

Cysteine 111 Affects Aggregation and Cytotoxicity of Mutant Cu,Zn-superoxide Dismutase Associated with Familial Amyotrophic Lateral Sclerosis*

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Converging evidence indicates that aberrant aggregation of mutant Cu,Zn-superoxide dismutase (mutSOD1) is strongly implicated in familial amyotrophic lateral sclerosis (FALS). MutSOD1 forms high molecular weight oligomers, which disappear under reducing conditions, both in neural tissues of FALS transgenic mice and in transfected cultured cells, indicating a role for aberrant intermolecular disulfide cross-linking in the oligomerization and aggregation process. To study the contribution of specific cysteines in the mechanism of aggregation, we mutated human SOD1 in each of its four cysteine residues and, using a cell transfection assay, analyzed the solubility and aggregation of those SOD1s. Our results suggest that the formation of mutSOD1 aggregates are the consequence of covalent disulfide cross-linking and non-covalent interactions. In particular, we found that the removal of Cys-111 strongly reduces the ability of a range of different FALS-associated mutSOD1s to form aggregates and impair cell viability in cultured NSC-34 cells. Moreover, the removal of Cys-111 impairs the ability of mutSOD1s to form disulfide cross-linking. Treatments that deplete the cellular pool of GSH exacerbate mutSOD1s insolubility, whereas an overload of intracellular GSH or overexpression of glutaredoxin-1, which specifically catalyzes the reduction of protein-SSG-mixed disulfides, significantly rescues mutSOD1s solubility. These data are consistent with the view that the redox environment influences the oligomerization/aggregation pathway of mutSOD1 and point to Cys-111 as a key mediator of this process.

More than 100 different mutations in the gene encoding Cu,Zn-superoxide dismutase (SOD1)² have been causally linked to familial amyotrophic lateral sclerosis (FALS), and

many of these mutants (mutSOD1s), when introduced in the genome of rodents, were able to produce the progressive degeneration of cortical, bulbar and spinal motor neurons typical of the human disease (1). Many mutSOD1s have been characterized *in vitro*, and they appeared to differ widely in their biochemical and biophysical properties, thus making difficult the identification of their shared toxic properties (2). However, aggregation is emerging as the common fate shared by all the mutants in pathological conditions. Aggregation of the mutant proteins has been described in neuronal cells transfected with a large set of mutSOD1s (3, 4), and mutSOD1 is indeed a major component of inclusions found both in various transgenic mice and in human patients with FALS (5). Whether aggregation is the primary mode of pathogenesis, is an issue that still remains to be resolved.

Compelling evidence has accumulated that abnormal disulfide cross-linking plays a pivotal role in the mechanism of mutSOD1 aggregation. Disulfide-linked mutSOD1 oligomers have been described in the spinal cord of symptomatic ALS transgenic mice (6) as well as in the mitochondrial fraction of both spinal cord of ALS transgenic mice and cultured motor neuronal-derived cells (3, 7), and disulfide-reduced mutSOD1 accumulated in CNS of transgenic ALS mice (8), suggesting that an incorrect handling of cysteines may underlie SOD1 protein oligomerization. Human SOD1 has four cysteine residues that have different reactivities: Cys-111 is exposed on the protein surface near the dimer interface, Cys-6 is packed tightly within the interior of the β -barrel, and Cys-57 and Cys-146 are involved in the disulfide bridge. In this study, we have investigated the contribution of cysteine residues in the mechanism of aggregation of wild-type and mutant human SOD1. Using a cell transfection assay for SOD1 solubility and aggregation we highlight a previously unappreciated role for Cys-111 in affecting *in vivo* mutSOD1 aggregation and cell toxicity. Our data further indicate that the redox state of the cell may represent a key factor in regulating the folding stability of both native and non-native, aggregated SOD1, and suggest that an approach based on GSH-mediated reduction of disulfides in aggregated SOD1, possibly targeting Cys-111, should be considered as a therapeutic strategy to counteract neurodegeneration in the pathogenesis of SOD1-linked FALS.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The tetracycline-responsive vectors pTRE-wtSOD1 or G93A, A4V, and H46R mutSOD1s were

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² The abbreviations used are: SOD1, Cu,Zn-superoxide dismutase; ALS, amyotrophic lateral sclerosis; FALS, familial ALS; mutSOD1, mutant Cu,Zn-superoxide dismutase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; GEE, cell-permeable ethyl ester form of reduced glutathione; Grx, glutaredoxin.

described elsewhere (3). GFP-wtSOD1 or GFP-G93A SOD1 were a kind gift of Zuoshang Xu (University of Massachusetts Medical School). For the mutagenesis of hSOD1 cysteine residues, PCR site-directed mutagenesis was performed, using pTRE2-wtSOD1 and G93A, A4V, or H46R SOD1, or GFP-wtSOD1 and GFP-G93A SOD1 as templates, followed by digestion with Dpn1. The following primers were used: C6F: forward 5'-ACTAAAGCTGTGTTCTGCTGAAGGGCGAC-3'; reverse 5'-GTCGCCCTTCAGCACGAACACAGCTTTAGT-3'; C6S: forward 5'-ACTAAAGCTGTGTCCGTGCTGAAGGGCGAC-3'; reverse 5'-GTCGCCCTTCAGCACGGACACAGTTTGTAGT-3'; C57S: forward 5'-TAATACAGCAGGCTCTACCAAGTGCA-GGTCC-3'; reverse 5'-GGACCTGCACTGGTAGAGCCTGCTGTATTA-3'; C111S: forward 5'-TCTCAGGAGACCATTCC-ATCATTGGCCGCA-3'; reverse 5'-TGCGGCCAATGATGGAATGGTCTCCTGAGA-3'; C146R: forward 5'-GGAAGTCG-TTTGGCTCGTGGTGTAATTGGG-3'; reverse 5'-CCCAATT-ACACCACGAGCCAAACGACTTCC-3'; and C146S: forward 5'-GGAAGTCGTTTGGCTAGTGGTGTAATTGGG-3'; reverse 5'-CCCAATTACACCACTAGCCAAACGACTTCC-3'. Double, triple, and quadruple cysteine mutants were produced from single, double, and triple cysteine mutants, respectively.

Mouse glutaredoxin-1 (*glrx1*, accession number NM053108) was cloned by reverse transcription-PCR from a mouse brain cDNA, using the forward primer 5'-AAAAAGCTTATGGCT-CAGGAGTTTGTGAAC-3' and the reverse primer 5'-AAAG-GATCCTTATAACTGCAGAGCTCCAATC-3'. The resulting PCR fragment was inserted into HindIII/BamHI restriction sites of pcDNA3 (Invitrogen). All the plasmid constructions were verified by automated sequencing.

Cell Culture and Transfection—Mouse motoneuronal cell line NSC-34 stably transfected with the pTetON plasmid (NSC-34/pTetON clone 7), coding for the reverse tetracycline controlled transactivator, were described elsewhere (3). Cells were grown in Dulbecco's modified Eagle's/F-12 medium supplemented with 10% fetal calf serum tetracycline free (FCS Tet-free, Cambrex), at 37 °C in an atmosphere of 5% CO₂ in air. Transfection for transient expression of each vector (1.5 μg of DNA/3 × 10⁵ cells) was obtained with Lipofectamine Plus reagent (Invitrogen) according to manufacturer's instruction. In the experiments with Grx-1, 0.5 μg of pTRE2-SOD1s plasmids was cotransfected with 1.5 μg of Grx-1 plasmid. After a 3-h incubation with transfection reagents, cells were shifted in normal growth medium. Induction of SOD1 expression was obtained by adding to culture medium 1 μg/ml doxycycline for 48 h.

