

Aggregate Formation in Cu,Zn Superoxide Dismutase-related Proteins*

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Marjatta Son‡, C. Dyan Cloyd‡, Jeffrey D. Rothstein§, Bhagya Rajendran‡,
and Jeffrey L. Elliott‡¶

From the ‡Department of Neurology, University of Texas, Southwestern Medical Center, Dallas, Texas 75390 and the §Department of Neurology, Johns Hopkins University, Baltimore, Maryland 21287

Aggregation of Cu,Zn superoxide dismutase (SOD1) protein is a pathologic hallmark of familial amyotrophic lateral sclerosis linked to mutations in the *SOD1* gene, although the structural motifs within mutant SOD1 that are responsible for its aggregation are unknown. Copper chaperone for SOD1 (CCS) and extracellular Cu,Zn superoxide dismutase (SOD3) have some sequence identity with SOD1, particularly in the regions of metal binding, but play no significant role in mutant SOD1-induced disease. We hypothesized that it would be possible to form CCS- or SOD3-positive aggregates by making these molecules resemble mutant SOD1 via the introduction of point mutations in codons homologous to a disease causing G85R SOD1 mutation. Using an *in vitro* assay system, we found that expression of wild type human CCS or a modified intracellular wild type SOD3 does not result in significant aggregate formation. In contrast, expression of G168R CCS or G146R SOD3 produced aggregates as evidenced by the presence of high molecular weight protein complexes on Western gels or inclusion bodies on immunofluorescence. CCS- and SOD3-positive inclusions appear to be ubiquitinated and localized to aggresomes. These results suggest that proteins sharing structural similarities to mutant SOD1 are also at risk for aggregate formation.

Mutations in Cu,Zn superoxide dismutase (SOD1)¹ cause one form of familial amyotrophic lateral sclerosis via a toxic gain of function (1–4). Disease-causing mutations span the entire SOD1 molecule and encompass virtually every structural and functional domain within the protein (5). Although the precise mechanisms underlying mutant SOD1 toxicity remain unclear, abnormal SOD1 protein folding that leads to aggregate formation is a pathologic hallmark of the disease and may be related to pathogenesis via inhibition of proteasome function (6–8). SOD1 aggregates can be visualized readily either as proteinaceous inclusions (immunofluorescence) or as high molecular weight complexes (Western blots) in the spinal cords of both autopsied familial amyotrophic lateral sclerosis patients and transgenic mice expressing mutant SOD1, which serve as an

animal model of the human disease (8–13). These SOD1-positive inclusions and complexes also may be generated in culture even in non-neuronal cells after transfection with mutant SOD1 and proteasome inhibition (12, 13). Because overexpression of wild type SOD1 in either culture or transgenic mice does not produce aggregates, the formation of aggregates may be related to structural motifs found in mutant SOD1. If this hypothesis is correct, then such aggregate-inducing motifs, if known, could be mimicked in proteins resembling SOD1 with the prediction that these proteins subsequently would form aggregates.

SOD1 is not the only superoxide dismutase requiring copper and zinc binding for normal activity. The copper chaperone for SOD1 (CCS), a 274-amino acid protein, has three structural domains and, like SOD1, is expressed ubiquitously in the central nervous system (14–16). Wild type CCS normally interacts with SOD1, allowing for the transfer of copper to SOD1, but does not play a role in mutant SOD1-induced disease (17–18). The first (residues 1–85) and third (residues 234–274) domains of CCS have no real sequence or structural homology with SOD1. Domain 2 (residues 86–233) does exhibit a 50% sequence identity with human SOD1, including three of the four histidine residues that bind copper as well as the three histidines and one aspartic acid that bind zinc, all arranged in a pattern similar to SOD1. Wild type CCS is catalytically inert. However, a D200H substitution, which reconstitutes the fourth copper binding site, is capable of restoring dismutase activity to CCS (19). Human extracellular SOD (SOD3) encoded by a gene distinct from *SOD1* is composed of 240 amino acids and harbors an 18-amino acid-long signal peptide targeting the protein for the extracellular compartment (20). In SOD3 neither the first 60 amino acids after the signal peptide nor the carboxyl-terminal portion (residues 212–240) bears any sequence or structural homology to SOD1. SOD3 does share a 40% sequence identity with SOD1 in the central portion of the molecule (residues 78–212), including all copper and zinc binding sites. Naturally occurring mutations in CCS or SOD3 have not been reported, nor have these proteins been linked to human disease.

Because the central domains in both CCS and SOD3 bear strong homology to SOD1, these proteins are ideal for testing whether the aggregate potential of mutant SOD1 can be mimicked in related proteins. In this work, we demonstrate that both CCS and SOD3 will form aggregates if modified to resemble mutant SOD1. By making a single amino acid substitution in CCS or SOD3 at a position homologous to a disease-causing site in SOD1, we can induce prominent CCS and SOD3 aggregates. These results indicate that proteins bearing structural motifs similar to mutant SOD1 are also at risk for aggregate formation.

