INSOLUBLE MUTANT SOD1 IS PARTLY OLIGOUBIQUITINATED IN AMYOTROPHIC LATERAL SCLEROSIS MICE*
Manuela Basso1,2, Tania Massignan1,2, Giuseppina Samengo1,2, Cristina Cheroni3, Silvia De Biasi2, Mario Salmona2, Caterina Bendotti3, Valentina Bonetto1,2
From the 1Dulbecco Telethon Institute, Milan, Italy; 2Department of Molecular Biochemistry and Pharmacology, Mario Negri Institute for Pharmacological Research, Milan, Italy; 3Department of Neuroscience, Mario Negri Institute for Pharmacological Research, Milan, Italy and 4Department of Biomolecular Sciences and Biotechnology, University of Milan, Italy
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Address correspondence to: Valentina Bonetto, Dulbecco Telethon Institute and Mario Negri Institute for Pharmacological Research, Via Eritrea 62, 20157 Milan, Italy; Tel.: +390239014548; Fax: +39023546277; E-Mail: bonetto@marionegri.it

Mutations in the Cu, Zn superoxide dismutase (SOD1) gene cause a familial form of amyotrophic lateral sclerosis (fALS) through an unknown gain-of-function mechanism. Mutant SOD1 aggregation may be the toxic property. In fact, proteinaceous inclusions rich in mutant SOD1 have been found in tissues from fALS patients and in mutant SOD1 animals, before disease onset. However, very little is known of the constituents and mechanism of formation of aggregates in ALS. We and others have shown that there is a progressive accumulation of detergent-insoluble mutant SOD1 in the spinal cord of G93A SOD1 mice. To investigate the mechanism of SOD1 aggregation we characterized by proteome technologies SOD1 isoforms in a Triton X-100-insoluble fraction of spinal cord from G93A SOD1 mice at different stages of the disease. This showed that at symptomatic stages of the disease part of the insoluble SOD1 is unambiguously mono- and oligoubiquitinated, in spinal cord and not in hippocampus, and that ubiquitin branches at K48, the major signal for proteasome degradation. At presymptomatic stages of the disease only insoluble unmodified SOD1 is recovered. Partial ubiquitination of SOD1-rich inclusions was also confirmed by immunohistochemical and electron microscopy analysis of lumbar spinal cord sections from symptomatic G93A SOD1 mice. On the basis of these results we propose that ubiquitination occurs only after SOD1 aggregation and that oligoubiquitination may underline alternative mechanisms in disease pathogenesis.

Amyotrophic lateral sclerosis (ALS)1 is a fatal neurodegenerative disease that specifically affects motor neurons in the spinal cord, brain stem and motor cortex. Mutations in the Cu, Zn superoxide dismutase (SOD1) gene cause a familial form of ALS (fALS) (1) through a gain-of-function mechanism, which remains unclear. Human SOD1 is a homodimeric antioxidant enzyme that catalyzes the dismutation of superoxide radicals to hydrogen peroxide and dioxygen. Each subunit contains an eight-stranded β-barrel motif, an active site that binds a catalytic copper ion and a structural zinc ion, and an intramolecular disulfide bond. This bond, a peculiarity for a cytosolic protein, is adjacent to the dimer interface and seems to be important in stabilizing the overall structure and in the function of the protein (2,3 ). To date, more than 100 different SOD1 mutations have been linked to fALS. SOD1 mutations, primarily missense, are scattered throughout the gene and are believed to influence different functions of the protein (4). However, at least some mutant enzymes share common properties in vitro. In comparison with wild-type (WT) SOD1, mutants display general structural instability (5), the disulfide bond being more susceptible to reduction (6), with an enhanced propensity to form aggregates (7). In vivo, proteinaceous inclusions rich in mutant SOD1 have been found in tissues from fALS patients, mutant SOD1 animal and cellular models (8). In fALS mice, SOD1 aggregates localize in neurons and astrocytes (9), as perikaryal deposits and as macromolecular complexes associated with various mitochondrial compartments (10) and inside the endoplasmic reticulum (11). In fALS mice SOD1 insoluble protein complexes are formed before the onset of motor dysfunction (12) and are found exclusively in tissues affected by the disease (13). All these observations indicate that mutant SOD1 aggregation may play a role in the pathogenesis and that aggregation may be a toxic property acquired by SOD1.
What we know about the aggregate protein constituents comes principally from immunohistochemistry studies on post-mortem tissues of ALS patients or spinal cords of mutant SOD1 mice (8). In sporadic and fALS patients the most widely seen inclusions (in almost all cases) immunostain for ubiquitin. In mutant SOD1 mice protein inclusions mainly immunoreactive for SOD1 and ubiquitin have been detected (14-16). However, a clear co-localization between SOD1 and ubiquitin signals was never showed. Ubiquitinated inclusions have been found in other neurodegenerative diseases (17), and might represent the overwhelmed cellular defense against misfolded and/or abnormally modified proteins, which are normally linked to polyubiquitin chains, then degraded by the proteasome system. Mutant SOD1 is catabolized by the proteasome in vitro, in transfected cells and mice spinal cord tissues (18-20). One possible explanation for the protein inclusions is that mutant SOD1 accumulates as a consequence of a low level or reduced activity of the proteasome. We and other groups have investigated the proteasome degradation pathways in connection with ALS, but it is still inexplicably contentious whether the constitutive proteasome activity is inhibited or not, and whether the induction of immunoproteasome subunits eventually compensates for proteasome dysfunction (16,21,22). In line with previous observations (12,23), we have shown that in spinal cord of G93A SOD1 mice there is progressive accumulation of high-molecular-weight and detergent-insoluble mutant SOD1 (16).

