Copper-binding-site-null SOD1 causes ALS in transgenic mice: aggregates of non-native SOD1 delineate a common feature

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Cu/Zn superoxide dismutase (SOD1), a crucial cellular antioxidant, can in certain settings mediate toxic chemistry through its Cu cofactor. Whether this latter property explains why mutations in SOD1 cause FALS has been debated. Here, we demonstrate motor neuron disease in transgenic mice expressing a SOD1 variant that mutates the four histidine residues that coordinately bind Cu. In-depth analyses of this new mouse model, previously characterized models and FALS human tissues revealed that the accumulation of detergent-insoluble forms of SOD1 is a common feature of the disease. These insoluble species include full-length SOD1 proteins, peptide fragments, stable oligomers and ubiquitinated entities. Moreover, chaperones Hsp25 and aB-crystallin specifically co-fractionated with insoluble SOD1. In cultured cells, all 11 of the FALS variants tested produced insoluble forms of mutant SOD1. Importantly, expression of recombinant peptide fragments of wild-type SOD1 in cultured cells also produced insoluble species, suggesting that SOD1 possesses elements with an intrinsic propensity to aggregate. Thus, modifications to the protein, such as FALS mutations, fragmentation and possibly covalent modification, may simply act to augment a natural, but potentially toxic, propensity to aggregate.

INTRODUCTION

Mutations in Cu/Zn SOD1 have been linked to familial amyotrophic lateral sclerosis (FALS) (1), a degenerative disease characterized by loss of lower and upper motor neurons in the motor cortex, brain stem and spinal cord (2). Over 70 different missense substitutions at more than 50 residues of the 153 amino acid protein have been described in individuals and kindreds affected by SOD1-linked FALS (www.alsod.org). A small number of mutations lead to early translation termination in the last of the five exons. Although intensely studied, the nature of the common abnormality by which all these mutations in SOD1 cause FALS has not been definitively identified. Most investigators accept the notion that the SOD1 mutations cause the disease through a gain-of-property mechanism (3–7), but just what this toxic property is has not be resolved.

One hypothesis has focused on the metal binding properties of Cu/Zn SOD1. The ‘Cu hypothesis’ holds that mutations diminish the normal shielding of the Cu cofactor in the enzyme, enhancing entry of less favored substrates including H₂O₂ and *ONO.O. Reactions between the Cu cofactor and H₂O₂ can produce potentially toxic radical species (8–11) whereas reaction with *ONO.O can catalyze the covalent nitration of tyrosine residues (12–14). However, whether these reactions are central to the toxicity of mutant SOD1 has been debated (15,16).

Another hypothesis suggests that aggregation of SOD1 may selectively injure motor neurons. Abnormal inclusions have been observed in human ALS spinal cords from both familial and sporadic cases (17–19), in spinal cords from transgenic mice expressing FALS variants (7,19), and in cytoplasm of cultured COS cells, or motor neurons, expressing FALS-SOD1 variants (20,21). Detergent-insoluble forms of mutant SOD1 have been detected in cultured cells and mice expressing FALS-SOD1 variants (22–24). The principal criticisms of this hypothesis are that relatively few mutants have been examined in model

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systems, and in some of the transgenic mouse models, SOD1 positive inclusions in affected spinal cords are relatively rare (19,24).

To test the Cu hypothesis, we have focused on disease associated mutations that occur in the Cu-binding site of SOD1. There are four histidines that coordinate bind Cu [histidines 46, 48, 63, and 120 (25)], and disease causing mutations have been described at histidines 46 and 48 (H46R and H48Q). Experimental mutants of SOD1 that combine these two mutations can induce motor neuron disease in transgenic mice (26). To eliminate the Cu binding site, we combined the two disease-causing mutations at histidine 46 and 48 with two experimental mutations at histidines 63 and 120 (H63G and H120G), yielding a protein (SOD1-Quad) that was surprisingly stable but completely inactive. When we expressed this variant in mice, via a mutated version of the human genomic fragment of the sod1 gene (27), we observed motor neuron disease similar in clinical and pathological appearance to other mouse models of SOD1-linked FALS. We interpret this outcome as evidence that motor-neuron specific toxicity does not require a normally configured active site to produce a protein toxic to motor neurons. In analyzing diseased tissues from mice expressing SOD1-Quad, we found evidence of aggregated forms of mutant protein by both size exclusion filter assay, and differential detergent extraction and centrifugation. We utilized the latter approach to compare aggregating SOD1 species in this new model to four other previously characterized models, demonstrating that SOD1-Quad acquires characteristics similar to natural FALS-SOD1 mutants. We also developed cell culture assays to examine 11 different FALS variants, finding that misfolding to produce detergent insolubility is a common feature. We suggest that misfolded and aggregating species of SOD1 are strong candidates for the toxic species in the disease.