SOD1 Solubility and Aggregation Assay—5–7 × 10⁵ cells were scraped off the plate in culture medium, collected by centrifugation, washed in PBS, and resuspended in 70 μl of PBS containing a protease inhibitor mixture (Sigma). 100 mM iodoacetamide (Sigma-Aldrich) was used to avoid uncontrolled oxidation of cysteine residues. Cell suspension was subjected to three freeze/thaw cycles in liquid nitrogen and centrifuged at 20,000 × *g* for 10 min at 4 °C. The supernatant was collected as the soluble fraction, whereas the pellet (insoluble fraction) was washed in PBS and resuspended in 70 μl of Laemmli sample buffer (62 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue). For the detergent-solubility experiments, the insoluble fractions were resus-

pending in buffer A (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 100 mM NaCl, protease inhibitor mixture) containing either 0.5% Nonidet P-40 or 2% SDS. In this last case, lysates were heated to 100 °C for 5 min. Lysates were centrifuged at 20,000 × *g* for 10 min, and the supernatants were collected as detergent-soluble fractions. Protein content was determined using the BCA protein assay (Pierce).

Electrophoresis, Western Blotting, and Immunofluorescence Analysis—Standard SDS-PAGE was performed as described (3). Western blot was performed onto nitrocellulose membranes (Amersham Biosciences). For the analysis of SOD1 oligomers, fractions were resuspended in Laemmli sample buffer without β-mercaptoethanol. After electrophoresis the gels were soaked for 30 min in SDS-PAGE running buffer containing 5 mM Tris(2-carboxyethyl)phosphine (Molecular Probes) and blotted onto polyvinylidene difluoride membranes (Millipore), as described by others (6).

After incubation in Tris-buffered saline (TBS) solution containing 0.1% Tween 20 and 5% nonfat milk, filters were incubated for 2 h at room temperature with the indicated antibodies diluted in a 2% nonfat milk, 0.1% Tween 20/TBS solution. Immunoreactive SOD1 was detected with a rabbit polyclonal anti-SOD1 antibody (Stressgen), which detects both human and mouse SOD1. The GFP epitope was detected with a rabbit polyclonal anti-GFP antibody (Santa Cruz Biotechnology). For the analysis of voltage-dependent anion channel expression level, the mouse monoclonal antibody anti-voltage-dependent anion channel (Calbiochem) was used. A goat anti-Grx1 antibody (R&D Systems) was used to detect Grx-1. Following extensive washing in 0.1% Tween 20/TBS solution, filters were incubated with the appropriated peroxidase-conjugated secondary antibodies, washed in 0.1% Tween 20/TBS solution and developed using the POD chemiluminescence detection system (Roche Applied Science). Image analysis and quantifications were performed by using the Kodak Image Station (KDS IS440CF 1.1) with 1D Image Analysis software.

For immunofluorescence analysis of SOD1 aggregates, cells cultured in 35-mm Petri dishes were washed in PBS and fixed with 4% paraformaldehyde in PBS for 10 min. Fixed cells were washed in PBS followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min. Cells were blocked for 1 h in 2% horse serum in PBS and incubated for 1 h at 37 °C with mouse monoclonal anti-SOD1 antibody (clone S.D.-G6, Sigma-Aldrich). Cells were washed in blocking buffer and incubated for 1 h with an Alexa Fluor 488 goat anti-mouse antibody (Molecular Probes). After rinsing in PBS, cells were stained with 1 μg/ml Hoechst 33342 (Sigma-Aldrich) and examined under a reflected fluorescence microscope (BX51, Olympus) equipped with an F-View digital camera and the Cell-F Digital Imaging Software. Fluorescence images were processed using Adobe Photoshop.

Determination of Glutathione Content—Determination of intracellular glutathione levels was performed in high-performance liquid chromatography as previously described (9).

Cell Viability Assay—Cell viability was assessed by colorimetric assay using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 Aqueous one solution assay, Promega),

