Structural properties and neuronal toxicity of amyotrophic lateral sclerosis–associated Cu/Zn superoxide dismutase 1 aggregates

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The appearance of protein aggregates is a characteristic of protein misfolding disorders including familial amyotrophic lateral sclerosis, a neurodegenerative disease caused by inherited mutations in Cu/Zn superoxide dismutase 1 (SOD1). Here, we use live cell imaging of neuronal and nonneuronal cells to show that SOD1 mutants (G85R and G93A) form an aggregate structure consisting of immobile scaffolds, through which noninteracting cellular proteins can diffuse. Hsp70 transiently interacts, in a chaperone activity-dependent manner, with these mutant SOD1 aggregate structures. In contrast, the proteasome is sequestered within the aggregate structure, an event associated with decreased degradation of a proteasomal substrate. Through the use of time-lapse microscopy of individual cells, we show that nearly all (90%) aggregate-containing cells express higher levels of mutant SOD1 and died within 48 h, whereas 70% of cells expressing a soluble mutant SOD1 survived. Our results demonstrate that SOD1 G85R and G93A mutants form a distinct class of aggregate structures in cells destined for neuronal cell death.

Introduction

Familial amyotrophic lateral sclerosis (fALS) is a neurodegenerative disorder resulting in the selective loss of motor neurons (Cleveland and Rothstein, 2001; Bruijn et al., 2004). Although a number of genetic defects have been linked to the development of fALS, the autosomal-dominant inheritance of mutant Cu/Zn superoxide dismutase 1 (SOD1) accounts for ~20% of cases (Rosen et al., 1993). Although numerous potential mechanisms of cytotoxicity have been proposed, including the depletion of molecular chaperones, sequestration and dysfunction of the proteasome, impairment of axonal transport, aberrant dismutase activities, and mitochondrial disruption by SOD1 mutations (Bruijn et al., 2004), the contribution of each proposed mechanism remains unclear. Although more than 100 different SOD1 mutations cause fALS, some of which retain complete enzymatic activity (Borchelt et al., 1994; Bowling et al., 1995; Gaudette et al., 2000), it has been well established that mutant SOD1-mediated toxicity is caused by a toxic gain-of-function rather than the loss of SOD1 activity (Bruijn et al., 1998, 2004; Cleveland and Rothstein, 2001). A common feature, shared by most SOD1 mutations both in mutant SOD1 transgenic mouse models (Bruijn et al., 1997; Johnston et al., 2000; Watanabe et al., 2001; Wang et al., 2002) and in tissue samples from fALS patients (Shibata et al., 1996; Kato et al., 2000), is the appearance of intracellular inclusions containing detergent-insoluble protein aggregates. Such proteinaceous inclusions also occur in many other neurodegenerative disorders (Taylor et al., 2002) and their presence suggests an inability of the protein quality control machinery (including molecular chaperones and the ubiquitin–proteasome system) to efficiently recognize, fold, and degrade this class of abnormal proteins (Kopito, 2000; Goldberg, 2003). Association of the proteasome, various molecular chaperones (i.e., Hsp40 and Hsp70), and other cellular proteins including transcription factors with aggregates (Bence et al., 2001; Watanabe et al., 2001; Kim et al., 2002; Takeuchi et al., 2002; Holmberg et al., 2004; Bennett et al., 2005) has been proposed as a common mechanism for aggregate-associated cytotoxicity (Cleveland and Rothstein, 2001; Sherman and Goldberg, 2001; Taylor et al., 2002; Goldberg, 2003; Bruijn et al., 2004).

The role of aggregates in cytoprotection or cytotoxicity of neuronal cell dysgenesis is a point of some controversy. Some studies have shown no correlation between polyglutamine-containing aggregates and increased cell death (Saudou et al., 2001; and others have suggested a role for polyglutamine-containing aggregates in the neurodegenerative process (Watanabe et al., 2001; Johnston et al., 2000; Cleveland and Rothstein, 2001). The appearance of protein aggregates is a characteristic feature of many neurodegenerative diseases, including familial amyotrophic lateral sclerosis (fALS), a neurodegenerative disorder caused by inherited mutations in Cu/Zn superoxide dismutase 1 (SOD1). Here, we use live cell imaging of neuronal and nonneuronal cells to show that SOD1 mutants (G85R and G93A) form an aggregate structure consisting of immobile scaffolds, through which noninteracting cellular proteins can diffuse. Hsp70 transiently interacts, in a chaperone activity-dependent manner, with these mutant SOD1 aggregate structures. In contrast, the proteasome is sequestered within the aggregate structure, an event associated with decreased degradation of a proteasomal substrate. Through the use of time-lapse microscopy of individual cells, we show that nearly all (90%) aggregate-containing cells express higher levels of mutant SOD1 and died within 48 h, whereas 70% of cells expressing a soluble mutant SOD1 survived. Our results demonstrate that SOD1 G85R and G93A mutants form a distinct class of aggregate structures in cells destined for neuronal cell death.