RESULTS

Motor neuron disease and SOD1 aggregates in H46R/H48Q/H63G/H120G mice

Previous characterizations of SOD1 reactivity with H2O2 and -ONOO have dealt with enzymes where Cu is loaded into its normal active site (8–11,13,14,28). There are four histidine residues that coordinate bind Cu, residues 46, 48, 63 and 120 (25). Mutations at histidines 46 and 48 have each been reported in FALS patients (www.also.org), and mice expressing an SOD1 with a double mutation at histidines 46 and 48 (H46R/H48Q) develop motor neuron disease (26). Enzymes harboring H46R and H48Q mutations alone, or in combination, lack detectable superoxide scavenging activity (26). Hence, partial destruction of the Cu-binding site in SOD1 renders the enzyme inactive, while retaining the capacity to cause motor neuron disease.

To completely destroy the Cu-binding site, we combined the H46R/H48Q double FALS mutation with additional mutations at histidines 63 and 120 (H46R/H48Q/H63G/H120G; the Quad mutant). Two lines (87 and 125) were identified that developed typical motor neuron disease, which progressed to hindlimb paralysis by 8–12 months of age (Fig. 1A and B). The levels of SOD1-Quad expression that were required to induce disease at these ages were similar to that of other FALS-SOD1 variants (Fig. 1D). In a native gel assay of SOD1 activity in spinal cords of non-transgenic mice (NTg), mice expressing high levels of wild-type human SOD1 (WT), and mice expressing SOD1-Quad, no bands corresponding to human SOD1 homodimers was neither diminished in its intensity nor altered in its migration in the SOD1-Quad mice, suggesting the SOD1-Quad protein may not dimmerize with the mouse enzyme.

Pathological examination of symptomatic SOD1-Quad mice demonstrated marked motor neuron loss (by silver impregnation) and dramatically increased glial fibrillar acidic protein (GFAP) immunostaining (Supplementary Material Fig. S1). Spinal cords and brain stems from affected mice also showed conspicuous inclusion body pathology, which was revealed by immunostaining with ubiquitin antibodies or thioflavin-S staining (Fig. 2 and Supplementary Material Fig. S2). These pathologies were absent in cerebral cortex, cerebellum, or other parts of the forebrain. As controls, age-matched wild-type SOD1 transgenics and non-transgenic littermates remained healthy and free of abnormal inclusions. Although immunostaining with SOD1 antibodies failed to detect obvious inclusions (data not shown), by size exclusion filter assay aggregated species of SOD1-Quad were abundant in homogenates of spinal cord and brain stem tissues from paralyzed SOD1-Quad mice (Fig. 2E). These structures were detected in cerebellum and cerebral cortex, but at much lower levels.

Figure 1. Transgenic mice expressing H46R/H48Q/H63G/H120G (Quad) mutant SOD1 develop motor neuron disease. (A) Hindlimb paralysis in SOD1-Quad mice (line 125). (B) SOD1-Quad mice (lines 125 and 87) developed complete hind limb paralysis at the age of 9–12 months, and 8–11 months, respectively. (C) Mice expressing SOD1-Quad do not produce excess superoxide scavenging activity. Only activity associated with endogenous SOD1 equal to that in nontransgenic (NTg) control mice is detected; 100 μg of protein from spinal cord homogenates of 2-month-old mice were tested for SOD1 activity in the native gel assays. (D) Disease progression is correlated with protein expression levels in different lines of SOD1-Quad mice; the higher the expression level (line 87>125>121), the earlier the onset of paralysis. A similar dose dependency for disease onset in mice expressing other SOD1 mutants including, the H46R/H48Q mutant (e.g. line 139>58), has been noted previously (26). Protein levels were determined by western blotting of spinal cord homogenates from 2-month-old mice.
The appearance of motor neuron disease and aggregated species of mutant SOD1 in the SOD1-Quad mice suggested to us that aggregation of the mutant protein may be the key to disease. To characterize further the SOD1 species accumulating in the symptomatic mice, we used differential detergent extraction and high speed centrifugation to enrich for forms of the mutant protein in non-native structure (see Materials and Methods). Proteins insoluble in non-ionic detergent (P2) and SDS (P3) were then solubilized by boiling in the SDS sample buffer before being analyzed by western blotting with two polyclonal antibodies: hSOD1 antibody, a peptide antiserum binding to amino acids 24–36 (not conserved between mouse and human SOD1); and m/hSOD1 antibody, a peptide antiserum recognizing amino acids 124–136 (completely conserved between mouse and human protein; Fig. 3A).