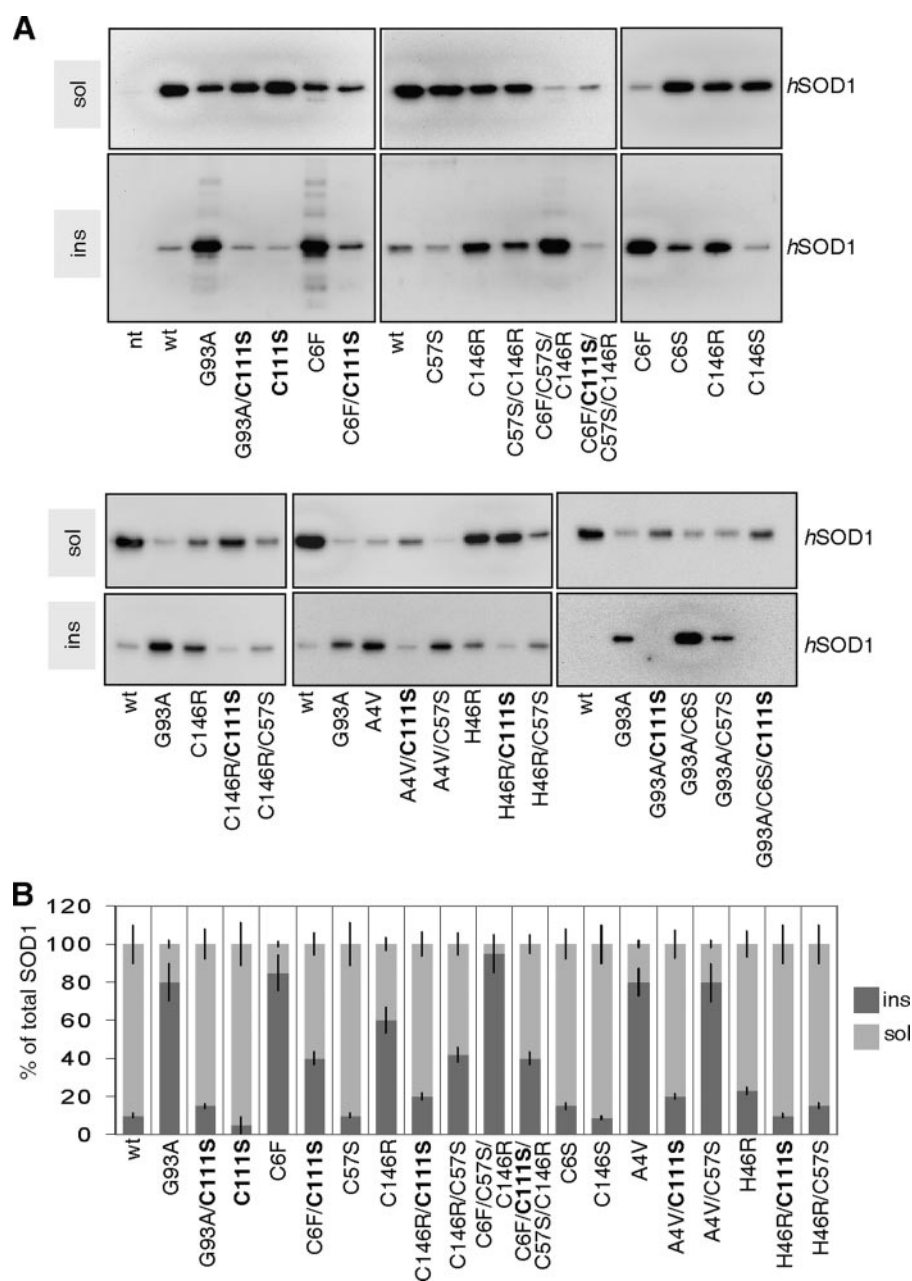


FIGURE 1. The effect of cysteine mutations on the solubility of human SOD1. A, NSC-34/pTetON cells were transfected with pTRE plasmids coding for wtSOD1 or the indicated mutSOD1s. To induce the expression of SOD1, 1 μ g/ml doxycycline was added. After 48 h of culture, insoluble (ins) and soluble (sol) fractions were isolated as described under "Experimental Procedures." Equal volumes from each fraction were subjected to standard, reducing SDS-PAGE and analyzed by Western blotting with an antibody anti-SOD1. Transient transfection elicits high expression levels of human SOD1, and endogenous mouse SOD1 is detectable only after longer exposure. nt, untransfected cells. One typical experiment is shown. B, histogram of the distribution of SOD1s between the soluble and insoluble fractions in NSC-34 cells as determined in $n = 4$ independent Western blot experiments. The total expression level was considered for each line as 100%, and the soluble and insoluble percentages from data in densitometric arbitrary units are expressed as mean \pm S.D.

accordingly to manufacturer's instructions. Absorbance at 490 nm was measured in a multilabel counter (Victor3-V, PerkinElmer Life Sciences).

RESULTS

Cysteine 111 Affects mutSOD1 Aggregation and Toxicity—To study the specific contribution of cysteine residues in the mechanism of SOD1 aggregation, we mutated human SOD1 in each

of its four cysteines (cysteines 6, 57, 111, and 146) and tested the ability of these mutations to affect SOD1 solubility in a cell transfection assay for SOD1 aggregation. Serine was chosen to substitute for Cys as a conservative mutation, whereas the mutation C6F and C146R were chosen because they have been associated with FALS. After transfection, cells were mechanically lysed in a buffer without any detergent, and the soluble and insoluble fractions isolated, subjected to reducing SDS-PAGE and analyzed by Western blotting. When any Cys residue of wtSOD1 is substituted with Ser, solubility of SOD1 is comparable to wtSOD1; however, both the FALS-associated G93A and C6F mutSOD1s were strongly insoluble (Fig. 1, A and B). For both mutSOD1s, high molecular weight species immunoreactive to anti-SOD1 antibodies are detected, as well as low molecular weight fragments, which have been described in transgenic mice and patients (4). Also, a large proportion of A4V and C146R mutSOD1s is accumulated in an insoluble form, while a lesser, but significant, fraction of the H46R mutSOD1 was insoluble (Fig. 1, A and B). Interestingly, the removal of Cys-111 was sufficient to shift all the FALS-associated mutSOD1s analyzed toward a soluble form (Fig. 1, A and B). On the contrary, a serine substitution of Cys-57 or Cys-6 had no effect on the insolubility of mutSOD1s. Moreover, the elimination of both the disulfide cysteines did not affect C6F behavior, whereas again C111S mutation was able to recover the solubility of the insoluble triple mutant C6F/C57S/C146R, although the quadruple cysteine mutSOD1 was much less abundant in cell extracts than the other mutSOD1s, most probably

because of a major effect on overall protein stability.

The insoluble fraction of G93A mutSOD1 was not solubilized by non-ionic detergents, because it sediments in an insoluble form even when treated with 0.5% Nonidet P-40, a condition that was able to solubilize the transmembrane mitochondrial protein voltage-dependent anion channel (Fig. 2), whereas insoluble mutSOD1 could be almost completely recovered under strong denaturing conditions, such as boiling in

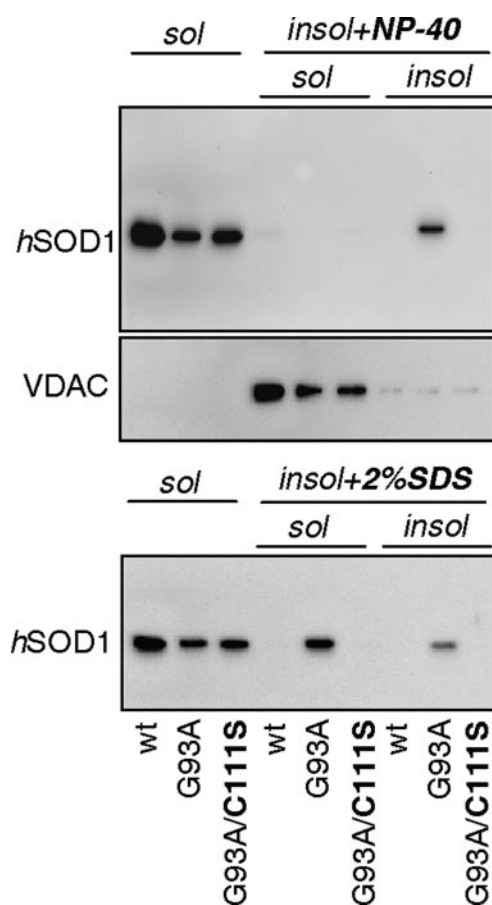


FIGURE 2. MutSOD1 insolubility is reversed by extraction with SDS, but not with non-ionic detergents. NSC-34/pTetON cells were transfected with pTRE plasmids coding for the indicated SOD1s in the presence of 1 μ g/ml doxycycline. Cells were mechanically lysed in a buffer without any detergents, and the soluble and insoluble fractions were isolated. Insoluble fractions were then resuspended in a buffer containing 0.5% Nonidet P-40 (upper panel) or 2% SDS (lower panel). In this last case, lysates were heated to 100 $^{\circ}$ C for 5 min. Detergent-insoluble proteins were resuspended in Laemmli sample buffer. Equivalent amounts of each fraction were subjected to standard, reducing SDS-PAGE and analyzed by Western blotting with an anti-SOD1 antibody or an antibody recognizing the transmembrane mitochondrial protein voltage-dependent anion channel.

2% SDS (Fig. 2). Similar results were obtained with all mutSOD1s used in this study (data not shown).

Mutation of Cys-111 was also tested *in vivo* for its ability to inhibit the formation of intracellular aggregates by mutSOD1s. NSC-34 cells were transfected with wtSOD1 or mutSOD1s in a normal or mutated cysteine background. As shown in Fig. 3, the expression of mutSOD1s induced the appearance of intracytoplasmic inclusion bodies in NSC-34 cells (Fig. 3, A and B), with the notable exception of cells transfected with the H46R mutant, where inclusions are not significantly more abundant compared with those that occasionally appear in cells overexpressing wtSOD1 (not shown), suggesting the existence of a threshold for insoluble mutSOD1 to form structures in a morphologically detectable aggregate. The number of aggregates is significantly reduced in cells transfected with mutSOD1s lacking Cys-111, whereas again, the removal of Cys-6 or Cys-57 was not able to inhibit the formation of inclusions by the G93A mutant (Fig. 3, A and B).