We have now thoroughly characterized SOD1 isoforms in a detergent-insoluble fraction from spinal cord of G93A SOD1 mice at different stages of the disease, with a view to elucidating the mechanism of SOD1 aggregation and the possible link to the disease pathogenesis. We unambiguously established that SOD1 accumulates in spinal cord detergent-insoluble aggregates of symptomatic G93A SOD1 mice in part as mono- and oligoubiquitinated forms.

**Experimental procedures**

**Transgenic mouse models**—Transgenic mice originally obtained from Jackson Laboratories and expressing a high copy number of mutant human (h) SOD1 with a Gly-93-Ala substitution, or wild-type (WT) hSOD1 mice, were bred and maintained on a C57BL/6 mouse strain at the Consorzio Mario Negri Sud, S. Maria Imbaro (CH), Italy. Transgenic mice are identified by PCR (1). The mice were housed at 21±1°C with relative humidity 55±10% and 12 h of light. Food (standard pellets) and water were supplied ad libitum. In this study, female G93A SOD1 mice were killed at 7, 12, 17 and 26 weeks of age, corresponding to early presymptomatic, presymptomatic, early symptomatic and end stage of the disease. Female WT SOD1 or non transgenic mice were used as controls. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. No. 116, G.U. Suppl. 40, Feb. 18, 1992, Circolare No. 8, G.U., 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, Dec.12, 1987; NIH Guide for the Care and use of Laboratory Animals, U.S. National Research Council, 1996).

**Extraction of detergent-insoluble proteins**—Tissues were processed as previously described (16), with some modifications. Briefly, they were homogenized in ice-cold homogenization buffer, pH 7.6, containing 15 mM Tris-HCl, 1 mM dithiothreitol, 0.25 M sucrose, 1 mM MgCl2, 2.5 mM EDTA, 1 mM EGTA, 0.25 M sodium orthovanadate, 2 mM sodium pyrophosphate, 5 µM MG132 proteasome inhibitor (Sigma), 1 tablet of Complete™/10 mL of buffer, Mini Protease Inhibitor Mixture (Roche Applied Science), and in some experiments 5 mg/mL of iodoacetamide, to ensure inhibition of ubiquitin-cleaving isopeptidases (24). The samples were centrifuged at 10000×g at 4°C for 15 minutes. The supernatant was centrifuged at 100000×g for 1 h to obtain the cytosolic fraction. The pellet was suspended in ice-cold homogenization buffer with 2% of Triton X-100 (TX) and 150 mM KCl added, sonicated three times for 10 sec and shaken for 1 hour at 4°C. Samples were then centrifuged twice at 10000×g at 4°C for 10 minutes to obtain TX-resistant pellets. Proteins were quantified by the Bradford assay. Pellets were frozen at −20°C until further analyses. Cytosolic fractions were analyzed in some experiments after stable modification of Cys residues: (i) reduction in presence of 10 mM dithiothreitol and alkylation with 5 mg/mL iodoacetamide, or (ii) oxidation with performic acid (25).

**Two-dimensional gel electrophoresis (2DE)**—Samples were dissolved in 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) immobilized-pH gradient (IPG) buffer...
(Amersham Biosciences) and 12 µL/mL DeStreak™ Reagent (Amersham Biosciences), which form stable disulfide bonds and prevent unspecific Cys residue oxidation during isoelectric focusing (IEF) (26). Samples were loaded by in-gel rehydration (1 h at 0 V, 270 Vhr at 30 V) on pH 4-7 linear or pH 3-10 non-linear 7-cm IPG strips (Amersham Biosciences). IEF was done on an IPGphor (Amersham Biosciences) according to the following schedule: 200 Vhr at 200 V, 10500 Vhr at 3500 V, 14375 Vhr of a linear gradient up to 8000 V, 48000 Vhr at 8000 V. Strips were then re-equilibrated in NuPAGE LDS Sample Buffer (Invitrogen) and second dimension was run on precast, 4-12% polyacrylamide gradient gel, NuPAGE® Bis-Tris (Invitrogen). Gels were stained with SYPRO® Ruby protein gel stain (Molecular Probes).

Western blotting (WB)—Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). Detection of ubiquitinated proteins required pre-treatment of the membrane to expose the ubiquitin epitope and favor antibody recognition. After blotting, membranes were treated with 6 M guanidine-HCl, 20 mM Tris-HCl, pH 7.5, 1 mM PMSF and 5 mM dithiothreitol for 30 minutes at room temperature (RT). For the reaction with primary antibodies, membranes were incubated for 1 h at RT with a blocking buffer (5% milk in Tris-buffered saline containing 0.1% Tween 20) and probed overnight at 4°C with rabbit polyclonal antibody anti-hSOD1 (Upstate), diluted 1:2000 in blocking buffer, or with rabbit polyclonal antibody anti-ubiquitin (DakoCytomation), diluted 1:800 in blocking buffer. The membrane was then washed and incubated for 1 h at RT with goat anti-rabbit peroxidase-conjugated secondary antibody diluted 1:5000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Blots were developed by the ECL-plus protein detection system (Amersham Biosciences). Immunoreactivity was normalized to the actual amount of proteins loaded on the membrane as detected after Coomassie blue staining. In double immunostaining experiments membranes were stripped by Restore™ Western blot stripping buffer (Pierce).