Spinal cord extracts from five different strains of FALS mice were examined; G37R (line 29), G85R (line 164), G93A (line 1—high expression), H46R/H48Q (line 139), and SOD1-Quad (line 125). As compared with either non-transgenic mice, or mice expressing high levels of wild-type human SOD1 (line 76), spinal cords from the paralyzed mutant mice contained large amounts of detergent-insoluble SOD1 species (Fig. 3A). However, the majority of both wild-type and mutant SOD1, from all animals, was soluble in non-ionic detergent [Fig. 3B; the soluble (S1) fraction contained ~600 μg total protein of which only 1 μg was analyzed by SDS-PAGE; the P2 fraction contained ~50 μg of total protein of which 3 μg was analyzed by immunoblot—see legend to Fig. 3].

Although the predominant SOD1 species solubilized after boiling in SDS consisted of full-length SOD1 monomers (open arrow), there were distinct immunoreactive peptide fragments (solid arrow) and high molecular weight (solid arrowhead) species in these insoluble fractions (Fig. 3A). Notably, the G85R variant always migrates, in SDS–PAGE, slightly faster than wild-type SOD1 or other FALS variants (4). This characteristic was also evident in 28 and 38 kDa species detected in the G85R mice. Interestingly, detergent-insoluble, SOD1-immunoreactive species in the spinal cords of the paralyzed mice displayed patterns that were not entirely identical when examined by immunoblot with the two different antibodies. The hSOD1 antibody recognized, with variable abundance, a set of ~7 kDa fragments that were particularly abundant in the H46R/H48Q mice. Additionally, there were several species that appeared to be slightly smaller than the full-length protein; these fragments were not recognized by the m/hSOD1 antibody, suggesting that these fragments lack C-terminal residues. By contrast, the m/hSOD1 antibody specifically recognized several fragments in the range of 9–15 kDa; these fragments were particularly abundant in spinal cords from the G85R and H46R/H48Q mice. Both antibodies recognized a pair of prominent SOD1 species (marked by asterisks), which have relative molecular masses of ~28 and ~38 kDa. Note that the hSOD1 antibody detected, at lower abundance, additional species just below these 28 and 38 kDa species and since these were not recognized by the m/hSOD1 antibody we infer that these molecules are truncated at the C-terminus.

Next, we examined spinal cord tissues from an FALS patient known to harbor an SOD1-A4V mutation, together with two non-SOD1 FALS patients, 12 sporadic ALS patients, and three controls. Only tissue homogenates from the patient harboring the A4V mutation showed enhanced levels of detergent-insoluble SOD1 monomers, fragments, and high molecular weight species (Fig. 3C). By contrast, little or no insoluble SOD1 was detected in spinal cord tissues from patients with sporadic and non-SOD1 familial ALS (Supplementary Material Fig. S3).

To determine whether covalent ubiquitination of any of these SOD1 species might be contributing to the complexity of the SOD1-immunoreactive profiles seen in immunoblots probed
with SOD1 antibodies, we probed immunoblots of the SDS insoluble pellet (P3) with monoclonal antibodies to ubiquitin. In all five of the SOD1 FALS models we examined, we found that the insoluble pellets contain ubiquitinated entities including two species with apparent molecular mass of mono and di-ubiquitinated mutant SOD1 (Fig. 4A). To confirm whether ubiquitinated SOD1 was indeed present in these fractions, we dissociated the P2 pellet fraction by boiling in 2% SDS, and then diluted the solubilized protein to 0.2% SDS and immunoprecipitated SOD1 protein with the m/hSOD1 antibody. Immunoprecipitated proteins were then subjected to immunoblot with ubiquitin antibody (Fig. 4B), noting the detection of two prominent proteins with relative molecular masses of 28 and 38 kDa (Fig. 4B), which are roughly the predicted sizes for mono- and di-ubiquitinated SOD1 proteins.