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¶ To whom correspondence should be addressed. Tel.: 214-648-2871; Fax: 214-648-6320; E-mail: jeffrey.elliott@utsouthwestern.edu.

¹ The abbreviations used are: SOD1, Cu,Zn superoxide dismutase; CCS, copper chaperone(s) for SOD1; SOD3, extracellular Cu,Zn superoxide dismutase; IC, intracellular; PBS, phosphate-buffered saline; WT, wild type; HSP 70, heat shock protein 70; Cy, cyanine.

EXPERIMENTAL PROCEDURES

Plasmids and DNA Constructs—Wild type and G85R cDNAs for human SOD1 were provided by Dr. Don Cleveland (University of California, San Diego). Wild type human CCS cDNA was a gift from Dr. Jonathan Gitlin (Washington University, St. Louis, MO). G168R and D200H CCS cDNAs were created via PCR primer-directed mutagenesis using the following primers: CCS D200H, 5'-GGC CTC CCC GGC CCA GGT CAT CTT CTC CCT CAT GAA-3'; CCS G168R, 5'-CAC CGC GGA GAC CTG CGC AAT G-3'. Wild type cDNA for SOD3 was generated by reverse transcription PCR from human cerebellar mRNA provided by Dr. Lawrence Honig (University of Texas, Dallas, TX). This full-length SOD3 cDNA was used as a template to create an intracellular (IC) version of SOD3 lacking the first 54 bp that encode an 18-amino acid signal peptide. Two variations of IC-SOD3 were made, including a long form with the full-length carboxyl-terminal end and a short form lacking the last 42 bp, which encode a polybasic heparin binding motif. In both long and short IC-SOD3, we made a G146R mutation via PCR primer-directed mutagenesis. The following primers were used in generating the various SOD3 constructs: 5'-CAA GCT TGC CAT GTG GAC GGG CGA GGA CTC G-3' and 5'-AAG CGG CCG CCA TGT GGA CGG GCG AGG ACT CGG C-3', forward primers for IC-SOD3; 5'-ATC TAG ATC ACT CTG AGT GCT CCC GCG CCT-3' and 5'-AAA CTC GAG TCA CTC TGA GTG CTC CCG CGC CTG-3', reverse primers for the short form of SOD3; 5'-CGT CGC GGA CCG CGA AGT TGC GG-3', the primer for SOD3 G146R. All final constructs were confirmed by sequencing. For protein expression, all cDNAs were cloned into pBUDCE4 (Invitrogen), a eukaryotic expression vector containing both cytomegalovirus and elongation factor 1 α promoters. Two copies of each cDNA were inserted into the vector; one copy was inserted into the cytomegalovirus site, and the second copy was inserted into the elongation factor 1 α site.

Transfection and Cell Extracts—COS1 (ATCC) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin and streptomycin. For Western blot assays, COS1 cells were grown to 80–100% confluency, subcultured at a density of 2.5×10^5 cells/35-mm culture well, and transfected with the constructs of pBUDCE4 with cDNA inserts for SOD1, CCS, or SOD3 according to the protocol for FuGENE 6 reagent (Roche Molecular Biochemicals). COS1 cells were treated 48 h after transfection with 6.5 μ M lactacystin (Calbiochem) or with Me₂SO as a vehicle control for 20 h. Cells were harvested 68 h after transfection by trypsinization followed by three PBS washes, and pellets were stored at -80°C . Pellets were thawed in homogenization buffer consisting of 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 0.5% Triton X-100 (Sigma), 0.5% IGEPAL CA-630 (Sigma), and 1% protease inhibitor mixture (P 8340, Sigma) and homogenized. Samples were taken from the homogenates, and the rest was centrifuged at $15,000 \times g$ for 2 min. Pellets were washed once with the buffer, centrifuged again, and sonicated with a Fisher sonic dismembrator at 10% power with short bursts. Homogenates and sonicated pellet fractions were kept at -80°C . Protein concentrations were determined by BCA protein assay (Pierce).

Western Blotting—Extracts were boiled for 5 min in 250 mM Tris-HCl, pH 6.8, containing 2.5% β -mercaptoethanol, 1% SDS, 10% glycerol, and dye. The extracts then were resolved on 4–20% Tris-glycine SDS-PAGE and transferred onto a polyvinylidene difluoride membrane according to the manufacturer's instructions (XCell SureLock system, Invitrogen). After transfer, the membranes were blocked for overnight at 4°C in blocking buffer (PBS containing 0.2% I-Block (Applied Biosystems) and 0.1% Tween 20 (Sigma)). Membranes were incubated with primary antibody in blocking buffer for 2–3 h at room temperature, washed four times with blocking buffer, incubated with alkaline phosphatase-conjugated secondary antibody, diluted 1:5000 in blocking buffer (Applied Biosystems) for 2 h at room temperature, and washed again. The immunoreactive signals were detected using the CDP-Star chemiluminescent detection system (Applied Biosystems).