Image analysis—SYPRO Ruby-stained 2D gel images were captured by the laser scanner Molecular Imager® FX (Bio-Rad) using a 532-nm laser as excitation source, and 530 nm longpass as emission filter. 2D WB images were captured by an Expression 1680 Pro scanner (Epson) at 16 bit and 300 dpi resolution. Densitometry and image analysis were done by the Progenesis PG240 v2006 software (Nonlinear Dynamics). Gel/blot matching was done by using the specific warping algorithm of the software in the manual mode, placing seeding points on recognizable, intense immunopositive spots.

Mass spectrometry—Protein spots were located and excised with an EXQuest™ spot cutter (Bio-Rad). Spots were processed and gel-digested alternatively with trypsin or endoprotease V8 (V8) (Sigma), essentially as previously described (27). Peptide mass fingerprinting was analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) on a ReflexIII™ (Bruker Daltonics) instrument using α-cyano-4-hydroxycinnamic acid as matrix. The mass spectra were internally calibrated with trypsin or V8 autolysis fragments, routinely obtaining accuracy better than 30 ppm. For the detection of additional post-translational modifications of SOD1, LC-MS/MS analysis was done on a reverse-phase microbore LC Surveyor system coupled to an ion trap mass spectrometer LCQ Deca XP™ Plus (Thermo Finningan), as described (28).

Immunohistochemical confocal analysis—Three WT SOD1 mice and five G93A SOD1 mice at a late symptomatic stage of disease were analyzed in each experiment. Mice were anesthetized, transcardially perfused, and spinal cords were removed, as previously described (27). Immunolabeling was done on lumbar spinal cord sections (30-µm thick floating cryosections). In each experiment, some of the sections were processed without primary antibody, in order to verify the specificity of the staining. Sections were blocked in phosphate-buffered saline containing 5% normal goat serum and 0.05% TX for 60 min at RT, then incubated with anti-ubiquitin antibody (DakoCytomation) diluted 1:750. The sections were incubated with the anti-rabbit biotinylated antiserum (Vector Laboratories, diluted 1:500), and the immune reaction was revealed by the TSA amplification kit (Cy5, Perkin Elmer), as described (29). After the ubiquitin staining, a second staining was done with the anti-hSOD1 antibody (Upstate Biotechnology) diluted 1:2000. The reaction was revealed by incubation with anti-rabbit antibody conjugated to Alexa 488 (1:500 dilution, Molecular Probes). No direct reaction was detected between this secondary fluorescent antibody and the anti-ubiquitin primary antibody.
using the dilution of antibodies above described. For the co-localization with glial fibrillary acidic protein (GFAP), the sections were first labeled with anti-ubiquitin and anti-hSOD1 antibodies, incubated with the anti-GFAP antibody (1:2500 dilution, Chemicon) and then with anti-mouse Alexa 546 antibody (1:500 dilution, Molecular Probes). For labeling with the motor neuron marker choline acetyl-transferase (ChAT), the sections were probed with the anti-ChAT antibody (1:200 dilution, Chemicon) and then with anti-mouse Alexa 546 antibody (1:500 dilution, Molecular Probes). Fluorescence-labeled sections were mounted with Fluorsave (Calbiochem) and analyzed under an Olympus Fluoview laser scanning confocal microscope. Wavelengths of 488 nm (Laser Ar-Kr), 546 nm (Laser He-Ne green) and 647 nm (Laser He-Ne red) were used to excite Alexa 488 and 546 and Cy5 fluorophore, respectively. Emission radiations (510-550 nm for Alexa 488 and 670 nm for Cy5) were collected on separate detectors. To eliminate the risk of cross-talk between channels, the sections were scanned in a sequential mode.

RESULTS

2DE characterization of TX-insoluble G93A SOD1 isolated from spinal cord of fALS mice at different stages of the disease—We have recently shown that in the spinal cord of G93A SOD1 mice there is a progressive accumulation of mutant SOD1 correlated with a decrease of its solubility in TX, forming evident aggregates at a late stage of the disease (16). In this study we further analyzed, using 2DE, SOD1 in TX-insoluble extracts of spinal cord from G93A mice, with a view to elucidating the mechanism of SOD1 aggregation. We analyzed samples from spinal cord of G93A mice at different stages of the disease, two presymptomatic stages, 7 and 12 weeks of age, and two late symptomatic stages, 17 weeks of age, and 26 weeks of age. As controls, we analyzed samples from spinal cord of 26-week-old WT SOD1 mice and hippocampus, tissue not affected by the disease, of late symptomatic G93A SOD1 mice. Fig. 1 shows anti-hSOD1 2D WB of 2DE-separated TX-insoluble proteins from spinal cord of fALS mice at the different stages of the disease and control samples. There is a marked difference between the 2D WB of the samples from presymptomatic G93A SOD1 mice (Fig.1 A-B), where only one spot (panel A) or three (panel B) spots are visible, and the 2D WB of the samples from mice at symptomatic stages of the disease (panel C-D), where several and highly intense spots are present. In panel A-B, the spots at about 16 KDa correspond to monomeric SOD1 isoforms. In panel C-D, two trains of spots in the 5-6 pl range are most intense, one with at least three spots at 16 kDa, corresponding to monomeric SOD1 isoforms, and one with at least four spots at an apparent Mr of 37 kDa, corresponding to dimeric SOD1 isoforms. Although proteins are dissolved in highly denaturing conditions (7 M urea, 2M thiourea) and with reducing agents, some dimeric SOD1 isoforms are detected, due to resistance to...
complete disassembly of the multimeric SOD1 complexes abundantly present in symptomatic fALS mice. Dimeric SOD1 isoforms are formed through intermolecular disulfide bonds, and in fact they disappeared when the sample was drastically denatured and stably alkylated at Cys residues with iodoacetamide before IEF (data not shown). Only in symptomatic fALS mice there are other four trains of spots, focalized at slightly higher pI than monomeric SOD1, at about 24, 32, 40, 48 kDa. Since these four trains differ by 8 kDa, which is a sign of ubiquitination, we did double immunostaining (Fig. 2) with antibody anti-ubiquitin and anti-hSOD1 on the same membrane to see whether there was any overlapping of signals. There was a clear overlap between the signals at 24 and 32 kDa in the anti-SOD1 and anti-ubiquitin 2D WB, and possibly also at 40 and 48 kDa. Monomeric SOD1 is therefore probably mono- and oligoubiquitinated in TX-insoluble spinal cord extracts of symptomatic G93A SOD1 mice. In conclusion, an increasing amount of TX-insoluble mutant SOD1 was recovered from spinal cord of fALS mice as disease progressed. Only at symptomatic stages of the disease part of the insoluble SOD1 is recovered as oligoubiquitinated. Small amount of TX-insoluble nonoligoubiquitinated SOD1 was present in hippocampus of late symptomatic fALS mice (Fig. 1E) and in spinal cord of 26-week-old WT SOD1 mice (Fig.1F).