**FALS mutations promote non-native folding to form detergent-insoluble complexes**

As noted above, and from our previous work (24), high-molecular weight, detergent-insoluble, mutant SOD1 is most abundant in tissues that develop visible pathology: brain stem and spinal cord. In this setting, it is impossible to determine whether the formation of these altered species of SOD1 is an inherent property of the protein or consequence of the disease process. To address this issue, we studied the detergent solubility of different SOD1 mutants expressed at high levels in cultured cells. Wild-type SOD1 and 11 FALS mutant cDNAs [A4V, G37R, G41D, H46R, H48Q, H46R/H48Q, G85R, G93C, L113T, Quad, and FS126 (frame shift 126–stop 131)] were inserted into the pEF-BOS expression vector (4) before transient transfection of human HEK-293 cells. Northern analysis of the transfected cultures showed similar SOD1 mRNA levels (data not shown) for each variant. As expected, the accumulated steady-state levels of the different mutants varied according to the intrinsic half-life of the individual proteins (Fig. 5A) (4,29).

Twenty-four hours after transfection, cells were harvested and differentially extracted as described in Materials and Methods. The non-ionic detergent-insoluble pellets (P2) and supernatant (S1) were separated, and SOD1 levels were quantified by western blotting with the hSOD1 antibody and [125]I-labeled secondary antibodies, followed by Phosphorimager detection and quantification (Fig. 5A and B). All of the cells transfected with FALS variants contained forms of mutant SOD1 that were insoluble in non-ionic detergents (Fig. 5A) and SDS (data not shown). Notably, however, wild-type human SOD1 was barely detectable in the insoluble fractions, even though the wild-type protein accumulated to the highest steady-state level in the soluble fraction. Comparing the relative amounts of SOD1 in the pellet and supernatant fractions, we found that each mutant has a statistically higher aggregation potential than wild-type SOD1 (Fig. 5B). Interestingly, the A4V, G41D, G85R and FS126 variants, all short-lived (4,29), produced more detergent insoluble species than any of the other mutants. The FS126 variant, the only truncated protein, appeared to be the most aggregation prone. These results strongly suggest that FALS variants are prone to adopting non-native conformations that lead to detergent-insoluble structures, and that this property is intrinsic to the mutants and does not require extrinsic events such as may occur in degenerating and diseased tissues.

Intrigued by the appearance of detergent insoluble fragments of SOD1 in the mouse and human tissues (Fig. 3), we used the cell culture transfection system to ask whether these molecules may also be inherently prone to adopt non-native...
conformations. We examined the aggregation potential of several experimental variants of wild-type SOD1 truncated at the N- and C-terminus to approximate the sizes of fragments observed in mouse and human tissues. Although the steady-state levels of each recombinant fragment were much lower than wild-type protein, each fragment produced detergent insoluble species (Fig. 6). These results support the notion that structural elements in SOD1 have an unusually high potential to aggregate, and that in the wild-type protein these elements must be effectively ordered in the native structure to avoid aggregation.

**Up-regulation of Hsp25 and αB-crystallin in symptomatic SOD1 mutant mice**

Because molecular chaperones are crucial participants in protein folding, degradation and aggregation, we used a panel of antibodies against heat shock proteins to determine whether any of the known chaperones were up-regulated in the spinal cords of FALS mice. Using non-ionic detergent extraction protocols described above, we also asked whether any of these chaperones were also associated with the detergent-insoluble SOD1 complexes (Fig. 7). Although we found no appreciable differences between control and symptomatic mice in analyzing the levels and solubility of Hsp40, Hsp60, Hsp70 and Hsp90, we observed a dramatic up-regulation in the levels of Hsp25. In affected mice from five different SOD1 mouse models, the level of Hsp25 was elevated in both the soluble and insoluble fractions. In the soluble fraction, Hsp 25 appeared as a doublet band, whereas in the pellet fraction only a single species of HSP25 was detected (Fig. 7A). Another small chaperone protein, αB-crystallin, was found to be significantly up-regulated in the mutant mice, with the majority of the induced component being localized to the detergent-insoluble fraction. Because these two proteins are known to form highly organized structures (30), we do not know whether they are integral components of SOD1 aggregates. However, both chaperones co-fractionated with SOD1 aggregates in the SDS insoluble fraction P3 (data not shown).