Immunofluorescence—COS1 cells were seeded at a density of 3.5×10^4 cells/chamber in 4-cell Lab-Tek II chamber slides (Nalge Nunc International). Cells were treated 48 h after transfection with 6.5 μ M lactacystin or with Me₂SO as a vehicle control for 20 h. 68 h after transfection, cells were washed three times in PBS, fixed with 100% methanol for 15 min at -20°C , washed again in PBS, and incubated for 3 to 4 h at room temperature in blocking buffer containing PBS with 5% normal serum from the same species as the secondary antibody (Jackson ImmunoResearch Laboratories, Inc.), 1% bovine serum albumin (fraction V, Sigma), 0.1% gelatin (fish skin gelatin, Sigma), and 0.05% sodium azide. Slides were incubated with primary antibodies in block-

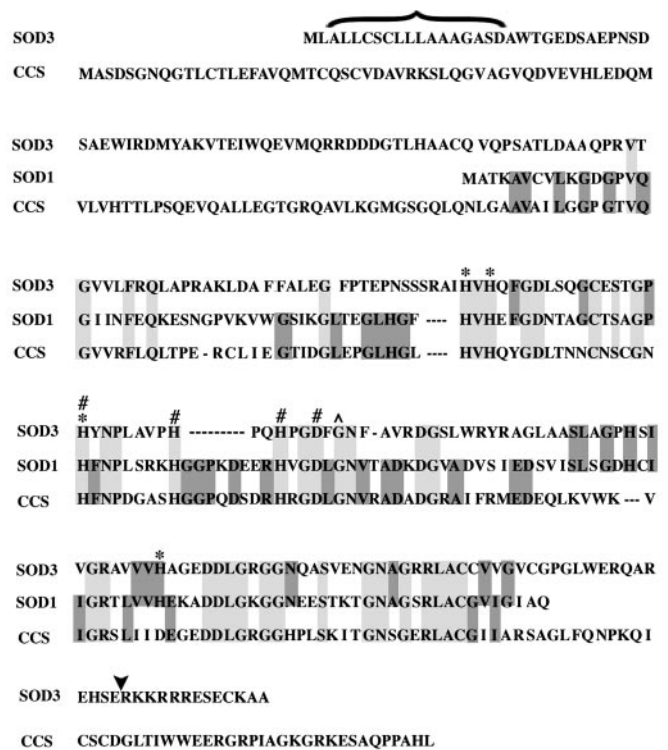


FIG. 1. Alignment of human SOD1, CCS, and SOD3 sequences. The amino acid residues shared by either SOD1 and CCS or SOD1 and SOD3 are dark gray; the residues shared by all three are light gray. An asterisk marks copper-binding amino acids, a number sign marks zinc-binding amino acids, a carat marks the Gly-85 codon in SOD1, a bracket indicates the SOD3 leader sequence, and an arrowhead indicates the site where the carboxyl-terminal heparin-binding residues were removed in SOD3.

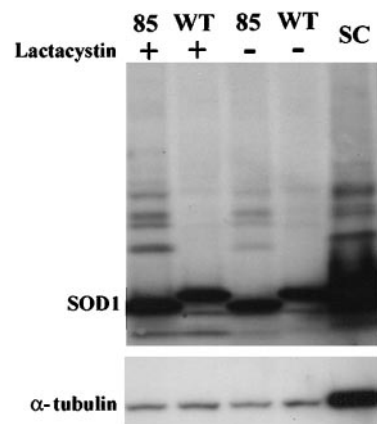


FIG. 2. SOD1-positive complexes in cells expressing human G85R SOD1. Pellet fractions of COS1 cell extracts transfected with WT or G85R human SOD1 and treated with lactacystin (+) or vehicle (-) were loaded on a Western gel (30 μ g of protein). Blots were probed with anti-human SOD1 antibody for detection of complexes and with anti- α -tubulin antibody after stripping for assessment of protein loading. Transfected, pelletized cells were homogenized and centrifuged at $15,000 \times g$ for 2 min. High molecular weight complexes were enriched in the pellet fractions, which represented 10–16% of the total protein. Spinal cord (SC) homogenate from a weak 7.5-month-old G93A SOD1 transgenic mouse was used as a control for complex formation. Expression of G85R SOD1 but not wild type SOD1 results in complex formation.

ing buffer overnight at 4°C , washed with PBS four times, incubated with cyanine-conjugated secondary antibodies (goat anti-rabbit or anti-mouse IgG labeled with Cy3 or Cy2, or donkey anti-sheep IgG labeled with Cy3 or Cy2, Jackson ImmunoResearch Laboratories, Inc.) in blocking buffer for 3 to 4 h, again washed with PBS four times, and mounted with Gel/Mount (Biomedica Corp.). Slides were viewed with a Nikon

