. (1997) Neuron 18, 327–338

5. Bruijn, L. I., Houseweart, M. K., Kato, S., Anderson, K. L., Anderson, S. D., Ohama, E., Reaume, A. G., Scott, R. W., and Cleveland, D. W. (1998) Science 281, 1851–1854

6. Johnston, A. J., Dalton, M. J., Gurney, M. E., and Kopito, R. R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 12571–12576

7. Watanabe, M., Dykes-Hoberg, M., Cullotta, V. C., Price, D. L., Wong, P. C., and Rothstein, J. D. (2001) Neurobiol. Dis. 8, 933–941

8. Wang, J., Xu, G., Gonzales, V., Coonfield, M., Fromholt, D., Copeland, N. G., Jenkins, N. A., and Borchelt, D. R. (2002) Neurobiol. Dis. 10, 128–138

9. Wang, J., Slunt, H., Gonzales, V., Fromholt, D., Coonfield, M., Copeland, N. G., Jenkins, N. A., and Borchelt, D. R. (2003) Hum. Mol. Genet. 12, 2753–2764

10. Ross, C. A., and Poirier, M. A. (2004) Nat. Med. 10, (suppl.) S10–S17

11. Wong, P. C., Pardo, C. A., Borchelt, D. R., Lee, M. K., Copeland, N. G., Jenkins, N. A., Sisodia, S. S., Cleveland, D. W., and Price, D. L. (1995) Neuron 14, 1105–1116

12. Zhu, S., Stavrovskaya, I. G., Drozda, M., Kim, B. Y., Ona, V., Li, M., Sarang, S., Liu, A. S., Hartley, D. M., Wu du, C., Gullans, S., Ferrante, R. J., Przedborski, S., Kristal, B. S., and Friedlander, R. M. (2002) Nature 417, 74–78

13. Menzies, F. M., Cooke, M. R., Taylor, R. W., Turnbull, D. M., Chrzanowska-Lightowlers, Z. M. A., Dong, L., Figlewicz, D. A., and Shaw, P. J. (2002) Brain 125, 1522–1533

14. Liu, J., Lillo, C., Jonsson, P. A., Vande Velde, C., Ward, C. M., Miller, T. M., Subramanian, J. R., Rothstein, J. D., Marklund, S., Anders, P. M., Brannstrom, T., Gredal, O., Wong, P. C., Williams, D. S., and Cleveland, D. W. (2004) Neuron 43, 5–17
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15. Pasinelli, P., Belford, M. E., Lennon, N., Bacskaí, B. J., Hyman, B. T., Trotti, D., and Brown, R. H., Jr. (2004) *Neuron* 43, 19–30

16. Urushitani, M., Kurisu, J., Tsukita, K., and Takahashi, R. (2002) *J. Neurochem.* 83, 1030–1042

17. Zhang, F., and Zhu, H. (2006) *Biochim. Biophys. Acta* 1760, 404–414

18. Vale, R. D. (2003) *Curr. Opin. Cell Biol.* 15, 33, 455–456

19. Guzik, B. W., and Goldstein, L. S. (2004) *EMBO J.* 23, 808–812

20. Holzbaur, E. L. (2004) *Trends Cell Biol.* 14, 233–240

21. Hafezparast, M., Klocke, R., Ruhrberg, C., Marquardt, A., Ahmad-Annuar, A., Bowen, S., Lalli, G., Witherden, A. S., Hummerich, H., Nicholson, S., Morgan, P. J., Oozageer, R., Priestley, J. V., Averill, S., King, V. R., Ball, S., Peters, J., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, M., Toda, T., Yamamoto, A., Hiraoka, Y,