G93A SOD1 is present in TX-insoluble aggregates as mono- and K48-linked oligoubiquitinated isoforms—We further confirmed SOD1 ubiquitination, assessing the site of ubiquitination of SOD1 and the site of branching of the polyubiquitin chains using a combination of 2DE and MALDI mass spectrometry (MS). TX-insoluble extracts from 26-week old G93A SOD1 mice were separated by 2DE in the 4-7 pI range. The gel image was matched and warped with the anti-ubiquitin and the anti-hSOD1 2D WB by computerized image analysis. Fig. 3 reports a representative SYPRO Ruby-stained 2D gel of aggregated proteins where the spots matched with 2D WB are indicated. Spots 6-14 matched concomitantly with anti-SOD1 and anti-ubiquitin immunoreactive spots. On the basis of the Mr in 2DE, spot 6-8 correspond to monoubiquitinated SOD1, spots 9-11 to biubiquitinated SOD1, spots 12-13 to triubiquitinated SOD1, and spot 14 to tetraubiquitinated SOD1. Spots 6-14 were gel-digested alternatively with trypsin or V8 and their peptide mass fingerprints were analyzed by MALDI MS to confirm and better characterize SOD1 ubiquitination. Table 1 shows the m/z of the ions that matched with SOD1 and ubiquitin tryptic and V8 fragments. All spots analyzed (6-14) contained ubiquitin- and SOD1-derived fragments. A larger number of fragments were found in correspondence with monoubiquitinated SOD1, spots 6 and 7, with respectively 13 and 16 fragment ions, and diubiquitinated SOD1, spot 9, with 12 fragment ions.

Proteolysis of ubiquitin-protein conjugates using trypsin or V8 produces protein fragments with characteristic tails, resulting from cleavage of the attached ubiquitin. The GG-tryptic tail increases the mass of the modified peptide by 114.04 Da. This strategy gives an indication of the attachment site if the protein sequence is known and the fragment contains only one K (the potential attachment site). Spots 6 and 7 of monoubiquitinated SOD1 contained an ion with m/z 1578.71, which is compatible with the calculated m/z of SOD1 129-143 fragment with the GG-tail added, GGNEESTK^{136}(GG)TGNAGSR (1646.67+114.04). Since fragments 129-143 contain a single K, we could conclude that SOD1 is linked to the ubiquitin chain through K136. Only mass fingerprints of spots 9,10,11,12 and 14, corresponding to bi-, tri- and tetraubiquitinated SOD1, contained a fragment ion with m/z of 1460.79, which is a signature peptide ion for K48-branched polyubiquitinated proteins, LIFAGK^{48}(GG)QLEDGR (31,32). Representative MALDI mass spectra for mono- and biubiquitinated G93A SOD1 are shown in Fig. 4. We conclude that SOD1 is mono- and oligoubiquitinated, and the C-terminus of the ubiquitin chain is linked to SOD1 at least through K136 and ubiquitin chain branches at K48.