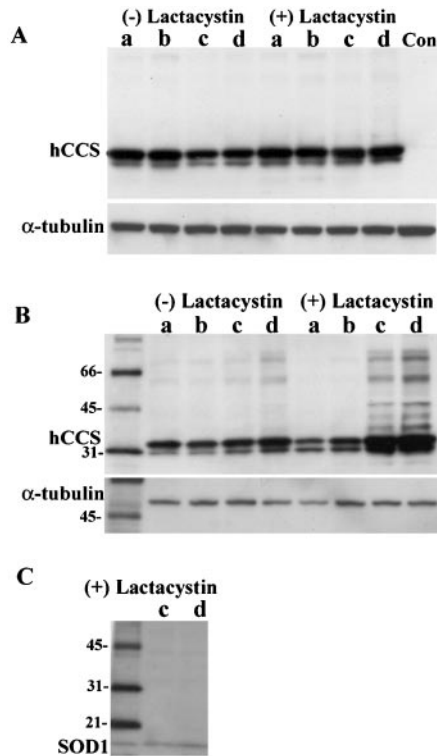


FIG. 3. Cells expressing G168R or G168R/D200H CCS form CCS-positive complexes. COS1 cells were transfected either with human WT (lane a), D200H (lane b), G168R (lane c), or G168R/D200H CCS (lane d) or with vector alone (Con), and the cells were treated with lactacystin (+) or vehicle (-). Transfected, pelletized cells were homogenized, and aliquots were taken for homogenate samples; the rest were centrifuged at $15,000 \times g$ for 2 min. High molecular weight complexes were enriched in the pellet fractions, which represented 10–16% of the total protein. **A**, 35 μ g of total cell homogenates from transfected COS1 cells were loaded on Western gels and probed with anti-CCS antibody (hCCS) and then with anti- α -tubulin antibody after stripping. Similar levels of human CCS protein were expressed for the differing CCS constructs. **B**, 30 μ g of pellet fractions of cell extracts were loaded on Western gels and probed with anti-CCS antibody (hCCS) and then with anti- α -tubulin antibody. Five independent experiments were performed with similar results; G168R and G168R/D200H mutations in CCS lead to CCS-positive complexes, whereas WT or D200H mutations do not. **C**, 30 μ g of pellet fractions of lactacystin-treated cell extracts were loaded on Western gels. Blots were probed with anti-human SOD1 antibody. Cells transfected with CCS mutations do not form SOD1-positive complexes.

fluorescent microscope under a fluorescein isothiocyanate, rhodamine, or dual excitation cube.

Antibodies—For Western blots, the following primary antibodies were used: sheep anti-human SOD1 (diluted 1:1000, Calbiochem), rabbit anti-CCS (diluted 1:4000, from Dr. Jeffrey Rothstein), rabbit anti-SOD3 (diluted 1:5000, gift from Dr. James Crapo), and mouse anti- α -tubulin (clone B-5-1-2, monoclonal, diluted 1:3000, Sigma). For immunofluorescence, rabbit anti-CCS and anti-SOD3 antibodies were used as primary antibodies as in the Western blots, and both were diluted 1:333. Also used as primary antibodies for immunofluorescence were the following mouse antibodies: anti-vimentin (clone V9, monoclonal, diluted 1:600, Dako), anti-HSP 70 (monoclonal, diluted 1:300, Stressgen), anti- γ -tubulin (clone GTU-88, monoclonal, diluted 1:300, Sigma), and anti-ubiquitin (clone FK2 against mono- and polyubiquitinated proteins, monoclonal, diluted 1:200, Biotrend).

RESULTS

Generation of Mutant CCS and Mutant SOD3—The alignment of amino acid sequences for human wild type SOD1, CCS, and SOD3 is shown in Fig. 1. A glycine to arginine change at codon 85 of SOD1 is capable of producing motor neuron disease both in humans and in transgenic mice characterized by prominent SOD1-positive aggregates (10). This glycine residue is conserved in both wild type CCS (codon 168) and wild type