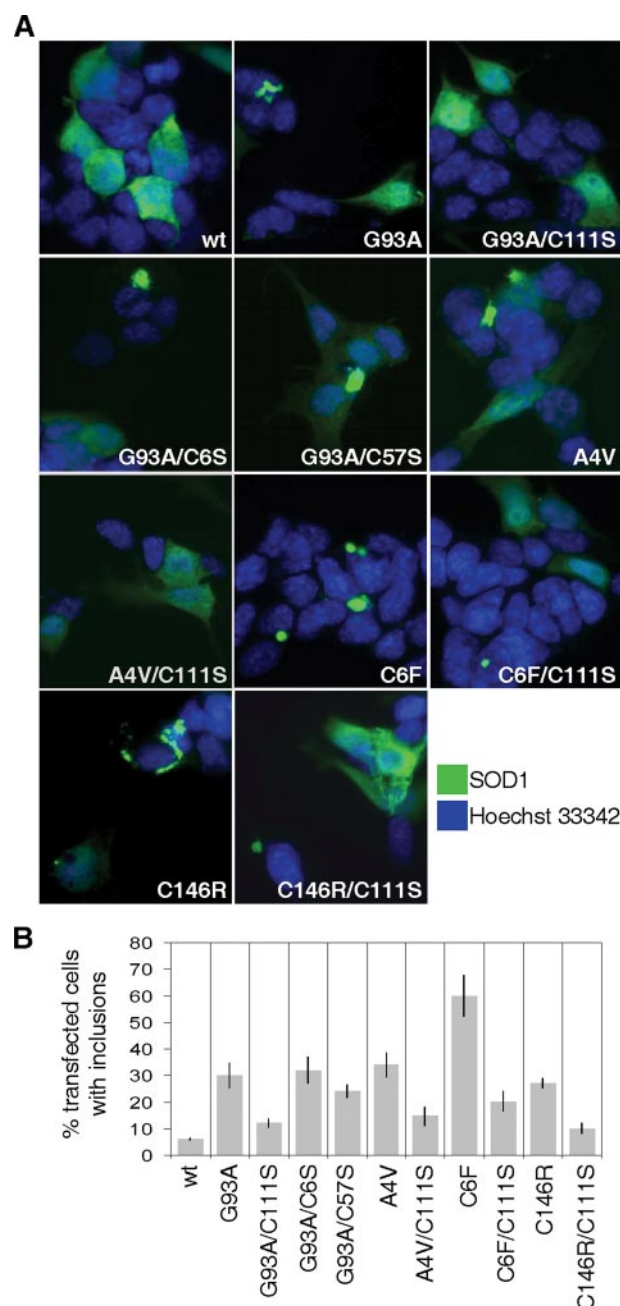


FIGURE 3. C111S mutation inhibits the formation of intracellular aggregates by mutSOD1s. A, NSC-34 cells were transfected with plasmids coding for wtSOD1 and the indicated mutSOD1s. After 48 h of culture, cells were subjected to immunofluorescence analysis with an antibody anti-SOD1. B, the proportion of SOD1-transfected cells bearing intracytoplasmic inclusions was scored ($n = 3$).

Given that the tendency of mutSOD1 to form intracellular aggregates has been linked to cell toxicity, we analyzed the effect of the transient expression of wtSOD1 and mutSOD1s, as well as the double mutants lacking Cys-111, on viability of NSC-34 cells. As reported in Fig. 4, the expression of mutSOD1s impairs cell viability as indicated by MTS assay, whereas expression of the same mutants with a replaced Cys-111 did not.

Redox Control of Mutant SOD1 Aggregation in NSC-34 Cells—The above data indicate that mutSOD1s aggregation and toxicity mostly depend upon a feature that is removed with

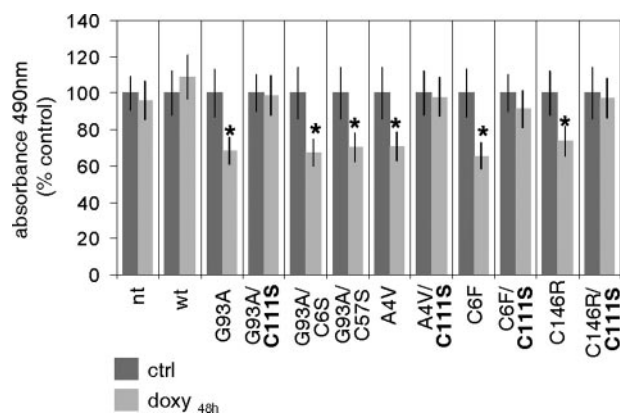


FIGURE 4. The replacement of Cys-111 protects NSC-34 cells from mut-SOD1-induced cell toxicity. NSC-34/pTetON cells were left untransfected (nt) or transfected with pTRE plasmids coding for wtSOD1 and the indicated mutSOD1s in the absence (ctrl) or presence of 1 μ g/ml doxycycline. After 48 h of culture, cell viability was assessed by an MTS assay. Absorbances at 490 nm are expressed as the percentage of the relative control cells. Values significantly different from relative controls are indicated with an asterisk when $p < 0.01$ ($n = 3$).

the single elimination of Cys-111. Because the formation of intermolecular disulfide bridges between SOD1 monomers involving the oxidation of cysteine residues has been proposed as a leading mechanism for SOD1 aggregation (3, 6, 10), we analyzed whether this feature could be related to the formation of disulfide-linked SOD1 oligomers. Soluble and insoluble fractions of transfected NSC-34 cells were therefore denatured in boiling 2% SDS, and subjected to denaturing, nonreducing electrophoresis in the presence of 100 mM iodoacetamide to avoid uncontrolled oxidation of cysteine residues. As shown in Fig. 5A (top panel), SOD1-immunoreactive bands accumulated in the high molecular weight range of the insoluble fraction of cells transfected with G93A and C6F mutSOD1s, with a proportional decrease in the amount of SOD1 migrating in the monomeric form. In contrast, the soluble fraction of mutSOD1s was monomeric as wtSOD1. In the presence of the reductant β -mercaptoethanol, the high molecular weight bands disappeared (Fig. 5A, bottom panel), demonstrating that mutSOD1 oligomers are stabilized through formation of intermolecular disulfide bonds. Most importantly, oligomeric SOD1 species were almost absent in both insoluble or soluble fractions of cells transfected with the double mutSOD1s G93A/C111S and C6F/C111S, whereas the substitution of Cys-6 or Cys-57 with a serine did not have an effect on the formation of disulfide-linked oligomers by G93A mutSOD1 (Fig. 5B). This indicated that the removal of Cys-111 is sufficient to abolish cysteine-dependent oligomerization of mutSOD1. These results prompted us to test whether the solubility of mutSOD1 could be affected by factors controlling the intracellular pool of glutathione, which is the cellular determinant of the thiol redox state (11). As shown in Fig. 6, treatment of cells with the cell-permeable ethyl ester form of reduced glutathione (GEE), which results in an increase in intracellular GSH (Fig. 6A), significantly improved G93A mutSOD1 solubility (Fig. 6B), whereas depletion of cytoplasmic GSH with L-buthionine-(S,R)-sulfoximine (BSO, Fig. 6A) enhanced its insolubility (Fig. 6C). Similar results were