Immunocytochemical examination of paralyzed mice localized Hsp25 to both neuronal and glial cells and αB-crystallin to oligodendrocytes. In neither case, were neuropil inclusions (recognized by ubiquitin antibodies) specifically marked (data not shown). Hence, we are uncertain as to whether these chaperones are directly binding protein contained in large visible inclusions, or protein organized in smaller structures not visible by light microscopy.

**DISCUSSION**

Our demonstration of pathologic and behavioral symptoms of motor neuron disease in strains of mice expressing SOD1-Quad proves that disease can be induced by mutant forms of SOD1 lacking the histidine residues that coordinately bind Cu in the active site. The pathologic features of disease in the SOD1-Quad mice were identical to that of other FALS-SOD1 mice; these features include ubiquitin-immunoreactive inclusions, thioflavin-S positive inclusions, robust astrogliosis, and loss of motor neurons. As we have previously demonstrated in other FALS-SOD1 mouse models (24), diseased tissues contained high molecular weight forms of SOD1-Quad that could be trapped by size exclusion filter trap. Similarly, behavioral phenotypes were identical; initial symptom of hindlimb weakness progressing to hindlimb paralysis before involvement of forelimbs. Thus by all criteria we have used to characterize FALS-SOD1 mice, the SOD1-Quad mice appear to have authentic motor neuron disease.

**Have we definitively disproved the Cu hypothesis?**

The Cu hypothesis is an umbrella term that covers hypotheses that involve the ability of SOD1 to covalently nitrate tyrosine residues in protein (12) or to react with H₂O₂ to produce radical species (8). These toxic reactions have been described for...
wild-type SOD1, with evidence that FALS mutations augment these activities. The nitration chemistries attributed to both wild-type SOD1 (28) and FALS variants (13,14) by other investigators were described in enzymes with intact active sites. There is no evidence that inactive enzymes can catalyze these reactions.

Similarly, the peroxidative activity of SOD1 was originally described for wild-type SOD1 with Cu bound in the active site (9,31). As was the case for the nitration reaction, mutations distal to the active site were found to augment the peroxidative reaction (8,10). Moreover, enzyme harboring the H48Q mutation within the active site was found to retain the peroxidative chemistry. Although altering one of the histidine residues responsible for binding Cu is not sufficient to eliminate this chemistry, there is no evidence that enzyme lacking all four of the histidine residues necessary for the coordinate binding of Cu could perform the peroxidative chemistry.

When one sums all of the studies that have tested the Cu-hypothesis by experimentally lowering the level of Cu binding in the active site [the present study, the work by Subramanian et al. (15) to delete CCS and diminish the loading of Cu into mutant SOD1, and our previous study of mice expressing a double histidine mutant (H46R/H48Q) (26)], then the weight of evidence does not favor the hypothesis as it pertains to Cu correctly loaded in the Cu-binding pocket. It remains to be determined whether Cu bound to other parts of the protein (the Zn site or elsewhere) could produce SOD1 enzymes that are capable of generating the same type, or some other type, of toxic chemical reaction (16).

Are aggregated forms of mutant SOD1 the toxic species?

We have examined, in great detail, five different mouse models of SOD1-linked FALS and found that disease is always associated with the accumulation of high molecular weight forms of mutant SOD1 that are retained by size exclusion filtration [Fig. 2 and (24)]. We have also found that the tissues that are most devastated in mice (brain stem and spinal cord) contain non-native, detergent insoluble, species of mutant protein. We have never observed full-length wild-type protein to possess these properties. Further, in a cell culture assay, we have found that of 11 different FALS mutants tested, all were found to be inherently prone to adopt non-native, detergent-insoluble, structures. In many other neurodegenerative disease
Elevated levels of Hsp25 were observed in spinal cords from all mutant SOD1 mice, but not in cords from mice expressing wild-type SOD1 or from NTg controls. Mutant SOD1 mice and normal control mice were extracted in non-ionic detergent before immunoblot analysis with antibodies specific to Hsp25. Substantially Hsp25 was up-regulated in both supernatant and detergent-insoluble pellet fractions. (a) B-crystallin was located in the detergent-insoluble fractions. Although we have not been able to visualize large SOD1-immunoreactive aggregates in the FALS mice with our SOD1 antibodies, we have noted that affected tissues contain large amounts of thioflavin-S positive inclusions. Thioflavin-S is thought to be relatively specific for β-sheet structures and will recognize β-amyloid peptide aggregates and aggregated forms of tau protein (39,40). The abundance of detergent insoluble forms of SOD1 in the brain stem and spinal cord of mice that show thioflavin-S positive inclusions leads us to believe that aggregated forms of mutant SOD1 are one of the targets for this dye in tissue sections from the FALS mice.