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et al., 1998; Zhou et al., 2001; Arrasate et al., 2004) whereas other studies have identified a direct relationship between aggregates and cellular toxicity (Nucifora et al., 2001; Wyttenbach et al., 2001; Jiang et al., 2003; Schaffar et al., 2004). Whether this controversy is due to biologically relevant differences associated with specific features of an aggregation-prone protein or technical differences (i.e., level of protein overexpression, cell type, method of detection/characterization) is unclear. The absence of a uniform definition for an aggregate or inclusion body may further contribute to such differences. Cell biological studies have typically used visual inspection and immunolocalization using fixed cells, complemented by biochemical methods that detect insoluble proteins using PAGE or a filter-trap assay of cell extracts. Other methods using fixed cells have used immunohistochemistry analysis and electron microscopy to visualize aggregate and inclusion structures at higher resolution (Bruijn et al., 1997, 1998). From such approaches, aggregates formed by expression of either mutant SOD1 or huntingtin have been suggested to have similar morphologies and structure. However, the recent applications of dynamic live cell imaging techniques (i.e., fluorescent loss in photobleaching [FLIP], and fluorescence resonance energy transfer [FRET]) have revealed unexpected differences in the aggregate structure of the polyglutamine proteins, ataxin 1 and mutant huntingtin (Chai et al., 2002; Kim et al., 2002; Stenoien et al., 2002; Holmberg et al., 2004), leading to the suggestion that other neurodegenerative disease-associated proteins might also form distinct classes of aggregates.

In this study, we assess the composition and dynamic properties of aggregates formed by G85R and G93A, two well-characterized mutant SOD1 proteins, to gain insight into the mechanistic events associated with SOD1 aggregate formation and cytotoxicity. Using live-cell imaging of individual PC12 neuronal and HeLa cells, we find that mutant SOD1 proteins form aggregates containing two distinct populations of protein, one highly aggregated and immobile forming a scaffold, and a second population mobile and diffusing within cavities present throughout the immobile scaffold. Additionally, in vivo these aggregates recruit, sequester, and decrease activity of the proteasome. In contrast, the molecular chaperone Hsp70 interacts transiently with mutant SOD1 aggregates. Using an automated time-lapse imaging technique to follow the fate of individual PC12 cells, we find that aggregate formation is lethal to the cell. These studies reveal that the aggregate structures formed by mutant SOD1 proteins, distinct from previously observed aggregates formed by other misfolded proteins, are associated with decreased cell survival.

Results

Mutant SOD1 forms aggregates containing mobile and immobile fractions

We monitored the in vivo dynamics of wild-type (WT) and mutant SOD1 proteins, as well as their molecular interactions in differentiated PC12 neuronal cells and HeLa cells, by expressing CFP and YFP fusions with WT (WT-CFP and WT-YFP) and mutant (G85R-CFP, G85R-YFP, G93A-CFP, G93A-YFP) SOD1 proteins. WT, G85R, and G93A fused to CFP (Fig. 1 A) or YFP (unpublished data) accumulated to equivalent levels in transiently transfected PC12 cells and both mutant proteins were detected as full-length products of expected molecular
Table I. Quantitative FRAP analysis of YFP-tagged proteins