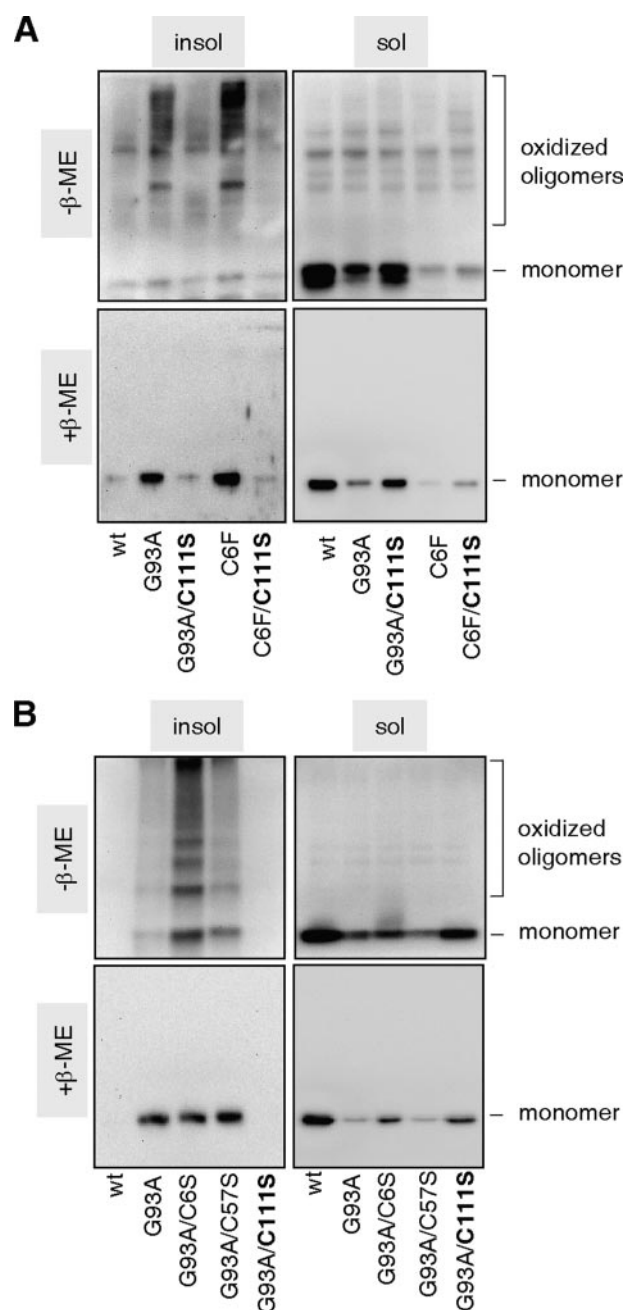


FIGURE 5. G93A and C6F mutSOD1s, but not G93A/C111S or C6F/C111S mutSOD1, accumulate as a disulfide-linked oligomeric form in the insoluble fractions of NSC-34 transfected cells. A, soluble and insoluble fractions from NSC-34 cells transiently transfected as indicated, were collected as described. Insoluble fractions were solubilized in a buffer containing 2% SDS. Soluble and insoluble fractions were then boiled for 5 min at 100 $^{\circ}$ C in the presence of 100 mM iodoacetamide. 15 μ g of proteins from each sample was subjected to a denaturing PAGE, either non-reducing (without β -mercaptoethanol, β -ME, upper panel) or reducing (with β -ME, lower panel). Western blot analysis was performed with an anti-SOD1 antibody. B, soluble and insoluble fractions from NSC-34 cell transiently transfected as indicated were treated as in A and analyzed by Western blotting with an anti-SOD1 antibody.

obtained when analyzing the effects of GSH supplementation/depletion on the solubility of A4V, H46R, C6F, and C146R mutSOD1s (Fig. 6, B and C).

It has been recently shown (12) that yeast glutaredoxins (Grxs), the thiol disulfide oxidoreductases that resolve both protein-SSG-mixed disulfides and intramolecular disulfides in

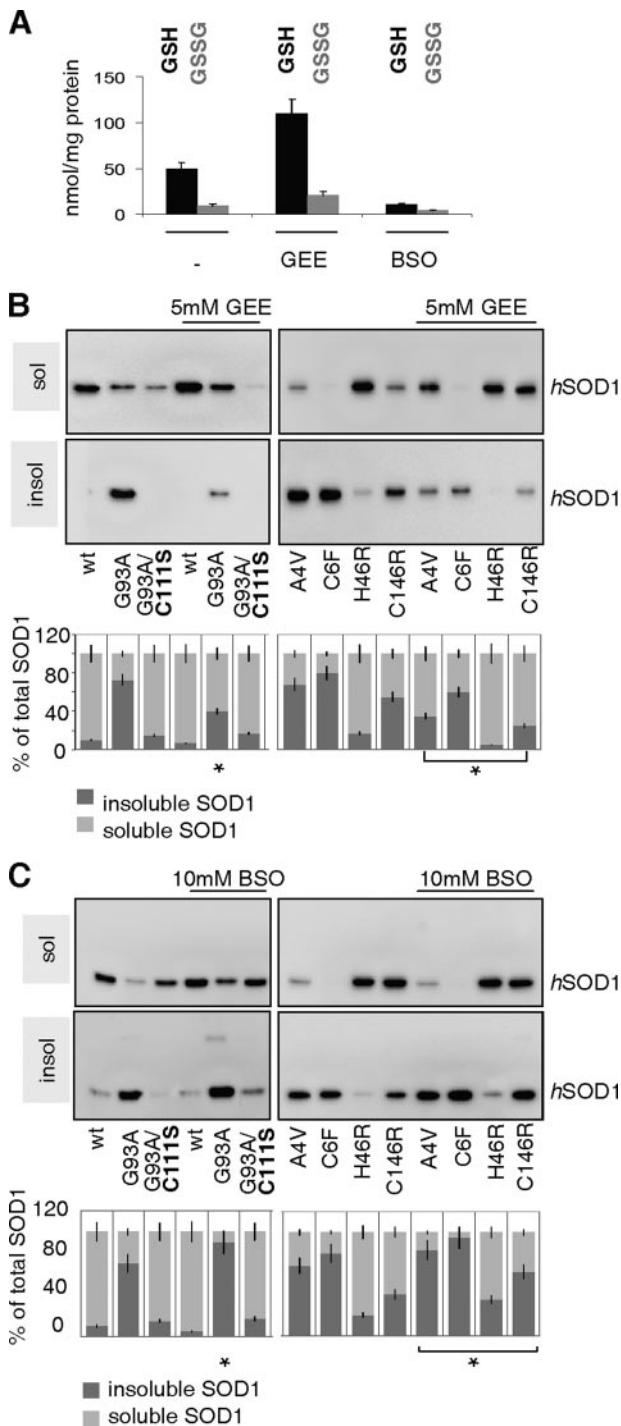


FIGURE 6. Redox control of mutant SOD1 aggregation in NSC-34 cells. A, reduced (GSH) and oxidized (GSSG) glutathione levels were measured by high performance liquid chromatography after a 48-h treatment of NSC-34 cells with 5 mM GEE or 10 mM BSO. B, NSC-34/pTetON cells were transiently transfected as indicated in the absence or in the presence of 5 mM glutathione ethyl ester (GEE). After 48 h, soluble and insoluble fractions were isolated and analyzed by Western blotting with an anti-SOD1 antibody. C, cells were transfected as in B and left untreated or treated with 10 mM L-buthionine-(S,R)-sulfoximine (BSO) for 48 h. Soluble and insoluble fractions were isolated and analyzed by Western blotting with an anti-SOD1 antibody. Histograms are calculated as in Fig. 1. Asterisks indicate values of insoluble mutSOD1s significantly different ($p < 0.01$, $n = 3$) from relative, untreated controls.