Features of the SOD1 FALS mouse models also implicate aggregation in the toxic process. Studies of β-peptide and polyglutamine protein aggregation have demonstrated that aggregation is a slow process where nucleation and seeding is the slowest component of the process, which is highly influenced by the concentration of the monomeric species (41–44). In studies of several lines of transgenic mice expressing varied levels of SOD1-G37R (6) and SOD1-H46R/H48Q (26), a strong correlation between the age of onset and the expression level of mutant proteins was observed. Moreover, in the G37R (6), G93A (3), H46R/H48Q (26) and SOD1-Quad mice, hyperexpression of the mutant protein was required to induce the disease. Although sometimes criticized as a flaw of these models, the requirement for high-level expression fits well with the notion that aggregation is crucial; at lower levels of expression the thermodynamics of the aggregation process would preclude any appreciable nucleation before mice reach their natural life expectancy. It is noteworthy that mice expressing the G85R variant develop disease, accompanied by aggregated species of mutant SOD1, when the steady state levels of protein are much lower—equal to or less than endogenous mouse SOD1 (7,24). Our demonstration that G85R variant has one of the highest aggregation potentials appears to explain how this mutant appears to possess a greater toxicity per unit of protein than other FALS variants tested in mice.

In our view, there is much evidence that SOD1-FALS shares features common to other neurodegenerative diseases where the aggregation of specific proteins is a pathologic hallmark. However, just what the toxic species is in these protein aggregation diseases has been hotly debated (reviewed in 45). Are the large aggregates the toxic species, or are there small oligomeric structures that are the real culprits? We note that, in every mouse model we have looked at, aggregated forms of mutant SOD1 increase in abundance as symptoms worsen (24). However, it remains possible that the large aggregates are biomarkers for another species of toxic protein whose detection is more elusive.

In our study of heat shock response in FALS mice, we noted that Hsp25 and β-crystallin were dramatically induced in the spinal cords of affected mice but that antibodies to these proteins did not decorate the large ubiquitin-positive inclusions that are prominent in affected spinal cords of the FALS mice (data not shown). It is possible that these chaperones are associated with much smaller SDS-insoluble structures; these small heat shock proteins are known to bind to proteins...
in non-native conformations (46). The cellular pattern of staining by antibodies to Hsp25 and zB-crystallin suggested that non-neuronal cell types, astrogia in the case of Hsp25 and oligodendrocytes in the case of zB-crystallin, were the major sources of these proteins. Hence, from these data we could argue that something other than a large aggregate has induced a response in non-neuronal cells.

One of the attractive aspects of the Cu-hypothesis was the notion that the gained-property (or activity) by SOD1 when carrying FALS mutations need not involve the acquisition of some entirely new function. Because wild-type SOD1 could, at low levels, catalyze toxic peroxidative and nitration activities (9,10,28), the disease associated mutations need only augment these natural secondary activities for the protein to acquire toxicity (8,12). Here, we demonstrate, through study of defined peptide fragments of wild-type SOD1, that there are elements in SOD1 that are prone to induce aggregation. Hence, once again we could argue that the FALS mutations simply augment a natural, potentially toxic, feature of the protein.

**Ubiquitin inclusion pathology in FALS mice**

The accumulation of ubiquitinated protein inclusions are hallmark pathologies of many neurodegenerative diseases (reviewed in 47). The appearance of ubiquitinated inclusions has been construed as evidence that there may be diminished proteasome function. A direct test of this possibility in a cell culture paradigm demonstrated that cells containing large aggregates of polyglutamine protein have lower proteasome function (48). We and others have noted that ubiquitin immunoreactive inclusions are frequently observed in spinal cords of paralyzed FALS mice (6,7,19,26). Our results indicate that most of the accumulated ubiquitin-immunoreactive material in the affected tissues of our mice fractionates in the detergent insoluble fraction (Fig. 4A). Some of this ubiquitin is conjugated to SOD1 protein (Fig. 4B), and thus it is possible that the ubiquitin-immunoreactive inclusion pathology seen in the mouse models reflects the abundance of ubiquitinated SOD1 in aggregates, which are likely to be poorly degraded by the cell. Whether the appearance of the large ubiquitinated inclusions reflects a general dysfunction of the ubiquitin/proteasome system is unclear.