SOD1-rich inclusions are partially ubiquitinated and mainly present in motor neurons of lumbar spinal cord sections of ALS-mice—To examine the cellular distribution (in neurons, astrocytes or microglia) of mutant SOD1 and the possible co-localization with ubiquitin accumulation, triple immunostaining experiments were done in the lumbar spinal cord sections of G93A SOD1 mice at the late symptomatic stage of disease (Fig. 5 F-S) in comparison with WT SOD1 mice (Fig. 5 A-E). Particularly intense labeling for hSOD1 was detected in G93A SOD1 mice at the edge of the vacuoles, characteristic of the ventral and intermediate regions of the grey matter of spinal cord, and in some degenerating neurons.
(Fig. 5 F,I,N,Q). In the same sections of G93A SOD1 mice, the ubiquitin immunoreactivity showed a pattern of distribution characterized by large spots of ubiquitin-positive accumulation scattered throughout the grey matter. In WT SOD1 mice, the ubiquitin labeling was very low and uniformly distributed in the neurons of the spinal cord, including the ChAT-immunopositive motor neurons (Fig. 5 B,D,E). Similar results were obtained with nontransgenic mice (data not shown). When we examined the distribution of hSOD1 and ubiquitin in the ChAT-positive motor neurons of G93A SOD1 mice, we noted that only occasional ubiquitin-positive spots were localized in vacuolized motor neurons where hSOD1 was highly expressed. However, in the merged images hSOD1 and ubiquitin showed no apparent co-localization. The analysis of hSOD1 and ubiquitin in GFAP labeled astrocytes of G93A SOD1 mice showed clearly that hypertrophic astrocytes do not accumulate hSOD1 (Fig. 5 N-S). However, some GFAP-positive cells, with a round morphology, had high levels of ubiquitin signal (Fig. 5 Q-R). The reactive microglial cells, labeled by the CD11b marker, showed no accumulation of hSOD1 or presence of ubiquitin-positive aggregates (data not shown).

The ultrastructural investigation confirmed the light microscopy data. In symptomatic G93A SOD1 mice, in fact, labeling of thin section of the lumbar ventral horn with the anti-hSOD1 antiserum resulted in the deposition of numerous gold particles on cytoplasmic aggregates, mainly consisting of irregularly oriented filaments, located in dendritic profiles (Fig. 6 A-B). Similarly, labeling of adjacent thin section with the anti-ubiquitin antiserum resulted in the deposition of numerous gold particles on cytoplasmic aggregates consisting of either irregularly oriented filaments or electron dense material (not shown). Double labeling with anti-hSOD1 and anti-ubiquitin allowed to demonstrate that some of the filamentous aggregates were positive for both markers (Fig. 6 C-D) whereas some others were intensely SOD1-positive but did not contain ubiquitin (Fig. 6 E-F). Samples from nontransgenic mice never contained SOD1-positive or ubiquitin-positive aggregates. In conclusion, we have shown that SOD1-rich inclusions are partially ubiquitinated and present mainly in motor neurons.

**SOD1 is widely microheterogeneous in IEF due to different conformers of the protein**—In all 2DE experiments SOD1 separated in IEF as trains of spots. However, by MS we did not find any modifications at least in the fragments analyzed that could explain the pI shift. Fig. 7 shows 2D WB of different preparations of SOD1 G93A. While soluble G93A SOD1 presented two major spots with 5.6 and 5.9 pI (Fig. 7A), TX-insoluble G93A SOD1 separated in at least three intense spots (5.3, 5.6, 5.9 pI) (Fig. 7B). Polydispersity in IEF using IPG strips can be caused by oxidation of the –SH groups in the protein. To exclude this, Cys residues were stably modified in two different ways before IEF separation. Fig. 7 reports the 2D WB of soluble G93A SOD1, alkylated with iodoacetamide (panel C) or irreversibly oxidized by performic acid (panel D). The polydispersity persisted with both treatments; after alkylation SOD1 separated in three abundant isoforms at the same pI as the untreated preparations, and after performic acid oxidation a train of at least five spots was observed although at a more acidic pI, as expected due to conversion of the Cys residue into cysteic acid. In agreement with previous observations on recombinant hSOD1 (33), we suggest that these isoforms are different conformational species of hSOD1 rather than post-translationally modified forms. We compared and quantified the distribution of monomeric SOD1 isoforms in soluble and TX-insoluble extracts from spinal cord of 26-week-old G93A SOD1 mice, using 2DE. Figure 6E shows that isoform 2 of soluble G93A SOD1 is the most abundant, and possibly the most stable isoform, comprising 75.5% of the total monomeric SOD1 and being five times the second most abundant one, isoform 3 (15.2%). In contrast, TX-insoluble G93A SOD1 is more evenly distributed among the three major isoforms 2, 3 and 4, respectively 50.2%, 29.3% and 11.7% (Fig. 7F) of total monomeric SOD1. In conclusion, hSOD1 appeared to be widely microheterogeneous in IEF. There is a different frequency distribution of the spots in soluble and TX-insoluble G93A SOD1, with a higher level of the acidic, most unstable, isoforms in the insoluble mutant SOD1 (33). This behavior might be linked to the existence of several conformational states of mutant SOD1 and may provide additional evidence that mutant SOD1 is structurally more unstable and therefore more prone to aggregation.

**DISCUSSION**

Protein aggregation is a hallmark of ALS pathogenesis for sporadic and familial forms.
Thus, ALS is considered a protein misfolding disease, like Alzheimer’s, Parkinson’s, polyglutamine and prion diseases. In all these neurodegenerative disorders the common feature is the aggregation of normally soluble proteins, due to the inability to assume the correct conformation. In familial forms the mutant protein is usually the major component of protein inclusions. SOD1 is one of the major components of the aggregates in mutant SOD1 fALS patients and animal models (8). However, not very much is known about the mechanism of SOD1 aggregation and its role in pathogenesis. Several contributing factors have been proposed for the formation of SOD1-rich protein inclusions in ALS: structural instability and aggregation-propensity of mutant SOD1 (5-7), inhibition of chaperone activity (34), and alteration of the ubiquitin-proteasome system (16,21).