proteins, specifically target the SOD1 disulfide cysteines, thereby affecting the folding stability of SOD1. To test whether Grxs could affect mutSOD1 solubility, NSC-34 cells were trans-

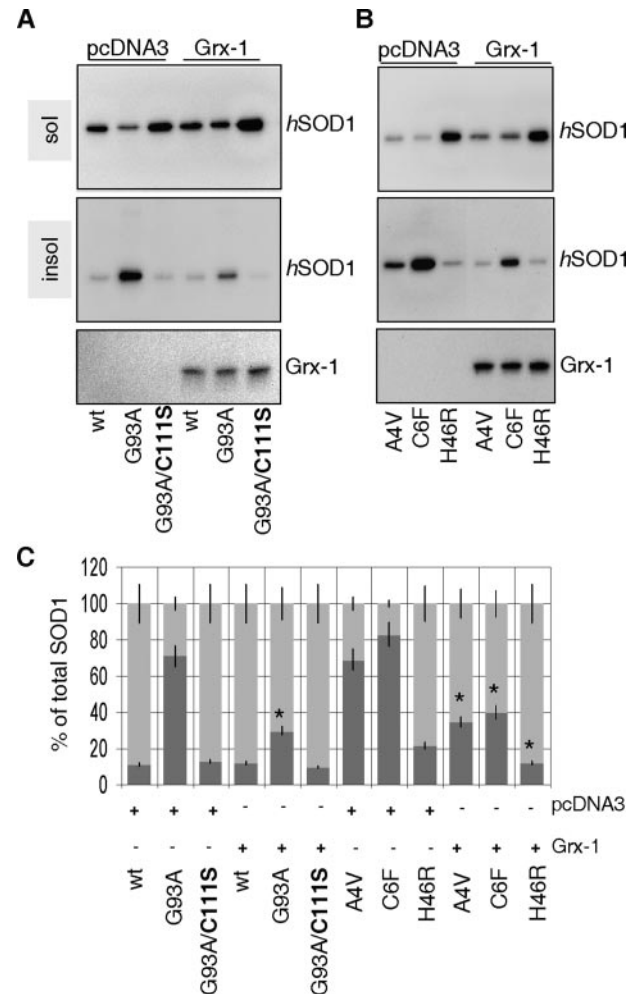


FIGURE 7. The overexpression of glutaredoxin-1 diminishes mutSOD1 insolubility. A and B, NSC-34/pTetON cells were transfected with wild-type or mutant SOD1s in the presence of a control plasmid (pcDNA3) or a plasmid coding for mouse cytosolic glutaredoxin-1 (Grx-1). Plasmids for SOD1 and Grx-1 were in a ratio of 1:3. Soluble and insoluble fractions were isolated after 48 h of culture in the presence of 1 μ g/ml doxycycline, and equivalent amounts were analyzed by Western blotting with an antibody anti-SOD1. An anti-Grx-1 antibody was used to reveal the levels of expression of Grx-1 in the soluble fraction. C, histograms are calculated as in Fig. 1. Asterisks indicate values of insoluble mutSOD1s significantly different ($p < 0.01$, $n = 3$) from relative controls.

fected with wild-type or mutant SOD1s, in the presence of a control plasmid or a plasmid coding for mouse cytosolic Grx-1. As shown in Fig. 7, the overexpression of Grx-1 strongly diminished G93A, A4V, C6F, and H46R mutSOD1 insolubility, further suggesting that GSH-mediated reduction of disulfides would be beneficial to prevent mutSOD1 aggregation.

Co-expression of G93A/C111S Double mutSOD1 Improves Solubility of G93A mutSOD1—It has recently been shown that human wtSOD1 exacerbates ALS-like disease in SOD1 G93A and SOD1 L126Z transgenic mice and converts unaffected SOD1 A4V transgenic mice to ALS disease (7). This phenomenon is accompanied by a conversion of the human wtSOD1 from a soluble form to a disulfide cross-linked, aggregated toxic form in the presence of mutSOD1s. On the basis of the results reported above, we hypothesized that C111S mutation could impair the ability of G93A mutSOD1 to convert soluble wtSOD1 to an insoluble form. To test this hypothesis, we devel-

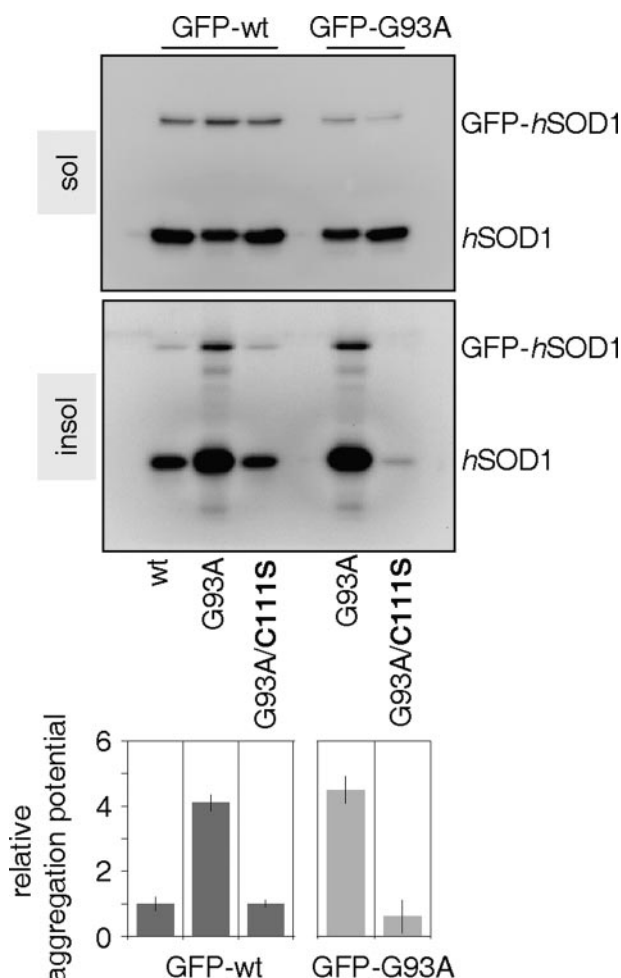


FIGURE 8. Cys-111 mediates the transfer of the aggregated phenotype from G93A mutSOD1 to wtSOD1. wtSOD1 and the mutSOD1 G93A and G93A/C111S were co-transfected for 48 h with GFP-tagged versions of wtSOD1 and the G93A mutant in NSC-34/pTetON cells. Soluble and insoluble fractions were isolated and analyzed by Western blotting with an anti-SOD1 antibody. Positions of GFP-tagged and untagged human SOD1s are indicated. Filters were stripped and reprobed with an anti-GFP antibody to check for the specificity of GFP-SOD1 signals (not shown). Histogram of the distribution of GFP-SOD1s in the insoluble fractions of NSC-34 cells as determined in $n = 3$ independent Western blot experiments. The relative levels of insoluble GFP-SOD1s were expressed as mean \pm S.D., considering GFP-wtSOD1 as one unit.

oped an assay where wtSOD1 and the mutSOD1 G93A and G93A/C111S were co-transfected with the GFP-tagged versions of wtSOD1 and the G93A mutant, so that they could be easily distinguished from the untagged enzymes because of their different electrophoretic mobility. As shown in Fig. 8, in the presence of G93A mutSOD1, GFP-wtSOD1 was much less soluble than in the presence of wtSOD1. The solubility of GFP-wtSOD1 was recovered when this protein was co-expressed with the G93A/C111S mutSOD1. Interestingly, the expression of G93A/C111S mutSOD1 converted the GFP-G93A mutSOD1 from an insoluble to a soluble form. These results not only indicate that the propensity to aggregate can be transferred from a mutSOD1 to the wild-type enzyme, but also the reverse, that protein solubility held by G93A/C111S mutant could be transferred to G93A mutSOD1, hampering its aggregation.