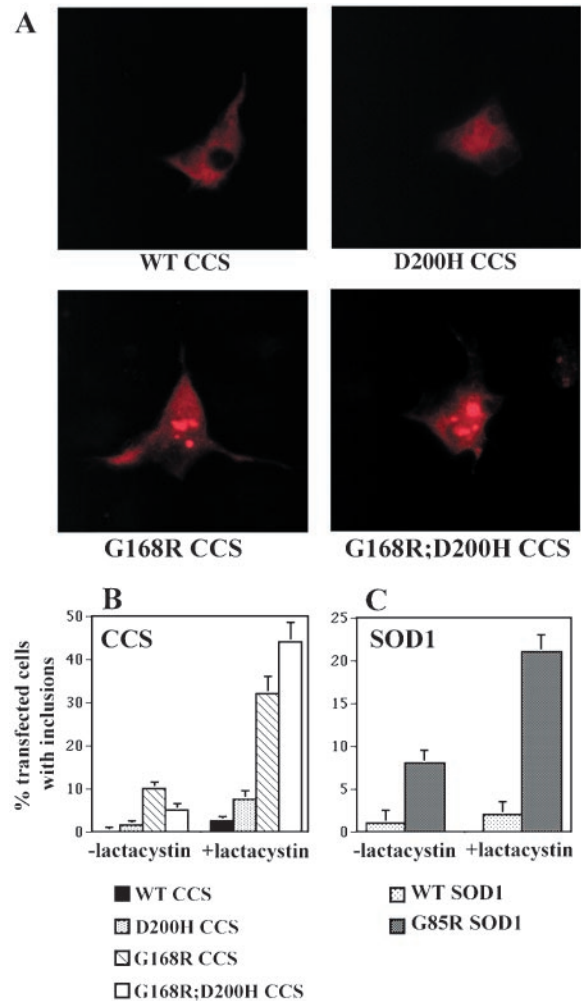


FIG. 4. CCS-positive inclusions are formed in cells expressing G168R-containing CCS mutants. COS1 cells were transfected with WT, D200H, G168R, or G168R/D200H CCS and treated with lactacystin. After fixation, transfected cells were identified with immunofluorescence by staining with rabbit anti-CCS antibody followed by Cy3-labeled secondary anti-rabbit antibody (red). **A**, CCS-positive inclusions form in cells transfected with G168R or G168R/D200H CCS but not with WT or D200H CCS. **B**, quantitation of CCS-positive inclusions in cells transfected with WT, D200H, G168R, or G168R/D200H CCS. Five separate experiments were performed, and in every experiment, at least 200 cells that were CCS-transfected and CCS-positive were counted in each group. Data show the percentage of CCS-transfected cells with CCS-positive inclusions in the presence (+) or absence (-) of lactacystin. **C**, quantitation of SOD1-positive inclusions in cells transfected with human WT or G85R SOD1. Data show the percentage of SOD1-transfected cells with SOD1-positive inclusions in the presence (+) or absence (-) of lactacystin.

SOD3 (codon 146). To test whether CCS and SOD3 can mimic mutant SOD1 and form aggregates, we made glycine to arginine mutations at this conserved codon (G168R in CCS and G146R in SOD3). We also made CCS bearing a D200H mutation either alone or in association with the G168R change. This D200H mutation restores the fourth copper binding site of CCS and establishes catalytic activity for the molecule. Because SOD3 is a secreted protein, we generated wild type and G146R SOD3 without the signal peptide to create an intracellular SOD3. The carboxyl terminus of SOD3 contains a polybasic heparin binding domain that may be cleaved or maintained depending on tissue type (21). For these experiments, we generated intracellular wild type and G146R SOD3 with and without these polybasic residues to give long and short forms of SOD3.

