Mutant Copper-Zinc Superoxide Dismutase Binds to and Destabilizes Human Low Molecular Weight Neurofilament mRNA*

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The mechanism by which mutated copper-zinc superoxide dismutase (SOD1) causes familial amyotrophic lateral sclerosis is believed to involve an adverse gain of function, independent of the physiological antioxidant enzymatic properties of SOD1. In this study, we have observed that mutant SOD1 (G41S, G85A, and G93A) but not the wild type significantly reduced the stability of the low molecular weight neurofilament mRNA in a dosage-dependent manner. We have also demonstrated that mutant SOD1 but not the wild type bound directly to the neurofilament mRNA 3'-untranslated region and that the binding was necessary to induce mRNA destabilization. These observations provide an explanation for a novel gain of function in which mutant SOD1 expression in motor neurons alters an intermediate filament protein expression.

Copper-zinc superoxide dismutase (SOD1)† is a small and abundant intracellular metalloenzyme that catalyzes the conversion of the superoxide anion to hydrogen peroxide (1, 2). The demonstration of a genetic linkage between mutations in the SOD1 gene and familial amyotrophic lateral sclerosis aroused interest in the role of SOD1 in motor neuron death (3). The mechanisms by which mutations in the SOD1 gene contribute to the pathogenesis of ALS remain to be defined crisply. A gain of function to the SOD1 enzyme, conferred by the mutation, remains a potential mechanism for this toxicity. Supporting this concept are the observations that many SOD1 mutants maintain normal enzymatic function (4), that SOD1 knock-out mice do not develop an ALS-like phenotype (5), and that the over-expression of wild type (WT) SOD1 in mutant SOD1 transgenic mouse models does not modify the disease state (5). A mechanism reported recently (6) for the underlying mutant SOD1 toxicity proposes that the formation of the monomeric mutant SOD1 protein is the result of oxidative stress, which leads to microaggregate formation. It was also shown that SOD1G93A but not WT co-localizes with neurofilamentous aggregates induced by low molecular weight neurofilament (NFL) overexpression in transfected Neuro2A cells (7).

Neuropathological hallmarks of ALS include the degeneration of the descending corticospinal tracts and spinal, bulbar, and cortical motor neurons (8). In the latter nerves, degenerating neurons characteristically contain a variety of inclusions, including neurofilamentous aggregates. Such neurofilamentous aggregates have been observed in a number of transgenic mouse models of motor neuron degeneration in which the stoichiometry of expression of the individual neurofilament (NF) subunits has been altered. These include alterations in the level of expression of each of the neurofilament subunit proteins (NFL and middle and high molecular weight neurofilaments) (9–11) and related intermediate filament proteins (12). It has been shown previously (13–15) by both in situ hybridization and single cell RT-PCR that the level of expression of NFL mRNA is reduced selectively in degenerating spinal motor neurons in ALS, suggesting that the alterations of NF stoichiometry are relevant to the disease process of ALS.

We observed recently (16) that the stability of human NFL (hNFL) mRNA was determined through mRNA-binding proteins interacting with the 3'-untranslated region (3'-UTR) of the hNFL mRNA and that these binding proteins were expressed differentially between control and ALS-derived spinal cord homogenates. It has been reported (15) that the transfected human SOD1G93A significantly deregulates steady state mRNA levels of NFL in the motor neuron hybridoma cell line (NSC34). The results suggest that the mutant SOD1 is associated directly with an alteration in the stoichiometry of NF expression, providing a link between mutant SOD1 and the formation of NF aggregates. In the present studies, we found that mutant SOD1 but not WT bound directly to hNFL mRNA on its 3'-UTR and led to an enhanced rate of mRNA degradation. This is the first report of mutant SOD1 functioning as a trans-acting mRNA-binding protein, which provides a novel gain of function for mutant SOD1 that leads directly to an alteration of neurofilament mRNA stoichiometry.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—NSC34 cells were grown as suggested previously (17) and transfected with Lipofectamine 2000 (Invitrogen). Stable transfections were selected by the constant presence of 1 mg/ml Geneticin (Invitrogen) and screened for high level expression using immunoblotting.

Cloning and Expression of hNFL Genes—Because of the limitations in the length of an RNA probe for RNA-protein binding assays, the last 1000 nt of hNFL mRNA, including the 3'-untranslated region (hNFL-1000), which contains the putative major stability regulatory elements (18), was RT-PCR amplified from an ALS patient RNA sample and cloned into pcDNA3.1(+) as the representative for the full-length hNFL (16). hNFL cDNA devoid of the 3'-untranslated region (hNFL-CDs) was also amplified and cloned.