DISCUSSION

Spinal cords from ALS mice contain mutSOD1 cross-linked via intermolecular disulfide bonds (6, 13), and disulfide bond involvement in aggregates formation has previously been reported for other neurodegenerative diseases (14, 15), reinforcing the notion that cysteines represent a susceptibility factor for protein stability, particularly in conditions where oxidative stress has been widely described (16).

The contribution of cysteine residues in the process of SOD1 aggregation has been extensively addressed almost exclusively *in vitro*. O'Halloran and co-workers have found that the demetallated, reduced monomers of human SOD1s cross-link with each other by forming intermolecular disulfide bonds under mild oxidative stress conditions, such as enhanced levels of oxidized glutathione (10). In this process, conserved Cys residues (cysteines 57 and 146) exhibit higher reactivity for the formation of the disulfide-linked multimers than that of the non-conserved Cys residues (cysteines 6 and 111). On the basis of these results, the authors proposed a model whereby the reduction of the conserved disulfide bond in human SOD1 predisposes the protein to formation of incorrect disulfide cross-links, oligomerization, and higher order aggregation in the presence of mild oxidants. This interpretation has been recently supported by the observation that mutant SOD1s accumulates in the CNS of ALS transgenic mice and in a human individual with an A4V SOD1 mutation in a monomeric/misfolded form with a reduced disulfide bond (8, 17).

Our results are in line with these reports in that the insoluble, aggregated mutSOD1s accumulated in a disulfide-linked oligomeric form in NSC-34 cells, but they also indicate the existence of a more complex interplay between cysteines and protein misfolding in the process of formation and/or stabilization of SOD1 aggregates. Cys-111 appears to be crucial to this process, because the mutation of this single residue, while having no effect on wtSOD1, recovers the solubility of the G93A, A4V, and H46R mutSOD1s and also of the FALS-associated cysteine mutants C146R and, to a lesser extent, C6F. This indicates that the presence of Cys-111 is essential for the toxic properties of mutSOD1s to become evident. The ability of SOD1 to sediment in an insoluble form does not merely depend on the number of cysteines available to form intermolecular disulfides bond, because SOD1 proteins devoid of two (Cys-57 and Cys-146) and three cysteine residues (Cys-6, Cys-57, and Cys-146) are still highly insoluble, whereas a double (Cys-6 and Cys-111) or quadruple cysteine mutant show a more soluble behavior. Rather, diverse cysteines affect solubility in different ways. The elimination of both Cys-57 and Cys-146 not only impairs wtSOD1 protein solubility, but is unable to affect the insoluble phenotype of C6F mutSOD1, indicating that the presence of reduced Cys-57 and Cys-146 is dispensable for SOD1 protein oligomerization. This interpretation is supported by recent findings *in vitro* showing that a dimeric wtSOD1 apoprotein, even with its intrasubunit disulfide bonds intact, forms high molecular weight oligomers under physiological conditions. This oligomerization occurs provided that metals ions are lacking and is abolished by the elimination of Cys-6 and/or Cys-111 (18).

The results reported in this work support a critical role for Cys-111 in the intermolecular disulfide formation and aggregation process of mutSOD1; however, it is likely that other cysteines are involved in the formation or stabilization of oligomers. This possibility is suggested by two different considerations: (i) mouse SOD1, which naturally lacks Cys-111, is able to produce an ALS-like disease when the G86R mutant (corresponding to G85R mutant of human SOD1) is overexpressed in mice (19), thus indicating that cysteine residues other than Cys-111 may be involved in aggregation once SOD1 is misfolded; (ii) mutations in each of the four cysteine residues of human SOD1 have been described in ALS, although data on familial inheritance or the causative link to ALS are not completely clear for all of them. This observation would imply that none of the cysteines is *per se* necessary for SOD1 to oligomerize, and other alterations in the overall folding of the protein are needed. This conclusion is further supported by the insoluble behavior of C6F *versus* C6S and C146R *versus* C146S mutSOD1s in the aggregation assay performed in this study, and by the observation that, in the case of C6F mutSOD1, which is the most insoluble and toxic mutSOD1 examined in this study, a small amount of insoluble protein is formed irrespective of the presence of Cys-111. It should also be noticed that previous studies have reported the presence of complexes containing high molecular weight SOD1 in cell and mouse models even in the presence of reducing agents (20–22), indicating that disulfide bonds may not be the only structures holding the SOD1 aggregates together. Therefore, the formation of mutSOD1 aggregates seems to arise as a consequence of covalent disulfide cross-linking and non-covalent interactions that provide the aggregate a strong resistance to denaturation.

MutSOD1s accumulation in an oxidized, aggregated state in the mitochondria of motoneuronal cells and has been recently described in the spinal cord of transgenic mutSOD1s mice (3, 7). This behavior has been linked to the more oxidizing redox environment of mitochondria in those cells. The results presented in this study indicate that glutathione-mediated modification of cysteine residues is a leading mechanism driving the formation of SOD1 aggregates. Although it is not surprising that the alteration of redox balance affects solubility of mutSOD1s, given the established connections between redox state, alterations of quaternary structure, and aggregation of SOD1 (10), the results on the effect of Grx-1 overexpression on mutSOD1 solubility *in vivo* are of particular interest. Glutaredoxins are GSH-dependent oxidoreductases that resolve both mixed disulfides between GSH and a polypeptide cysteine, and intramolecular disulfides in proteins, targeting selected cysteine residues (23). Mammalian cytosolic Grx-1, in particular, is implicated in several cellular processes that require strict redox control, for instance the regulation of transcription factors (24) and apoptosis (25, 26), and is regulated itself by oxidative conditions (27). In a recent report, Culotta and co-workers (12) demonstrated that the major cytosolic Grx of yeast is able to target the intramolecular disulfide of ALS human mutSOD1 A4V both *in vivo* and *in vitro*, thus decreasing mutSOD1 stability, when copper activation was impaired due to the absence of CCS1 (the copper chaperone for yeast SOD1). Our results in a mammalian model system suggest that in conditions where

CCS and metal cofactors are not limiting, Grx-1 can reduce the intermolecular disulfides that stabilize oligomeric mutSOD1 structures, thus favorably contributing to mutSOD1 solubility. Clearly, additional studies are needed to clarify the mechanism underlying Grx-1 action on SOD1.

The results presented here provide evidence of a tight link between the formation of cysteine-dependent oligomers, SOD1 aggregation, and cell toxicity. This conclusion is strengthened by the observation that the ability of mutSOD1 to convert a soluble wtSOD1 to an insoluble form might depend on Cys-111. The overexpression of human wtSOD1 exacerbates ALS-like disease in affected mutSOD1 transgenic mice and converts unaffected SOD1 A4V transgenic mice to ALS disease (7, 28), a phenomenon that has been linked to a requirement of a threshold of SOD1 expression to cause the disease in mice. When we analyzed the solubility of mixture of wild-type and/or mutSOD1 in a cell transfection assay, we were able to validate that the insoluble attribute of G93A mutSOD1 can be transferred to a wild-type enzyme, and we further demonstrated that C111S mutation abrogates this effect. Moreover, the concurrent expression of G93A/C111S mutSOD1 converts a G93A mutSOD1 from an insoluble to a more soluble form, clearly indicating that a disulfide cross-linked form of SOD1, whether or not mutated, is fundamental in the mechanism of aggregation and, possibly, cell toxicity of SOD1.