**Why do FALS variants of SOD1 tend to aggregate?**

One simple mechanism could involve interactions between specific domains exposed only when non-native structures are adopted. An example of this type of interaction has been termed domain swapping (49,50). If FALS mutations were to heighten spontaneous unfolding of the protein, then alternative conformations could form oligomeric species. We have noted that the distribution of disease-causing mutations is not entirely random. Mutations are most preponderant at residues conserved across many phyla and occur at a much higher frequency in β-strand domains (Wang and Borchelt, unpublished data), suggesting that these mutations may destabilize native structures. Consistent with this notion, Rodriguez et al. (51) reported that 14 FALS mutants have decreased thermal stability, a property augmented when Cu and Zn were removed (52). Thermal stability was also eroded by eliminating the single disulfide bond in the protein (53). In an unfolded state, interactions between hydrophobic β-strands could occur between two individual molecules rather than within individual molecules in a domain-swapping type of interaction (49,50). In such a scenario, highly stable oligomeric molecules could form (Fig. 8).

It is possible that ubiquitination or fragmentation of SOD1 increases its potential to form aggregating structures. Ubiquitinated forms of mutant SOD1 were detected in the detergent-insoluble fraction of affected spinal cords from each of the FALS mouse models tested. However, in our cell culture assay, ubiquitinated forms of SOD1 were less prevalent in the detergent insoluble fraction. Thus, how ubiquitination may affect solubility is not clear. By contrast, it appears that fragmentation can certainly change the solubility of the protein. The FS126, truncation, variant was the most aggregation-prone of all variants tested. Experimentally truncated variants of wild-type SOD1 likewise demonstrated very high aggregation potential. Therefore it is possible that fragmentation of mutant SOD1 may actually potentiate aggregation.

**Does aggregation potential correlate with a clinical aspect of SOD1-linked FALS?**

In studies of SOD1-linked FALS, it has been noted that disease onset is highly variable, even within a particular kindred, but disease severity (as indicated by duration) appears to be more predictable by the location of the mutation (54). However, with the exception of the A4V and D90A mutations, the number of affected individuals with a given mutation is usually quite small. It has been noted that patients harboring the A4V mutation tend to have very short durations (<2 years) (55–57), suggesting a more severe mutation. Likewise, patients harboring the FS126 mutation also show short duration (~3 years) (58). Both of these mutants show very high aggregation potential in our cell culture assay. However, disease of short duration has also been noted in patients with the H48Q (59) and I113T (54–57) mutations, variants that appear to have a lower aggregation potential in our cell culture assay. Notably, the measured half-lives of the H48Q and I113T variants are substantially longer than that of the A4V and FS126 variants (4,29). We note that, in our cell culture assay, the strongest correlate to aggregation potential was the measured half-life of the individual variant. The FS126 truncation variant, which has the shortest recorded half-life (<5h) (29), displayed the highest aggregation potential. Other short-lived variants, including the A4V, G41D and G85R variants, along with the FS126 variant, clearly segregated into a distinct group of proteins that were much more prone to form detergent-insoluble structures (Fig. 5). In previous studies, we have established that these four variants exhibit the shortest half-lives when expressed in cultured cells (29,60). If the accumulation of mis-folded, aggregating, SOD1 is a key pathologic process, then disease severity may be due to a complex interaction between the half-life of the protein and its potential to aggregate. What is most striking to us is that proteins with relatively short half-lives have the greatest aggregation potential. Hence, it becomes easier to explain how these unstable proteins could possess toxicity that is equivalent to the longer-lived variants.
CONCLUSIONS
We demonstrate that an experimental variant of SOD1, lacking all crucial residues for the coordinate binding of Cu, induces an ALS-like disease in mice that is indistinguishable from the disease phenotypes of mice expressing natural FALS variants of SOD1. Disease in these mice, as well as all other models of FALS mice expressing human SOD1, is accompanied by the accumulation of high-molecular-weight SOD1 aggregates, and non-native, detergent-insoluble species of mutant protein. In all of these respects, the SOD1 models of FALS bear striking resemblance to other neurodegenerative diseases where the accumulation of mis-folded and aggregating protein culprits features in the pathogenesis of disease.