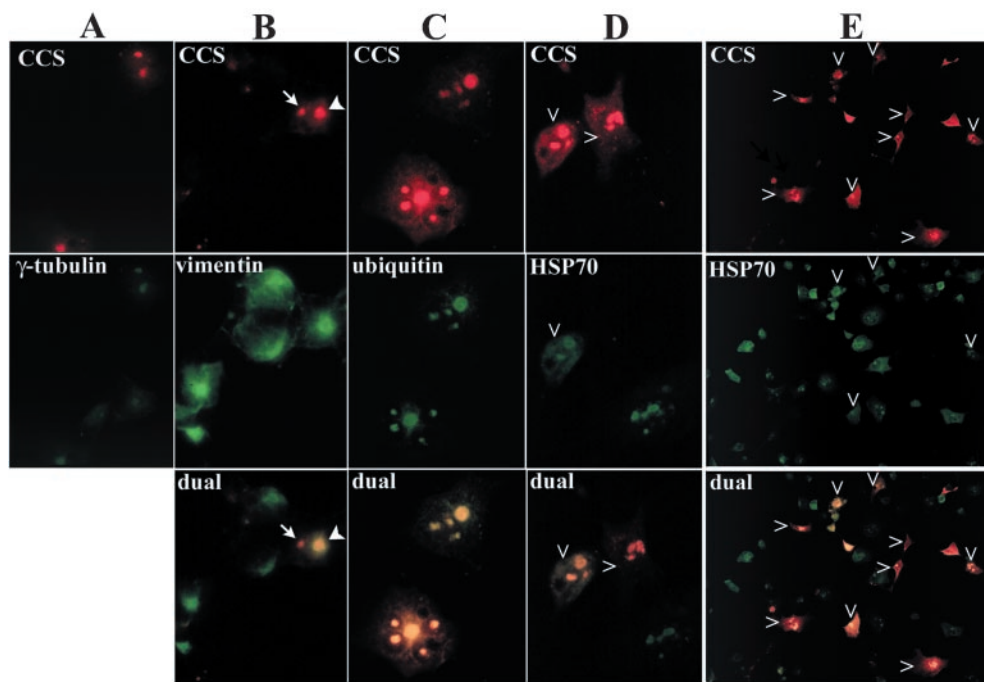


FIG. 5. **Characterization of CCS-positive inclusions.** COS1 cells were transfected with G168R/D200H CCS and treated with lactacystin. A, after fixation, cells were labeled with rabbit anti-CCS antibody and stained with secondary Cy3 anti-rabbit antibody (red) and with mouse anti- γ -tubulin (lane A), anti-vimentin (lane B), anti-ubiquitin (lane C), or anti-HSP 70 antibody (lanes D and E), all of which were stained with secondary Cy2 anti-mouse antibody (green). Several, but not all, of the CCS-positive inclusions appear to be in aggresomes. CCS-positive inclusions colocalize with ubiquitin but not always with HSP 70. Magnification, $\times 600$ (lanes A–D) or $\times 200$ (lane E).

Aggregate Formation in G168R CCS—To assess potential aggregate formation with CCS, we modified an *in vitro* method used previously to study SOD1 aggregates (12). In this assay, COS1 cells transfected with G85R SOD1 but not with wild type SOD1 form high molecular weight SOD1-positive complexes on Western gels. These SOD1 complexes are concentrated in pellet fractions of total cell lysate and can be accentuated by proteasome inhibition (Fig. 2). We therefore transfected COS1 cells with human wild type, D200H, G168R, and G168R/D200H CCS. Cells transfected with these four CCS constructs expressed similar levels of CCS protein when examined in Western blots on total cell homogenates using α -tubulin as a control (Fig. 3A). CCS antibody clearly recognized the various mutant forms of CCS in addition to wild type CCS. Base-line COS1 cell expression of CCS was extremely low and barely detectable in Western blots. No clear CCS-positive aggregates were observed in these total cell lysates even after treatment with the proteasome inhibitor lactacystin.

Because SOD1-positive complexes are concentrated in the pellet fraction rather than in total cell homogenates, we performed Western blots on the pellet fraction from CCS-transfected cells. Visible CCS-positive high molecular weight protein complexes were observed in the pellet fraction of COS1 cells transfected with G168R or G168R/D200H CCS but not with wild type or D200H CCS. After treatment with the proteasome inhibitor lactacystin, cells transfected with G168R or G168R/D200H CCS generated even more prominent CCS-positive high molecular weight protein complexes, whereas cells transfected with wild type and D200H CCS did not form these complexes (Fig. 3B). Thus, complex generation is not related to the overexpression of CCS protein but rather to the expression of CCS bearing the G168R mutation. The observation that D200H expression alone did not induce complexes indicates that a single mutation in CCS by itself does not automatically induce complex formation.

Because of the known interaction between CCS and SOD1, we next investigated whether cells with CCS-positive com-

plexes also developed SOD1-positive complexes. Western blot analysis did not confirm the presence of SOD1-positive complexes in CCS-transfected cells (Fig. 3C).

We used immunofluorescence to assay possible inclusion body formation with the various CCS constructs. Expression of G168R and G168R/D200H CCS yielded prominent CCS-positive inclusions (Fig. 4A). The quantitation of CCS-positive inclusions in COS1 cells transfected with wild type or mutant CCS is shown in Fig. 4B. In non-lactacystin-treated cultures, few cells transfected with G168R or G168R/D200H CCS formed CCS-positive inclusions. Virtually no inclusions were noted in cells expressing wild type or D200H CCS. After lactacystin treatment, close to 45% of transfected cells expressing either G168R or G168R/D200H CCS formed CCS-positive inclusions. Again, cells transfected with wild type or D200H CCS generally did not form CCS-positive inclusions. The quantitation of SOD1-positive inclusions in COS1 cells transfected with wild type or G85R SOD1 is shown in Fig. 4C. Expression of G85R SOD1 produced SOD1 inclusions, whereas WT SOD1 did not. These results indicate that CCS bearing G168R mutations will form CCS inclusions under conditions similar to those in which disease-causing SOD1 mutations have been found to cause SOD1-positive inclusions.

Characterization of CCS-positive Inclusions—To determine whether CCS inclusions were present in aggresomes, we used antibodies against γ -tubulin and vimentin. γ -Tubulin colocalized with CCS-positive inclusions, indicating that the inclusions were found in the organizing center of the microtubules (Fig. 5A). Many CCS-positive inclusions also were found within vimentin-positive cages, thereby confirming localization within aggresomes (Fig. 5B, arrowhead). However, occasional CCS-positive inclusions were located clearly outside of this vimentin-positive cage (Fig. 5B, arrow). CCS-positive inclusions in G168R/D200H-transfected cells were smaller after a shorter treatment with lactacystin (7 h), but their localization was similar (data not shown). It is therefore possible that CCS-positive inclusions not localized in aggresomes may be in tran-

sit either to or from aggresomes.