Other possibilities should be considered. In particular, it has been recently shown that mutSOD1 has higher affinity for copper than wild-type SOD1, and this affinity is strictly dependent upon Cys-111. A C111S substitution markedly attenuates copper affinity and increases protein stability, suggesting that mutSOD1 may have a toxic interaction with Cu^{2+} through Cys-111, and this interaction contributes to the pathogenesis of FALS (29). This hypothesis would also reconcile the idea that an aberrant copper chemistry is implicated in ALS with data showing that an ALS-like phenotype developed in transgenic mice overexpressing a mutSOD1 where all four copper ligands (His-46, -48, -63, and -120) are mutated (4).

Whatever the mechanism underlying the “toxic” feature provided by Cys-111, an approach based on GSH-mediated reduction of disulfides in aggregated SOD1, possibly targeting Cys-111, might be beneficial to neurodegeneration in the pathogenesis of SOD1-linked FALS.

Addendum—After submission of this manuscript, Niwa *et al.* (30) published a report that supports a role for intermolecular disulfide bonds between cysteines at positions 6 and 111 in high molecular weight aggregate formation, ubiquitylation, and neurotoxicity of mutSOD1.

REFERENCES

1. Carri, M. T., Grignaschi, G., and Bendotti, C. (2006) *Trends Pharmacol. Sci.* **27**, 267–273
2. Shaw, B. F., and Valentine, J. S. (2007) *Trends Biochem. Sci.* **32**, 78–85
3. Ferri, A., Cozzolino, M., Crosio, C., Nencini, M., Casciati, A., Gralla, E. B., Rotilio, G., Valentine, J. S., and Carri, M. T. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 13860–13865
4. 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
5. Bruijn, L. I., Houseweart, M. K., Kato, S., Anderson, K. L., and Anderson, S. D.,

- Ohama, E., Reaume, A. G., Scott, R. W., and Cleveland, D. W. (1998) *Science* **281**, 1851–1854
6. Furukawa, Y., Fu, R., Deng, H. X., Siddique, T., and O'Halloran, T. V. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 7148–7153
7. Deng, H. X., Shi, Y., Furukawa, Y., Zhai, H., Fu, R., Liu, E., Gorrie, G. H., Khan, M. S., Hung, W. Y., Bigio, E. H., Lukas, T., Dal Canto, M. C., O'Halloran, T. V., and Siddique, T. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 7142–7147
8. Jonsson, P. A., Graffmo, K. S., Andersen, P. M., Brannstrom, T., Lindberg, M., Oliveberg, M., and Marklund, S. L. (2006) *Brain* **129**, 451–464
9. Ciriolo, M. R., Palamara, A. T., Incerpi, S., Lafavia, E., Bue, M. C., De Vito, P., Garaci, E., and Rotilio, G. (1997) *J. Biol. Chem.* **272**, 2700–2708
10. Furukawa, Y., and O'Halloran, T. V. (2005) *J. Biol. Chem.* **280**, 17266–17274
11. Schafer, F. Q., and Buettner, G. R. (2001) *Free Radic Biol. Med.* **30**, 1191–1212
12. Carroll, M. C., Outten, C. E., Proescher, J. B., Rosenfeld, L., Watson, W. H., Whitson, L. J., Hart, P. J., Jensen, L. T., and Cizewski Culotta, V. (2006) *J. Biol. Chem.* **281**, 28648–28656
13. Wang, J., Xu, G., and Borchelt, D. R. (2006) *J. Neurochem.* **96**, 1277–1288
14. Welker, E., Wedemeyer, W. J., and Scheraga, H. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 4334–4336
15. Wong, E. S., Tan, J. M., Wang, C., Zhang, Z., Tay, S. P., Zaiden, N., Ko, H. S., Dawson, V. L., Dawson, T. M., and Lim, K. L. (2007) *J. Biol. Chem.* **282**, 12310–12318
16. Halliwell, B. (2006) *J. Neurochem.* **97**, 1634–1658
17. Rakhit, R., Robertson, J., Vande Velde, C., Horne, P., Ruth, D. M., Griffin, J., Cleveland, D. W., Cashman, N. R., and Chakrabarty, A. (2007) *Nat. Med.* **13**, 754–759
18. Banci, L., Bertini, I., Durazo, A., Girotto, S., Gralla, E. B., Martinelli, M., Valentine, J. S., Vieru, M., and Whitelegge, J. P. (2007) *Proc. Natl. Acad. Sci. U. S. A.* **104**, 11263–11267
19. Ripps, M. E., Huntley, G. W., Hof, P. R., Morrison, J. H., and Gordon, J. W. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 689–693
20. Johnston, J. A., Dalton, M. J., Gurney, M. E., and Kopito, R. R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 12571–12576
21. Liu, J., Lillo, C., Jonsson, P. A., Vande Velde, C., Ward, C. M., Miller, T. M., Subramaniam, J. R., Rothstein, J. D., Marklund, S., Andersen, P. M., Brannstrom, T., Gredal, O., Wong, P. C., Williams, D. S., and Cleveland, D. W. (2004) *Neuron* **43**, 5–17
22. Wang, J., Xu, G., and Borchelt, D. R. (2002) *Neurobiol. Dis.* **9**, 139–148
23. Holmgren, A., Johansson, C., Berndt, C., Lonn, M. E., Hudemann, C., and Lillig, C. H. (2005) *Biochem. Soc. Trans.* **33**, 1375–1377
24. Qanungo, S., Starke, D. W., Pai, H. V., Mielay, J. J., and Nieminen, A. L. (2007) *J. Biol. Chem.* **282**, 18427–18436
25. Daily, D., Vlamis-Gardikas, A., Offen, D., Mittelman, L., Melamed, E., Holmgren, A., and Barzilai, A. (2001) *J. Biol. Chem.* **276**, 21618–21626
26. Daily, D., Vlamis-Gardikas, A., Offen, D., Mittelman, L., Melamed, E., Holmgren, A., and Barzilai, A. (2001) *J. Biol. Chem.* **276**, 1335–1344
27. Hashemy, S. I., Johansson, C., Berndt, C., Lillig, C. H., and Holmgren, A. (2007) *J. Biol. Chem.* **282**, 14428–14436
28. Jaarsma, D., Haasdijk, E. D., Grashorn, J. A., Hawkins, R., van Duijn, W., Verspaget, H. W., London, J., and Holstege, J. C. (2000) *Neurobiol. Dis.* **7**, 623–643
29. Watanabe, S., Nagano, S., Duce, J., Kiaei, M., Li, Q. X., Tucker, S. M., Tiwari, A., Brown, R. H., Jr., Beal, M. F., Hayward, L. J., Culotta, V. C., Yoshihara, S., Sakoda, S., and Bush, A. I. (2007) *Free Radic. Biol. Med.* **42**, 1534–1542
30. Niwa, J., Yamada, S., Ishigaki, S., Sone, J., Takahashi, M., Katsuno, M., Tanaka, F., Doyu, M., and Sobue, G. (2007) *J. Biol. Chem.* **282**, 28087–28095

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