| Sample                        | Diffusing fraction | D    | Nondiffusing fraction | kD  |
|-------------------------------|--------------------|------|-----------------------|-----|
|                               | %                  | μm/s | %                     | s⁻¹ |
| WT-YFP                        | 95.0 ± 2.3         | 2.35 ± 0.60 | 5.0 ± 2.3 | N/A |
| G85R-YFP diffuse              | 92.1 ± 3.1         | 1.67 ± 0.29 | 7.9 ± 3.1 | 0.013 ± 0.008 |
| G93A-YFP diffuse              | 92.1 ± 2.9         | 1.47 ± 0.23 | 7.9 ± 2.9 | 0.011 ± 0.006 |
| G85R-YFP aggregate            | 23.2 ± 3.2         | 1.27 ± 0.72 | 76.8 ± 1.9 | <0.001 |
| G93A-YFP aggregate            | 38.4 ± 2.2         | 0.88 ± 0.26 | 61.6 ± 2.2 | <0.001 |
| LMP2-YFP (diffuse G85R-CFP)   | 94.6 ± 2.8         | 2.20 ± 0.39 | 5.4 ± 2.8 | 0.012 ± 0.009 |
| LMP2-YFP (G85R-CFP aggregate)| 76.7 ± 1.2         | 1.78 ± 0.50 | 72.4 ± 1.2 | <0.001 |
| Hsp70-YFP (diffuse G85R-CFP)  | 85.8 ± 1.9         | 1.76 ± 0.31 | 14.2 ± 1.8 | 0.008 ± 0.002 |
| Hsp70-YFP (G85R-CFP aggregate)| 65.2 ± 1.9         | 1.07 ± 0.12 | 34.8 ± 1.9 | 0.004 ± 0.001 |
| Hsp70-SBDx-YFP (diffuse G85R-CFP)| 92.6 ± 1.9        | 1.93 ± 0.28 | 7.4 ± 1.9 | 0.009 ± 0.006 |
| Hsp70-SBDx-YFP (G85R-CFP aggregate)| 76.4 ± 1.6     | 2.13 ± 0.34 | 23.6 ± 1.6 | 0.003 ± 0.001 |

FRAP data from Figs. 1F, 3B, and 4B were analyzed with GraphPad Prism software, as described in Supplementary Information, Methods online. Data are displayed as mean ± SEM. Two-tailed t-test analysis (95% confidence) was used to compare the statistical difference between data sets: *** P < 0.001; ns, P > 0.05.

To investigate the mobility of diffuse and aggregated SOD1 proteins, we performed quantitative and qualitative FRAP analysis in PC12 cells (Lippincott-Schwartz et al., 2001). Compared with WT-YFP and the diffusely localized G85R-YFP and G93A-YFP proteins, which were completely mobile, aggregated G85R-YFP and G93A-YFP proteins showed dramatically reduced mobility (Fig. 1, E and F, and Table I). Unexpectedly, the pattern of fluorescence recovery within either mutant SOD1 aggregate was not consistent with the presence of a single protein population, as reported with aggregates formed by the mutant huntingtin protein (Kim et al., 2002; Holmberg et al., 2004). The FRAP data suggested that both G85R and G93A mutant SOD1 aggregates are comprised of two protein populations. Mathematical analyses of FRAP data indeed supported these assertions, as both curves fit to a recovery consistent with two populations (see Materials and methods). An immobile (nondiffusing) protein population, with off-rates <0.001 s⁻¹, predominated and accounted for 76.8 ± 1.9% (G85R-YFP) and 61.6 ± 2.2% (G93A-YFP) of total fluorescent protein within the aggregates (Fig. 1, E and F, and Table I). These fractions remained immobile even when the analysis was extended to a 10-min period (unpublished data). The presence of a slow off-rate and a persistent immobile population reveals a long residency time (τ > 1,000 s) of mutant SOD1 protein in this immobile form, corresponding to stable SOD1 aggregate structures. In contrast, the remaining 23.2 ± 1.9% (G85R-YFP) and 38.4 ± 2.2% (G93A-YFP) of protein within each aggregate was diffused (Fig. 1, E and F, and Table I).

The “diffuse” fractions of the mutant SOD1 proteins are not the same as the “diffuse” WT protein, as the diffusion coefficients are 1.47 μm²/s (G85R-YFP) and 1.67 μm²/s (G93A-YFP), compared with 2.35 μm²/s (WT-YFP; Table I). These results together with additional analyses (see Materials and methods) suggest that the diffuse G85R-YFP corresponds to higher-order structures of approximate molecular weights of 250 kD. This could reflect the formation of oligomers of G85R-YFP or the stable association of other cellular proteins with G85R-YFP. These results suggest that mutant SOD1 may form oligomers that are transiently diffuse, followed by higher-order oligomerization and thus populations with slower diffusion, and finally aggregation and immobilization (no diffusion). Similar to differentiated PC12 cells, we observed that both a diffuse and immobile (nondiffusing) fraction of protein exist within mutant SOD1 aggregates in undifferentiated PC12 and HeLa cells (Fig. S1). From these results, we conclude that both G85R-YFP and G93A-YFP form immobile aggregates; however, differences in the fraction of each mutant SOD1 protein present in the aggregated state reveals subtle distinctions between the two mutant proteins.