SYBR Green Real Time Quantitative RT-PCR—Relative quantitative
PCR was performed using the SYBR Green method. By adopting one primer from the vector sequence, transfected hNFLs was differentiated from the endogenous homologous murine NFL gene.

Establishment of Tetracycline Tightly Controlled SOD1 Expression in the NSC34 Cell Line—First, NSC34 cells were transfected with pTet-Off vector (Clontech) and selected by the constant presence of 1 mg/ml Geneticin. Colonies with a high expression level of tTA were screened indirectly by measuring neomycin resistance gene expression with real time PCR. pTRE2 (Clontech) constructs with WT or G93A SOD1 coding regions were transfected subsequently into these selected colonies together with pTK-Hyg (Clontech) and selected by the constant presence of 1 mg/ml Geneticin and 150 μg/ml hygromycin B (Sigma). Tet-responsive suppression of SOD1 by doxycycline (Clontech) was monitored by using real time PCR assays and comparing the human SOD1 mRNA level from cultures with or without 2 μg/ml doxycycline.

Gel Shift, Supershift, and Cross-link Assays—In order to demonstrate the specific binding of SOD1 to hNFL mRNA, we developed a SOD1-hNFL mRNA binding system using gel retardation assays. hNFL 4×106 cpm 32P-labeled RNA probe in 20 μl buffer was incubated with 1 μg of sheared poly(rC)·poly(rG) as a nonspecific competitor or 2 μg of unlabeled hNFL or SOD1 transcripts competes for the binding sites for SOD1. The reaction mixture was incubated at room temperature for 1 h, 5 μl of unlabeled hNFL transcripts were added. Then, 4 μl of 5× sample buffer and 5 μl of DMSO were added, and a 15% polyacrylamide gel was loaded and electrophoresed. The gel was stained with ethidium bromide and photographed under ultraviolet light. The autoradiograph taken with Fuji X-ray film was analyzed using a densitometric scanner.

RESULTS

Mutant SOD1 Destabilizes hNFL mRNA—We developed stable transfections of the NSC34 cells with wtSOD1, SOD1G41S, SOD1G93A, or with pcDNA3.1(+) vector only. These cell lines were further transfected transiently with pcDNA/hNFL-1000 together with pEGFP-C1 (Clontech) (the latter served as a normalizing control for transfection efficiency when checked with flow cytometry). No significant difference was observed in transfection efficiency among different stably transfected NSC34 cells (data not shown). Total RNA was extracted using RNAeasy mini columns (Qiagen). Actin mRNA levels were used as the normalizing control for equal starting amounts of material. hNFL mRNA levels were reduced significantly in SOD1G41S- or SOD1G93A-transfected NSC34 cells compared with those in wtSOD1 or vector-transfected (p < 0.001). To determine whether this reflected an effect on the stability of hNFL mRNA as opposed to an alteration in transcriptional activity, we then suppressed RNA synthesis by adding 1 mg/ml actinomycin D. Stably transfected SOD1 NSC34 cells were further transfected with pcDNA/hNFL-1000 for 24 h, and actinomycin D was added for the indicated period of time. hNFL mRNA levels were measured using real time PCR (Fig. 1B). hNFL mRNA demonstrated a more rapid decay in the presence of mutant SOD1 (G41S and G93A) than with WT (p < 0.01). We also used a tetracycline-controlled SOD1 expression system to further demonstrate the dosage-dependent destabilization of mutant SOD1 on hNFL mRNA. The expression of heterogeneous WT or G93A SOD1 was inhibited by the presence of 2 μg/ml doxycycline and further transfected with pcDNA/hNFL-1000. After 12 h, cells were detached and suspended in fresh medium in 4 wells of a 6-well plate. The final concentration of doxycycline for each well was adjusted to 2, 1, 0.5, and 0 μg/ml, respectively.
respectively. Cells were harvested after 24 h, homogenized, and divided into two aliquots. One aliquot was loaded onto a SDS-polyacrylamide gel for protein level detection by anti-SOD1 Western blot (Fig. 2A), and the other was used for total RNA extraction and real time PCR to check the hNFL mRNA level. The hNFL mRNA amount was plotted against the corresponding SOD1 protein level (Fig. 2B), revealing that the level of hNFL mRNA was irrelevant to the levels of co-expressed human WT SOD1 protein levels, it decreased continuously with the increasing level of SOD1G93A protein. The results are representative of three independent replications.