Ubiquitinated protein aggregates are TX-insoluble (35) and we demonstrated that mutant SOD1 accumulated in TX-insoluble fractions as the disease progressed (16). Thus, TX-insoluble fractions are enriched in ubiquitinated protein aggregates and mutant SOD1, mimicking the composition of ALS protein inclusions. We thoroughly characterized SOD1 in TX-insoluble protein aggregates from spinal cord of G93A SOD1 mice at different stages of the disease to elucidate the mechanism of mutant SOD1 aggregation. Insoluble SOD1 is recovered already at an early presymptomatic stage of the disease (7 weeks of age) as unmodified protein. At symptomatic stages of the disease SOD1 was present as several isoforms at different molecular weights and pl and we elucidated some aspects of their structure using proteome-based technologies. We found that monomeric G93A SOD1 is mono- and oligoubiquitinated with a high prevalence of mono- and biubiquitinated forms. We identified a single potential site of linkage between ubiquitin and K163 of SOD1. However, we cannot exclude that ubiquitin could be linked to other K. We propose that the high-molecular-weight species comprise SOD1 conjugated to polyubiquitin chains rather than SOD1 monoubiquitinated at various sites. In fact, in the mass spectra of bi-, tri-, and tetraubiquitin conjugated SOD1 we found a signature peptide ion for K48-branched polyubiquitinated proteins. Polyubiquitin chains are built by formation of an isopeptide bond between the C-terminal G76 residue of one ubiquitin and the ε-amino group of the seven potential K of the preceding ubiquitin. Residue K48 is a major site of chain initiation and is the principal proteasome delivery signal, while the other major linkage, through K63, does not seem to play a role in protein turnover (36). Therefore, our finding suggested that the ubiquitin chains conjugated to SOD1 would target the protein to degradation. Why this does not happen needs further investigation, but it might be tentatively explained on the basis of our results. In our TX-insoluble aggregates we recovered mono- and oligoubiquitinated SOD1, but the majority of insoluble SOD1 exists as nonubiquitinated protein. This is one of the possible reasons why we could not detect a clear overlapping of the SOD1 and ubiquitin signals in the immunohistochemistry studies and we found only sporadic SOD1 positive aggregates co-labeled with ubiquitin by electron microscopy analysis. We did not find SOD1 linked to more than four ubiquitin chains. It is possible that misfolded polyubiquitinated mutant SOD1 is easily degraded by the proteasome, and therefore it does not aggregate and is not recovered in TX-insoluble material. We also found that insoluble SOD1 accumulates, as unmodified, at presymptomatic stages of the disease, while is recovered as partly oligoubiquitinated only at symptomatic stages of the disease. These observations support the idea that oligoubiquitination occurs after SOD1 aggregation, as a secondary event, not necessary for aggregate formation. A similar mechanism has been proposed for α-synuclein, a major component of pathological inclusions in patients with Parkinson’s and Lewy body diseases, also found as mono- and oligoubiquitinated, but principally as nonubiquitinated (37-39). This is in agreement also with the work by Gilchrist et al., in which were observed ubiquitin-positive lesions appearing 30 days later than SOD1 positive inclusions in the spinal cords of SOD1 G93A mice crossed with epitope tagged ubiquitins (40). As schematically described in Fig. 8, it may be that mono- and oligoubiquitination only occur at K sites of already aggregated SOD1, which are accessible to the ubiquitin-ligase machinery, and that additional ligations are impeded by steric hindrance or by an unknown mechanism. Since tetraubiquitin appears to be the shortest chain with high affinity for the proteasome (41), the mono- and oligoubiquitinated SOD1 may be inefficiently recognized by proteasomes, leading to aberrant accumulation and the consequent overload and dysfunction of the ubiquitin-
proteasomal system. A clogged degradation system may have effects on the elimination of aberrantly oxidized proteins (27) and the turnover of important regulatory proteins, linking protein aggregation to cellular degeneration and death (42). In our studies the accumulation of mutant SOD1 was observed almost exclusively in the motor neurons undergoing degeneration or already degenerated, rather than in the reactive astrocytes and microglia, further indicating that this mechanism may play a role in cell death. Moreover, oligoubiquitinated SOD1 was not recovered in hippocampus of late symptomatic fALS mice, linking SOD1 ubiquitination to tissue-specific neurodegeneration.

In line with recent publications it is also possible that alteration of other degradation pathways, such as the endosomal-lysosomal system, may be involved, as it was shown in a number of neurodegenerative diseases (43). In fact, it seems that the ubiquitin-proteasome system plays a major role in reducing the level of soluble misfolded proteins, while autophagy in clearing of cells from protein aggregates. In fact, suppression of autophagy in mice causes neurodegeneration with accumulation of ubiquitin-containing inclusion bodies, without obvious proteasome alteration (44,45). It has been also suggested that association of ubiquitin-proteasome with neuronal inclusions may be a “non-stick” coating that prevents further growth of the inclusion and toxic interactions, and enhances neuronal survival (40,46). Moreover, limited ubiquitin chain elongation, through the action of deubiquitinating enzymes found in association with ubiquitinated inclusions (47), would not excessively engage the proteasome and compromise further protein degradation pathways. The role of ubiquitin in masking aggregated proteins, as opposed to marking them for proteasome degradation, although intriguing, has not been well established and needs further investigation.