To test the hypothesis that two protein populations are indeed present within the mutant SOD1 aggregates, we used a complimentary imaging technique, FLIP. Fluorescence intensities...
of G85R-YFP and G93A-YFP within and outside the aggregate were measured while a region adjacent to the aggregate was continuously photobleached. We expected that a fraction of protein within the aggregate would remain intact while a second population would diffuse out of the aggregate and become photobleached. As with FRAP analyses, our FLIP data presented a better fit to a two-phase exponential decay curve, confirming the presence of two distinct populations of protein. FLIP revealed that 45% (G85R-YFP) and 38% (G93A-YFP) of the initial fluorescence within the aggregate persisted over a 10-min period (Fig. 1, G and H), supporting our hypothesis that one population of mutant SOD1 protein forms a stable aggregate structure. In contrast, the fluorescence intensities of WT-YFP, as well as the diffusely localized G85R-YFP and G93A-YFP proteins, were rapidly reduced and continued to decrease to basal fluorescence after 10 min of continuous photobleaching, indicative of a single mobile population (Fig. 1, G and H). Together, the FRAP and FLIP analyses suggest that mutant SOD1 (G85R and G93A) aggregates are composed of two protein populations, one mobile and diffusing and a second highly immobile and aggregated population.

### Mutant SOD1 aggregates form a disordered “honeycomb-like” porous structure

FRAP and FLIP analyses suggested that mutant SOD1 proteins formed porous aggregate structures through which other soluble cellular proteins may diffuse. This is in contrast to aggregates formed by mutant huntingtin and polyglutamine-YFP proteins, which we and others have shown form a solid and immobile core structure (Chai et al., 2002; Kim et al., 2002; Holmberg et al., 2004). To determine whether noninteracting soluble proteins can indeed diffuse through the mutant SOD1 aggregate structures, PC12 cells were transiently cotransfected with a construct encoding an aggregation-prone protein (G85R-CFP, G93A-CFP, or httQ78-CFP) and a soluble YFP or WT-YFP. YFP (Fig. 2 A) and WT-YFP (Fig. S2 available at http://www.jcb.org/cgi/content/full/jcb.200504050/DC1) proteins displayed a diffuse and uniform pattern of localization, both within and outside the mutant SOD1 aggregates. In contrast, aggregates formed by expression of an expanded polyglutamine-containing mutant huntingtin protein (httQ78-CFP) exhibited a solid core that completely excluded YFP (Fig. 2 A). These results suggested that soluble proteins can colocalize with the aggregate structures formed by mutant SOD1.

Whereas standard colocalization methods do not establish whether associated proteins are nonspecifically trapped or diffuse within the aggregate, FLIP analysis provides a direct test, as we would expect that soluble proteins can diffuse rapidly out of the aggregate structures and become photobleached. The initial fluorescence intensities of YFP, either within or outside the mutant SOD1 (G85R-CFP or G93A-CFP) aggregates, were rapidly reduced and continued to decrease to basal fluorescence after 10 min of continuous photobleaching (Fig. 2, B and C). These results were similar to the diffusion of YFP alone, confirming that YFP molecules remain diffuse even when localized within the mutant SOD1 aggregate structures. Experiments performed in parallel revealed that WT-YFP molecules also diffuse through the mutant SOD1 aggregate structures (Fig. S2). These results support our hypothesis that mutant SOD1 proteins form porous, almost honeycomb-like aggregate structures, consisting of a highly immobile scaffold through which soluble cellular proteins can diffuse.