Further dual localization studies confirmed that CCS-positive inclusions appear to be ubiquitin-positive (Fig. 5C). Protein chaperones such as HSP 70 have been suggested to be important in modulating mutant SOD1 protein folding and have been associated with SOD1-positive inclusions in mutant SOD1 transgenic mice (7). Treatment with lactacystin causes a nonspecific increase in HSP 70 expression with the formation of HSP 70-positive inclusions (data not shown). CCS inclusions were positive for HSP 70 in some cells (Fig. 5, D and E, marked with \vee) but not in others (Fig. 5, D and E, marked with $>$), suggesting that CCS inclusions are not always dependent on HSP 70 expression.

G146R IC-SOD3 Expression Causes Aggregate Formation—To assess possible aggregate formation with G146R IC-SOD3 expression, we used the same COS1 cell assay system. COS1 cells were transfected with intracellular wild type and G146R SOD3 (both short and long forms) and then treated with proteasome inhibitors. Western blots confirmed expression of IC-SOD3 within COS1 cells (Fig. 6A). However, we were not able to distinguish SOD3 complexes clearly in the pellet fractions on Western blots either in the presence or absence of lactacystin.

We then used immunofluorescence to determine whether expression of G146R IC-SOD3 would yield SOD3-positive inclusions. In the absence of lactacystin treatment, less than 4% of cells transfected with wild type intracellular SOD3 (either short or long forms) formed SOD3-positive inclusions (Fig. 6B). In contrast, 14% of G146R SOD3 short- and 10% of G146R SOD3 long-transfected cells exhibited SOD3-positive inclusions without proteasome inhibition. These percentages are considerably larger than those observed for COS1 cells transfected with either G168R CCS or G85R SOD1, suggesting that the intracellular expression of G146R IC-SOD3 may have greater aggregation potential.

We treated transfected cells with the proteasome inhibitor lactacystin to determine whether the percentage of inclusion-forming cells could be increased. As a control, we found that expression of wild type human native SOD3 (extracellular), even in the presence of lactacystin, did not yield inclusions. After lactacystin treatment, a large percentage of cells transfected with intracellular SOD3, either wild type or G146R, formed complexes. However, the percentage was significantly higher in cells expressing G146R compared with wild type SOD3, indicating that the presence of a G146R mutation contributed to a substantial increase in the aggregation potential of the SOD3 protein. Other changes in the protein, such as the presence or absence of the terminal heparin binding domain, had no appreciable effect on the inclusion formation of G146R SOD3.

We also performed colocalization studies of SOD3-positive inclusions. SOD3-positive inclusions appear to colocalize partially with vimentin cages (Fig. 6C) and with γ -tubulin (data not shown). SOD3 inclusions also appear to be ubiquitin-positive (Fig. 6C) but not always HSP 70-positive (data not shown). These results indicate that SOD3-positive and CCS-positive inclusions share overall similar characteristics.

DISCUSSION

Aggregate formation of mutant proteins is characteristic of many adult onset neurodegenerative conditions (22–25). This pattern holds true for SOD1-related disease, in which SOD1-positive aggregates are found in both human beings and transgenic mice that harbor a mutant *SOD1* gene. Unlike Huntington's disease, where disease-causing polyglutamine repeats represent an aggregate-forming motif found in proteins linked to neurodegenerative diseases, the aggregate-forming motifs