Mutant SOD1 Binds hNFL mRNA Directly and within Its 3′-UTR—The wtSOD1, SOD1G41S, SOD1G85A, or SOD1G93A cDNA was cloned in-frame into pHM6 plasmid, producing hemagglutinin (HA)-tagged SOD1 proteins on transfection of the NSC34 cells, which were co-transfected with pcDNA/hNFL-1000. Protein was extracted 24 h after transfection and mixed with anti-HA affinity matrix (overnight at 4 °C), and HA-tagged SOD1 protein was immunoprecipitated. Following washing with lysis buffer, binding elements to SOD1 protein were released from the agarose beads by boiling and centrifugation. The supernatant was then used as the template for reverse transcription and real time PCR to quantify the levels of hNFL mRNA that had been co-precipitated with HA-tagged SOD1 protein. We observed that mutant SOD1 (G41S, G85A, and G93A) had significantly higher affinity in binding hNFL mRNA compared with the WT (Fig. 3B). We further examined the binding by UV cross-linking (Fig. 4A), gel shift, and supershift assays (Fig. 4B) and observed the loss of the initial binding band in the supershift assay with the presence of anti-SOD1 antibody but no predicted super-shifted band from the incorporation of the antibody. It is possible that the binding sites for hNFL mRNA on the SOD1 protein may have been blocked by antibody, thus entirely eliminating the probe binding.

The major ribonucleoprotein binding and regulatory sequence on murine NFL mRNA is located in the 3′-UTR of the mRNA (18, 19), suggesting that possible binding sites for mutant SOD1 on hNFL could also fall into that region. We assessed this possibility with North-Western blotting (Fig. 5A), using radiolabeled hNFL-1000 and hNFL-CDS RNA probe and observed a significant reduction in the affinity of hNFL mRNA without 3′-UTR to mutant SOD1 proteins. Densitometry results (Fig. 5B) suggested that the locations of the major binding sites for mutant SOD1 on hNFL mRNA were in the 3′-untranslated region. Similar results were obtained from three independent replications.

Mutant SOD1 Destabilizes the hNFL mRNA through Its 3′-UTR—To demonstrate that the binding on its 3′-UTR by mutant SOD1 causes the instability of hNFL mRNA, we cloned hNFL 3′-UTR after a stop codon 3′ to the green fluorescence protein gene in pEGFP-C1 vector and transfected the construct into stably transfected SOD1 NSC34 cells. An irrelevant sequence of the same length was amplified from an ampicillin resistance gene complementary sequence on pcDNA3.1 vector and cloned as a scramble sequence after a stop codon 3′ to cross-linking (Fig. 4A), gel shift, and supershift assays (Fig. 4B) and observed the loss of the initial binding band in the supershift assay with the presence of anti-SOD1 antibody but no predicted super-shifted band from the incorporation of the antibody. It is possible that the binding sites for hNFL mRNA on the SOD1 protein may have been blocked by antibody, thus entirely eliminating the probe binding.

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green fluorescence gene in pEGFP-C1 vector, serving as a negative control. Wild type, G41S, or G93A SOD1 stably transfected NSC34 cells were further transiently transfected with pEGFP/hNFL-3'UTR-H11032-UTR or pEGFP/scramble. Cells were harvested 24 h after transfection, and the expression of eGFP reporter gene was measured with flow cytometry (Fig. 6A). Median values of green fluorescence intensity (Fig. 6B) showed that the expression of the eGFP reporter gene tagged with 3'UTR of hNFL mRNA was reduced significantly with the presence of mutant SOD1 (G41S or G93A) compared with WT. In contrast, the expression of reporter eGFP gene tagged with scramble sequence stayed steady regardless of the presence of either WT or mutant SOD1. The results shown were representative of three independent replicas.

**DISCUSSION**

Our experiments demonstrated for the first time that mutant SOD1 but not WT interacts directly with human hNFL mRNA. A, UV cross-link assays showing the formation of a binding complex (arrow) on 32P-labeled hNFL mRNA probe from mutant but not WT human SOD1-transfected NSC34 homogenates. B, to confirm the involvement of SOD1 protein in the binding complex, NSC34 protein homogenates were incubated with hNFL RNA probe with or without prior incubations with anti-SOD1 antibody (supershift assay). In the presence of the antibody, the intensity of the initial binding banding (arrow) was reduced dramatically.

The role for such regulatory proteins in modulating NF mRNA stability has been well established. Murine NFL mRNA cis-acting elements are present in the 3'-coding region (3'-CR) and 3'-UTR of the NFL mRNA (18, 19). Deletion of the 3'-UTR or 527 nt of the 3'-CR increased the stability of murine NFL mRNA compared with the full-length transcript in cell lines. Deletion of both the 3'-UTR and 3'-CR led to a further stabilization of the transcript. A major stability determinant was then localized to a 68-nt sequence that forms the junction between the 3'-CR and 3'-UTR of NFL.