### Hsp70 transiently interacts with mutant SOD1 aggregates

Although noninteracting proteins diffuse rapidly through the mutant SOD1 aggregate structures, we speculated that other proteins, which interact with aggregates formed by neurodegenerative disease-associated proteins, would display reduced mobility. To investigate the interaction of the molecular chaperone Hsp70 within the mutant SOD1 aggregates, PC12 cells were transiently transfected with constructs encoding G85R-CFP and a YFP-tagged Hsp70 (Hsp70-YFP) protein. This construct has been previously used in our laboratory and retains its...
activity as a molecular chaperone (Kim et al., 2002). FRAP analysis showed that 85.8 ± 1.9% of Hsp70-YFP was diffused in cells expressing soluble G85R-CFP (Fig. 3, A and B, and Table I). In contrast, in cells with G85R-CFP aggregates, 34.8 ± 1.9% of Hsp70-YFP was associated with the aggregates, with the remaining Hsp70-YFP in a soluble state (Fig. 3, A and B, and Table I). To address whether the association of Hsp70 reflected its chaperone function, we measured the mobility of a mutant Hsp70-YFP containing a mutation in the substrate-binding domain (Hsp70-SBDx-YFP). The mobility of Hsp70-SBDx-YFP was higher than for Hsp70-YFP with aggregated G85R-CFP (Fig. 3 B and Table I), revealing that a functional Hsp70 chaperone cycle is required for the interaction between Hsp70 and the aggregate. To further demonstrate that the Hsp70-aggregate interaction was transient, we used FLIP analysis, which showed that the fluorescence intensity of Hsp70-YFP within G85R-CFP aggregates declined rapidly to basal levels over a 10-min period of photobleaching (Fig. 3, C and D). From the results of FRAP and FLIP analysis, we conclude that Hsp70 is not sequestered, rather it is associated transiently with aggregate structures formed by mutant SOD1 proteins.

**Mutant SOD1 aggregates sequester the proteasome and impair its activity**

Aggregates formed by many neurodegenerative disease–associated proteins, including mutant SOD1, have been shown to interact with the proteasome (Bence et al., 2001; Urushitani et al., 2002; Goldberg, 2003; Kabashi et al., 2004). To monitor interactions between mutant SOD1 and the 20S proteasome, we introduced a YFP-tagged β subunit (LMP2-YFP) into PC12 cells together with a construct encoding G85R-CFP. LMP2 is quantitatively incorporated into active proteasomes and therefore provides an excellent indicator of the functional proteasome (Reits et al., 1997; Holmberg et al., 2004). FRAP analysis indicated that 94.1 ± 3.3% of LMP2-YFP was diffuse in cells containing diffusely localized G85R-CFP (Fig. 4, A and B, and Table I). These results were similar to the previously reported mobility of LMP2-GFP in cells expressing LMP2-GFP alone (Reits et al., 1997) or in cells containing a diffusely localized WT NH₂-terminal huntingtin protein (Holmberg et al., 2004). In aggregate-containing cells, the proteasome is relocated to aggregates, with 72.1 ± 1.8% of the total LMP2-YFP immobilized within the G85R-CFP aggregate with a slow off-rate < 0.001 s⁻¹ (Fig. 4, A and B, and Table I). The presence of a slow off-rate reveals a long residency time (τ = 1,000 s) for LMP2-YFP in this immobile state suggesting a tight interaction with the G85R-CFP aggregate.

To further establish the interaction of LMP2-YFP with the G85R-CFP aggregate, we used FLIP analysis. FLIP showed that 39.7 ± 10.7% of the initial fluorescence intensity of LMP2-YFP within G85R-CFP aggregates was retained after a 10-min period of photobleaching (Fig. 4, C and D), suggesting that the proteasome is sequestered within G85R-CFP aggregates. To verify that these results reflect the endogenous proteasome, we used indirect immunofluorescence analysis and showed that the endogenous 20S subunit of the proteasome is associated with G85R-CFP and G93A-CFP aggregates (Fig. S3 available at http://www.jcb.org/cgi/content/full/jcb.200504050/DC1). Taken together, these results demonstrate that the proteasome is sequestered into the immobile fraction of the mutant SOD1 aggregate structures.