Human SOD1 shows a peculiar behavior in 2DE, separating in several isoforms. Choi et al. recently identified four major SOD1 isoforms in brains of Alzheimer’s and Parkinson’s disease patients and proposed that at least some of them are differentially metallated forms (48). However, we exclude this as the reason, because at least in our experiments, proteins recovered from 2DE were metal-free, since they were extracted in the presence of metal chelating agents and dissolved in heavily denaturing buffers. Instead, we prefer the idea that the train of spots corresponds to different charge isoforms, representing different conformational species. In agreement with our hypothesis Wenisch et al. have reported that recombinant hSOD1 under nonadenating conditions separates in IEF as several isoforms with equal amount of metal bound, meaning that hSOD1 heterogeneity does not depend on the metal content (33). We propose that the wide IEF heterogeneity of TX-insoluble mutant hSOD1 is a structural feature linked to aggregation propensity. However, we cannot exclude that the most acidic and low-abundant isoforms of TX-insoluble SOD1 correspond to oxidized isoforms, which we were not able to identify because they were under the detection limit.

In conclusion, using proteomic technologies, we thoroughly characterized for the first time SOD1 and its ubiquitination in spinal cord of G93A SOD1 mice at different stages of the disease. This is a further step in the understanding of the mechanism at the basis of SOD1 aggregation and ubiquitination. Using a sensitive proteome-based approach we could unequivocally show that SOD1 is oligoubiquitinated, although at a low extent and only at symptomatic stages of the disease. In line with this, co-localization of ubiquitin in SOD1 aggregates is hardly detectable by immunohistochemistry and electron microscopy. It is possible that oligoubiquitination of SOD1 underlines alternative mechanisms in disease pathogenesis rather than indicating dysfunction of protein degradation pathways. An intriguing hypothesis would be that the short polyubiquitin chain has a protective role in limiting further aggregation and proteasome overload. It seems now urgent to biochemically characterize the proteins that co-aggregate with SOD1 in each compartment of the cell, including mitochondria and endoplasmic reticulum (10,11). In view of the recent advancements in proteome technology, it appears feasible a comprehensive analysis of all the constituents of the aggregates. This would better define the role of aggregation in ALS and disclose the molecular basis of the pathology. Proteomic studies on aggregates are in progress in our laboratory.
REFERENCES


**FOOTNOTES**

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1The abbreviations used are: ALS, amyotrophic lateral sclerosis; SOD1, superoxide dismutase; fALS, familial form of ALS; WT, wild-type; TX, Triton X-100; 2DE, two-dimensional gel electrophoresis; IPG, immobilized-pH gradient; IEF, isoelectric focusing; WB, Western blotting; h, human; V8, endoprotease V8; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; GFAP, glial fibrillary acidic protein; ChAT, choline acetyl-transferase.

**FIGURE LEGENDS**

Fig. 1. Anti-hSOD1 2D WB of TX-insoluble aggregates (75 µg) from spinal cord of G93A SOD1 mice at 7 (A), 12 (B), 17 (C) and 26 (D) weeks of age. Anti-hSOD1 2D WB were also done with TX-insoluble aggregates from hippocampus of 26-week-old G93A SOD1 mice (E) and spinal cord of 26-week-old WT SOD1 mice. Proteins were separated by 2DE on 4-7 pH IPG strips, blotted and probed with anti-hSOD1 antibody (Upstate). Arrows indicate the trains of spots of monomeric and dimeric SOD1. Asterisks indicate trains of spots at +8, +16, +32 and +48 kDa from monomeric SOD1 (16 kDa), which could correspond to mono- and oligoubiquitinated SOD1. The samples are from pools of three animals.
Fig. 2. Anti-ubiquitin 2D WB (A) and anti-hSOD1 2D WB (B) of TX-insoluble extracts from spinal cord of a 26-week-old G93A SOD1 mouse. After anti-ubiquitin WB, the membrane was stripped and re-probed with anti-hSOD1 antibody. Arrows indicate trains of spots corresponding to monomeric and dimeric SOD1. Asterisks highlight trains of perfectly overlapping spots, indicating that G93A SOD1 is at least mono- and biubiquitinated.

Fig. 3. SYPRO Ruby-stained 2D gel of TX-insoluble extracts from spinal cord of 26-week-old G93A SOD1 mice. Proteins (75 µg) were separated by 2DE on 4-7 pH IPG strips. The sample is from a pool of five mice. The map indicates the spots matched with anti-hSOD1 2D WB, corresponding to monomeric G93A SOD1 (spot 1-5), monoubiquitinated (spot 6-8), biubiquitinated (spot 9-11), triubiquitinated (spot 12-13) and tetraubiquitinated (spot 14) G93A SOD1, on the basis of their Mr. Spots 6-14, concomitantly matching with anti-hSOD1 and anti-ubiquitin 2D WB, were further analyzed. Spots were digested with trypsin or V8 and fingerprints were analyzed by MALDI MS.

Fig. 4. Representative mass spectra of regions of tryptic fingerprints of spot 6 (A) and spot 9 (B) are shown. Panels A-B highlight: an ion of a SOD1 tryptic fragment, fragment 10-23 (SOD1<sub>10-23</sub>), an ion of an ubiquitin tryptic fragment, fragment 30-42 (UBI<sub>30-42</sub>), in (A) the ion of the GG-tryptic tailed 129-143 SOD1 fragment (SOD1<sub>129-143</sub>GG), which indicates that ubiquitin is linked to K136 of G93A SOD1, and in (B) the ion of the GG-tryptic tailed 43-54 ubiquitin fragment (UBI<sub>43-54</sub>GG), which indicates that ubiquitin branches at K48.