MATERIALS AND METHODS
Transgenic mice, human tissues and cell culture models
The H46R/H48Q/H63G/H120G mutation was engineered into a 12 kb fragment of human genomic DNA, encompassing the entire SOD1 gene (27), by PCR-based oligonucleotide primer-directed mutagenesis. Each coding exon and at least 50 bp of intronic sequences on either side of each exon were subsequently verified by sequence analysis before injection into mouse embryos (C3H/HeJ × C57BL/6J F2). Transgenic founder mice were identified by PCR amplification of DNA extracted from tail biopsies. Lines were maintained by crossing transgenic males to non-transgenic (C57BL/6J × C3H/HeJ F1) females (Jackson Laboratories, Bar Harbor, ME, USA), the G85R variant (line 164) (7), the G37 variant (line 29) (6), the H46R/H48Q variant (lines 139 and 58) (26), and the wild-type protein (line 76) (6). The animal use protocol was approved by the Animal Care and Use Committee of The Johns Hopkins Medical Institutions.

Human post-mortem spinal cord tissues from a patient harboring the A4V variant, two familial ALS patients with unknown causes, 12 patients with sporadic ALS, and three control cases were obtained from Johns Hopkins Alzheimer’s Disease Research Center (n = 12) and J. Rothstein at Johns Hopkins University, School of Medicine (n = 6). HEK 293 cells were transfected with expression plasmids encoding engineered human SOD1 cDNAs. All point mutations and fragments were generated based on PCR strategies and expressed by the pEF-BOS vector (61); many of the constructs used here have been described previously (4). Disease mutant FS126 comprises a 2 bp deletion at codon 126 resulting in a shift in reading frame and termination five amino acids downstream from residue 125 (58). Confluent (90%) HEK 293 cells, cultured by standard procedures in 60 mm wells, were transfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and harvested 24 h later for analyses as described below.

Differential detergent extraction and centrifugation
Tissues and cultured cells were homogenized in 1× TEN (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, and 100 mM NaCl); (for tissues, volume : weight = 10 : 1; for 60 mm culture dishes, 100 µl lysis buffer per well). Tissue homogenates or cell lysates were mixed at 1 : 1 with TEN (1× TEN; 1% Nonidet P40; proteinase inhibitor cocktail 1 : 100 dilution (P 8340, Sigma, St Louis, MO, USA)). The mix was sonicated [50% output for 30 s with a probe sonicator (70 W; TEKMAR, Cincinnati, OH, USA)] and then centrifuged at >100 000g for...
5 min to separate supernatant S1 and pellet P1 in a Beckman Airfuge. The P1 pellet was washed with buffer B (1 × TEN; 0.5% Nonidet P40) by sonication (50% for 30 s), and centrifuged at >100,000g for 5 min to obtain pellet P2, which was resuspended in buffer C (1 × TEN; 0.5% Nonidet P40; 0.5% deoxycholic acid; 0.25% SDS). The P2 fraction was further extracted by sonication (50% for 30 s) and centrifugation (>100,000g for 5 min) to sediment P3, which was resuspended by buffer D (1 × TEN; 0.5% Nonidet P40; 0.5% deoxycholic acid; 2% SDS).

Western blotting and immunoprecipitation
Proteins were fractionated by SDS–PAGE on either 8–16% Tris-glycine or 16.5% tricine–peptide (for SOD1 fragments) Criterion gels (Bio-Rad, Hercules, CA, USA). Samples were boiled for 5 min in Laemmli sample buffer containing 2.5% β-mercaptoethanol. SOD1 was detected by immunoblotting with rabbit polyclonal antibody hSOD1 (7), or rabbit polyclonal antibody m/hSOD1 (4). A 125I-labeled donkey-anti-rabbit antibody (Amersham, Piscataway, NJ, USA) was used in a fashion similar to a standard immunoblot, before enhanced chemiluminescence detection.

Histopathology and immunocytochemistry
Mice anesthetized with ethyl ether were sacrificed by transcardial perfusion with 1 × PBS (pH 7.4), followed by 4% paraformaldehyde in 1 × PBS. Brains and spinal cords were removed, post-fixed in the same fixative, embedded in paraffin, and sectioned for histologic and immunologic staining. Sagittal sections of the brain and brain stem and cross sections of spinal cord (10 mm) were stained with hematoxylin and eosin, silver impregnation and thioflavin-S. Deparaffinized sections were processed for immunocytochemistry, using antibodies against ubiquitin (DAKO, Carpenteria, CA, USA). The immune reaction was visualized with diaminobenzidine, and sections were counterstained with hematoxylin.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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