We next examined whether sequestration of the proteasome within the mutant SOD1 aggregate has negative consequences on proteasome activity. To address this, we used an Ubi-YFP reporter construct (Holmberg et al., 2004) that was stably transfected into PC12 cells. The Ubi reporter consists of ubiquitin followed by a 40-amino acid lysine-containing linker region. The ubiquitin is rapidly cleaved off in vivo, promoting ubiquitination of the internal lysine residues and fostering degradation of the YFP-fusion protein by the proteasome. Cells in which proteasomal activity is impaired will show an accumulation of the Ubi-YFP protein. Differentiated Ubi-YFP/PC12 cells were transiently transfected with WT-CFP, G85R-CFP, or G93A-CFP. As a positive control, we transiently transfected the Ubi-YFP/PC12 cells with a construct encoding the mutant huntingtin protein (hntQ78-YFP). Similar
to previous reports (Bence et al., 2001; Bennett et al., 2005), we detected an increased level of Ubi-YFP accumulation (1.88 ± 0.23-fold increase; unpublished data) in cells containing httQ78-CFP aggregates. Compared with cells expressing WT-CFP or diffusely localized G93A-CFP, cells displaying a diffuse G85R-CFP localization showed an increased accumulation of Ubi-YFP (Fig. 4, E and F). A further and substantial enhancement in Ubi-YFP accumulation was observed in cells containing aggregates formed by either G85R-CFP (2.90 ± 0.19-fold increase) or G93A-CFP (2.59 ± 0.26-fold increase; Fig. 4, E and F). These findings suggest that the formation of mutant SOD1 aggregates is highly correlated with reduced in vivo proteasome activity.

**Cell death caused by mutant SOD1 aggregates**

To test whether aggregate formation is directly associated with neuronal cell death, differentiated PC12 cells expressing YFP, WT-YFP, G85R-YFP, or G93A-YFP were followed using live-cell time-lapse fluorescence microscopy. Individual cells were monitored, during a 48-h period (days 3 to 5 after transfection) for protein accumulation, aggregate appearance, and cell death, based on morphological change or propidium iodide (PI) staining (Fig. 5 and Video S1 available at http://www.jcb.org/cgi/content/full/jcb.200504050/DC1). Whereas WT-YFP-transfected cells did not form aggregates, the percentage of cells expressing aggregated G85R-YFP or G93A-YFP after 3 d of expression was 30 and 15%, respectively. Within the ensuing 48-h period, 15.3 ± 3.6% of nontransfected PC12 cells and 14.5 ± 3.5% of WT-YFP-transfected cells underwent cell death, whereas 88.5 ± 1.2% for G85R-YFP and 90.3 ± 4.1% for G93A-YFP of cells expressing a mutant SOD1 aggregate subsequently died (Fig. 5 A).

An additional 30 (G85R-YFP) and 15% (G93A-YFP) of cells, in which aggregates were initially not detected, formed an aggregate during this 48-h monitoring period. We followed the level of G85R-YFP accumulation (total YFP fluorescence) before overt aggregate appearance in these cells and compared it to G85R-YFP accumulation in cells without aggregate appearance during this 48-h period (Fig. 5 B). As aggregates appeared at varying times during this 48-h period, the time-point of overt aggregate appearance was normalized as the 48-h time-point. Certain cells maintained a constant level of G85R-YFP protein reflecting a balance between synthesis and degradation without detectable aggregate formation (Fig. 5 B), whereas other cells displayed an imbalance in these processes, with a rapid increase in the level of mutant SOD1 protein, followed by the formation of large, visible aggregates (Fig. 5 B, aggregate formed). Furthermore, 70% of these aggregate-forming cells died during the subsequent 48-h monitoring period with the majority of these cells dying within 6 to 24 h after initial aggregate appearance (Fig. 5 C). In contrast, of the 30% of PC12 cells expressing diffuse mutant SOD1 proteins that exhibited cell death, we did not observe the same striking correlation with cell death (Fig. 5 C). Likewise, cells expressing soluble WT SOD1 did not undergo cell death, thus ruling out negative effects due to a flux of heterologous expression of SOD1 (Fig. 5 C). We conclude from these time-lapse studies that there is a close correlation between the appearance of mutant SOD1 aggregates, and subsequent cell death.
species and toxicity. Using live-cell imaging, we observe that we observed similar biophysical properties of the aggregate death. Despite the difference in mutation type and in function, proteins form a novel class of aggregates that correlate with cell toxicity.

In this study, we show that mutant G85R and G93A SOD1 proteins form a novel class of aggregates that correlate with cell death. The structural characteristics for mutant SOD1 aggregates described here are observed in both neuronal (PC12) and nonneuronal (HeLa) cells, suggesting that these features may reflect common intrinsic properties associated with the expression of these mutant SOD1 proteins, independent of cell type.