Fig. 5. High magnification of laser scanning confocal micrographs of immunofluorescence for ubiquitin and hSOD1 in a triple staining with ChAT (A-M) or GFAP (N-S) in the ventral horn lumbar spinal cords from WT (A-E) or G93A SOD1 mice at the late symptomatic stage of the disease (F-S). In the sections from WT SOD1 mice, both hSOD1 and ubiquitin labeling are homogeneously distributed in the ChAT positive motor neurons (A-E). Sections from G93A SOD1 mice show intense hSOD1 immunostaining preferentially at the edges of the vacuoles (arrows in L,M) and inside degenerating motor neurons (arrowhead in I,L). Ubiquitin immunoreactivity is distributed as large, intensely labeled spots scattered through the ventral horn (H,P), however, almost no co-localization is found with hSOD1 immunostaining (I,L). Sections from G93A SOD1 mice also show intense immunoreactivity for GFAP (O) but there is no accumulation of hSOD1 in the hypertrophic astrocytes (Q, S); occasionally, GFAP-positive cells characterized by round morphology revealed high levels of ubiquitin (R). Scale bars: 40 micron (A-D,F-I,N-Q); 20 micron (E,L,M,R,S).

Fig. 6. Electron micrographs of immunogold labeling of the lumbar ventral horn of symptomatic G93A SOD1 mice. A-B Single labeling for human SOD1. Low (A) and high (B) magnification of a dendritic aggregate (delineated by arrows in A) containing disorganized filaments and several gold particles coding for human SOD1 (15 nm gold particles). C-F. Double labeling for human SOD1 (10 nm gold particles) and for ubiquitin (20 nm gold particles). C, D. Low (C) and high (D) magnification of a filamentous dendritic aggregate containing numerous gold particles coding for human SOD1 (small particles) and for ubiquitin (large particles). E, F. Low (E) and high (F) magnification of a filamentous dendritic aggregate enriched in gold particles coding for human SOD1 (small particles) but not for ubiquitin (large particles). Scale bars: A, B, C, E = 1 micron; D, F = 0.5 microns.

Fig. 7. Anti-hSOD1 2D WB of different preparations of G93A SOD1: TX-soluble cytosolic G93A SOD1 (A), TX-insoluble G93A SOD1 (B), TX-soluble cytosolic G93A SOD1 subjected to two different treatments to stably modify Cys residues before IEF separation, alkylation with iodoacetamide (C) and irreversible oxidation to cysteic acid with performic acid (D). Proteins were separated by 2DE on 4-7 pH IPG strips, blotted and probed with anti-hSOD1 antibody (Upstate). E and F are zoomed images of SYPRO Ruby-stained 2DE, focused on SOD1 isoforms, of cytosolic protein fraction (E) (same sample as in A) and TX-insoluble proteins (F) (same sample as in B) of spinal cords from 26-week-old G93A SOD1 mice. Proteins were separated by 2DE on 3-10 pH IPG strips. The sample is from a pool of five mice. The three-dimensional (3D) visualization of the densitometric measurements is shown on the top of the 2DE images. The contribution of the single
isoform 1-5 is expressed as a percentage of the total monomeric G93A SOD1 in the sample. Percentages are the means of three replicates.

Fig. 8. Schematic representation of the proposed mechanism of SOD1 aggregation. Immature SOD1 undergoes post-translational modifications assisted by molecular chaperones leading to the dimeric metallated form. Mutant forms are expected to be less stable and form protein aggregates through dimer dissociation (49). The ubiquitin-ligase machinery is activated by the cell in the attempt to eliminate SOD1-rich aggregates. Mono- and oligoubiquitin chains are linked to SOD1 at accessible K sites. The oligoubiquitinated SOD1-rich inclusions accumulate possibly because of a not efficient recognition by the proteasome system and/or alteration of other degradation pathways. Oligoubiquitinated protein inclusions may also have a protective role, preventing further growth of the inclusion and toxic interactions, and enhancing neuronal survival (46).
Table 1

MALDI MS analysis of tryptic and V8 mass fingerprints of protein 2DE spots matching concomitantly with anti-SOD1 and anti-ubiquitin immunoactive spots (6-14)

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m/z obs observed m/z; m/z cal calculated m/z; V8, fragment produced by proteolysis with V8, the others are obtained by proteolysis with trypsin; polyubiquitin, signature peptide for K48-branched polyubiquitinated proteins (31,32); *The m/z cal refers to the N-terminally acetylated fragment; #The m/z cal refers to the fragment with carbamidomethylated Cys; (GG), SOD1 tryptic fragment with characteristic -GG- (with a mass shift of 114.04) tail, resulting from the cleavage of the linked ubiquitin, which indicates SOD1 site of ubiquitination.
Figure 1
Figure 2

Anti-ubiquitin

Anti-hSOD1

Figure 2
Figure 3
Figure 4

A  Monoubiquitinated G93A SOD1 spot 6

B  Biubiquitinated G93A SOD1 spot 9
Figure 5
Figure 7
Figure 8

misfolded isoforms
folded
ubiquitin

monomeric G93A SOD1

chaperones

aggregated G93A SOD1

ligase

holo-dimeric G93A SOD1

proteasome

oligoubiquitinated-aggregated G93A SOD1

protective role?
Insoluble mutant SOD1 is partly oligoubiquitinated in amyotrophic lateral sclerosis mice
Manuela Basso, Tania Massignan, Giuseppina Samengo, Cristina Cheroni, Silvia De Biasi, Mario Salamera, Caterina Bendotti and Valentina Bonetto

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