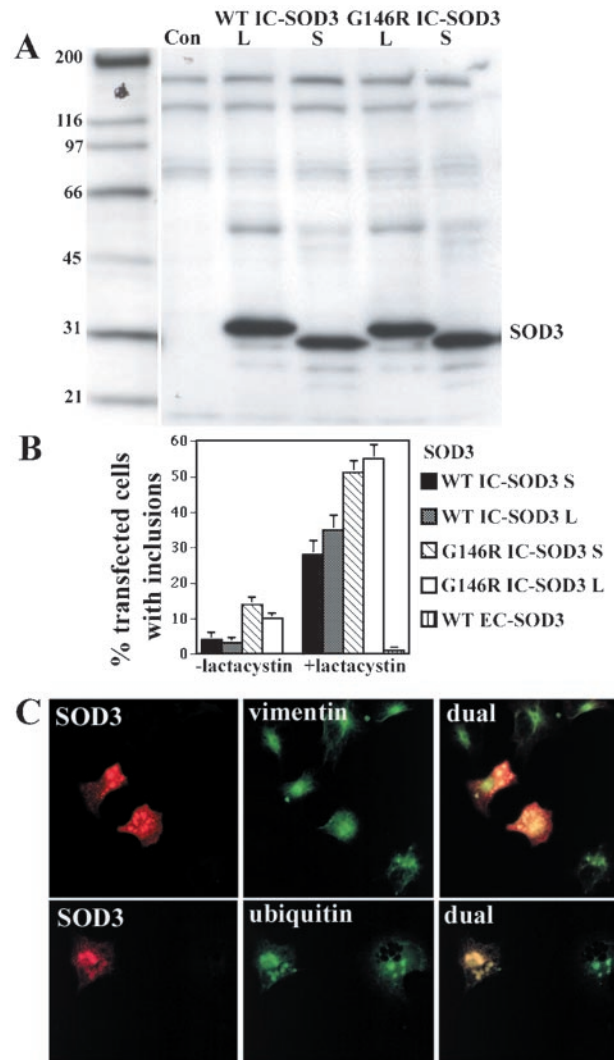


FIG. 6. Expression of human wild type and G146R intracellular SOD3 *in vitro*. COS1 cells were transfected with short WT IC-SOD3, long WT IC-SOD3, short G146R IC-SOD3, long G146R, or vector alone (Con). A, 35 μ g of cell homogenates were loaded on Western gels. Blots were probed with anti-human SOD3 antibody. B, quantitation of SOD3-positive inclusions. COS1 cells were transfected with WT IC-SOD3 or G146R IC-SOD3, either short form (S) or long form (L), or with WT extracellular (EC) SOD3. After fixation, transfected cells were identified with immunofluorescence by staining with rabbit anti-human SOD3 antibody followed by Cy3-labeled secondary anti-rabbit antibody. Five separate experiments were performed, and in every experiment, at least 200 SOD3-transfected and SOD3-positive cells were counted in each group. Data show the percentages of SOD3-transfected cells with SOD3-positive inclusions in the presence (+) or absence (–) of lactacystin. In the absence of lactacystin, G146R IC-SOD3 formed inclusions. After lactacystin treatment, cells transfected with all intracellular forms of SOD3 produce inclusions. However, significantly more inclusions are formed in cells transfected with G146R mutant forms of IC-SOD3 than with WT forms of IC-SOD3. C, characterization of SOD3-positive inclusions. Cells were transfected with the long form of G146R IC-SOD3 and treated with lactacystin. After fixation, cells were labeled with anti-human SOD3 antibody and stained with secondary Cy3 anti-rabbit antibody (red) and with either mouse anti-vimentin or anti-ubiquitin antibody with secondary Cy2 anti-mouse antibody (green). The overall pattern of SOD3-positive inclusions appears to be similar to what is observed for CCS inclusions. SOD3-positive inclusions appear to be ubiquitinated, and many appear to localize within vimentin cages.

within SOD1 remain obscure. It is also unclear whether proteins that are related to mutant SOD1 and harbor similar structural motifs form aggregates. In this work, we demonstrate that selective single amino acid mutations in proteins

related to SOD1 will induce aggregate formation of these proteins. These results suggest that motifs shared in mutant SOD1, CCS, and SOD3 can lead to aggregate formation.

Although the structural properties within SOD1, CCS, and SOD3 that predispose these proteins to form aggregates are unclear, there is evidence to suggest that metal binding may be important. These proteins share similar copper- and zinc-binding residues arranged in nearly identical spatial patterns. Although much attention was focused initially on copper within SOD1 as possibly related to disease pathogenesis, recent work has suggested that copper binding may not be relevant (18, 26). Our findings here provide additional evidence that copper binding may not be critical to aggregate formation in that mutations in the copper-binding Asp-200 residue of CCS do not alter the ability of CCS to form aggregates.

There is stronger evidence to support an important role for zinc binding to SOD1 for both disease pathogenesis and aggregate potential. Altering the levels of zinc-binding proteins can significantly affect zinc levels within the central nervous system and can change the disease course in mutant SOD1 transgenic mice (27). Although zinc binding sites may be preserved in SOD1 mutations causing disease, studies have shown that zinc binding to mutant SOD1 is abnormal and has consequences for both the enzymatic and structural integrity of the protein (28–29). Changes in zinc binding can enhance the tendency of certain proteins, including prion protein and β -amyloid, to form insoluble complexes, and such mechanisms may contribute to the formation of SOD1 aggregates observed in the disease (30–32). Prion protein is especially interesting in that like SOD1, it possesses dismutase activity and binds both copper and zinc (33–35). Mutations in prion protein are responsible primarily for several human dementias, but cases of motor neuron involvement with amyotrophy, similar to amyotrophic lateral sclerosis, have been reported (36).

The biologic consequences of mutant SOD1 aggregate formation are unclear. Protein aggregates may be directly harmful to cells. Recent work has found that protein aggregates interfere with proteasome function, thus potentially leading to a cycle of protein processing dysfunction, increased aggregate formation, and consequent further declines in proteasome protein clearing (6). However, the formation of protein aggregates also might be helpful for cells and might be used as a potential way to sequester harmful proteins (37). It also is possible that aggregates may be of little positive or negative consequence in disease; rather, they are only a marker of cellular pathology (38). Whether protein aggregates contribute to mutant SOD1-induced motor neuron disease is unclear, but certainly there exists a good correlation between aggregate formation and the disease state. Based on this fact, we would predict that expression of G168R CCS or G146R intracellular SOD3 *in vivo* would lead to a motor phenotype similar to that found in expression of G85R SOD1. Such experiments will test the biological significance of aggregate formation in motor neuron disease and are being pursued actively.

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