The regulation of the stability of labile mRNAs through unique trans-acting binding proteins is recognized increasingly as a key pathway in the regulation of gene expression. For instance, the stability of both c-myc and c-fos is modulated by the formation of ribonucleoprotein complexes between AU-rich cis elements and cellular RNA binding factors (20–22). Neuron-specific AU-rich cis element-binding proteins, including HuD, HuC, and HuR, have been shown to bind with high affinity to AU-rich cis elements of mRNA involved in cellular...
growth and differentiation (23). HuD regulates the stability of the microtubule protein tau mRNA (24) such that the treatment of PC12 cells with antisense oligonucleotide to HuD inhibits NGF differentiation and neuritic outgrowth. HuR, the ubiquitous embryonic lethal abnormal vision (ELAV) member, has been shown to modulate the stability of mRNAs involved in cell growth and angiogenesis, including interleukin-8, tumor necrosis factor-α, and vascular endothelial growth factor (25–28).

Multiple trans-acting binding protein factors to the murine major NFL mRNA stability determinant region were then identified by screening the rat brain cDNA expression library with the 68-nt RNA as a probe. Among factors with binding affinity to the probe is the p190RhoGEF (29), which has important functions in neural differentiation and maturation via the activation of Rho GTPases. It has a highly interactive C-terminal domain, providing potential connections to multiple pathways in the cell. p190RhoGEF has been shown to bind to an untranslated, differentiated neuron-specific BC1 RNA (30) and to proteins such as 14-3-3 (31) and focal adhesion kinase (32).

The consequences of mutant but not WT SOD1 protein altering the stability of the NFL mRNA remain to be defined. Although the most obvious consequence of this process would be to alter the stoichiometry of NF protein expression in a fashion known to be associated with both ALS and motor neuron degeneration, the reduction in NFL mRNA may also have indirect effects. In this case, by reducing the abundance of hNFL mRNA because of the direct destabilization of the mRNA, higher levels of endogenous free binding factors that would otherwise have bound to the available mRNA could be induced. Alternatively, the binding of mutant SOD1 itself to hNFL mRNA may induce the release of competing trans-acting binding factors such as p190RhoGEF from hNFL mRNA. In both of these postulates, mutant SOD1 may potentially alter the availability of these RNA binding factors to interact with other binding targets. This effect, termed a “transdominant effect,” is exemplified by myotonic dystrophy type 1 (DM1) (33). DM1 is the most common form of muscular dystrophy in adults and is dominantly inherited. The mutation responsible for DM1 is a CTG expansion located in the 3′-UTR of the dystrophia myotonica-protein kinase gene (DMPK) (34–38). Although the DMPK gene knock-out mouse does not develop features typical of DM1 (39, 40), a transgenic mouse with an untranslated CUG repeat in an unrelated mRNA (actin) develops myotonia and myopathy, suggesting that transcripts with expanded CUG repeats are sufficient to generate a DM phenotype (41). It has
been reported that mutant DMPK RNA binds and sequesters up to 90% of the observed transcription factors, leading to a subsequent reduction in the expression of multiple genes, including the ion transporter CIC-1 that has been implicated in myotonia (42). In this case, the transcription factors sequestered by mutant DMPK mRNA are transdominant mediators. This hypothesis of a transdominant effect for mutant SOD1 could have an additional advantage in explaining the prefer-

**FIG. 6.** Mutant SOD1 (G41S or G93A) destabilizes eGFP reporter gene mRNA via the interaction with 3' tagged with hNFL mRNA 3'-UTR. A, SOD1 stably transfected NSC34 cells were co-transfected with eGFP reporter gene that was 3'-tagged with either hNFL 3'-UTR sequence or a scramble sequence of the same length from ampicillin resistance gene. Flow cytometry was employed 24 h after transfection to determine the green fluorescence intensity as a measure of eGFP reporter mRNA stability. B, the graph shows the median value of green fluorescence intensity from each group. Comparing the presence of mutant SOD1 (G41S or G93A) with WT, green fluorescence median values were significantly lower from hNFL 3'-UTR-tagged eGFP. No effect was observed using scramble sequence-tagged eGFP.
ential vulnerability of motor neurons in ALS, despite the ubiquitous expression of the mutant SOD1, because the ultimate damage may be mediated by interacting binding factors and not by the mutant SOD1 itself. Both BCI RNA and p190RhoGEF could be considered candidates for this purpose because both have neuron-specific expression. Expressions are currently in place to examine this hypothesis.

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