Our live-cell analyses suggest that, even though the mutant SOD1 aggregate is structured, it is highly amorphous, with a disordered honeycomb-like scaffold through which other cellular proteins readily diffuse. This is in contrast to the aggregate structures formed by mutant huntingtin or polyglutamine-YFP that are immobile and densely packed, prohibiting the diffusion of certain soluble proteins (Chai et al., 2002; Kim et al., 2002; Holmberg et al., 2004). A third class of aggregate structures formed by expression of the polyglutamine protein, ataxin-1, is comprised of either fast or slow mobile components exchanging between the aggregate and the nucleoplasm (Stenoien et al., 2002). Taken together with our current results with mutant SOD1, we conclude that mutant huntingtin, ataxin-1, and SOD1 form aggregates that appear fundamentally distinct in their organization and structure. The molecular characteristics that distinguish these three classes were revealed only by the use of dynamic live cell imaging methods. Whether these results can be extrapolated to suggest an even more diverse family of in vivo aggregate structures, remains a question to be addressed in future studies on other neurodegenerative disease associated proteins.

Among the mechanisms proposed for aggregate-associated cytotoxicity is the association of cellular proteins with misfolded oligomers and aggregates (Bruijn et al., 1997, 2004; Cleveland and Rothstein, 2001; Pasinelli et al., 2004). Polyglutamine aggregates have been shown to recruit and sequester polyglutamine-containing transcription factors, such as TBP and CREB-binding protein (Steffan et al., 2000; Nuñofora et al., 2001; Kim et al., 2002). The interaction between TBP and polyglutamine aggregates is dependent upon the presence of a polyglutamine stretch in TBP (Kim et al., 2002); consistent with this is the lack of colocalization of TBP with the mutant SOD1 aggregates (unpublished data). Another commonly observed class of proteins associated with various aggregates are components of the protein quality control machinery, including Hsp70 and the proteasome (Bence et al., 2001; Cleveland and Rothstein, 2001; Watanabe et al., 2001; Kim et al., 2002; Takeuchi et al., 2002; Urushitani et al., 2002; Bruijn et al., 2004; Bennett et al., 2005; Holmberg et al., 2004; Kabashii et al., 2004). Our results show that, while Hsp70 interacts transiently with mutant SOD1 aggregates in a chaperone-independent manner, Hsp70 is not sequestered within the aggregate, similar to what was observed with mutant huntingtin (Kim et al., 2002). Likewise, both the endogenous proteasome and the transiently transfected LMP2 proteasomal subunit interact with the mutant SOD1 aggregate. However, FRAP and FLIP analyses showed that the proteasome was sequestered irreversibly.

Discussion

In this study, we show that mutant G85R and G93A SOD1 proteins form a novel class of aggregates that correlate with cell death. Despite the difference in mutation type and in function, we observed similar biophysical properties of the aggregate species and toxicity. Using live-cell imaging, we observe that both mutant SOD1 proteins form large perinuclear aggregates that contain two populations; an immobile fraction organized into an amorphous scaffold containing mutant SOD1 and other cellular proteins such as the proteasome, and another population of mobile (diffuse) protein including mutant SOD1 in a soluble state and molecular chaperones. The structural characteristics for mutant SOD1 aggregates described here are observed in both neuronal (PC12) and nonneuronal (HeLa) cells, suggesting that these features may reflect common intrinsic properties associated with the expression of these mutant SOD1 proteins, independent of cell type.
Coincident with the sequestration of the proteasome within the mutant SOD1 aggregate, we observe decreased proteasome activity; however, our results do not establish whether proteasome impairment occurred before or after the appearance of aggregates. Whereas we observed a slight increase in Ub-YPFP accumulation in cells expressing diffusely localized G85R-YPFP, a greater inhibitory effect on proteasome activity was observed in cells with visible aggregates, similar to what has been observed for mutant huntingtin (Bence et al., 2001; Bennett et al., 2005). However, this contrasts with mutant ataxin-1 aggregates that do not irreversibly sequester the colocalized proteasome (Stenoien et al., 2001). We conclude that the biochemical properties of each misfolded protein determine the intrinsic features of aggregate structure, the nature of interactions with other cellular proteins, and the dynamics of these interactions.

In our studies, mutant SOD1 aggregates localize near the microtubule organizing centers and are surrounded by α-tubulin (unpublished observations), consistent with previous studies showing that mutant SOD1 forms aggresomes through a microtubule-dependent process (Johnston et al., 1998; 2000; Kopito, 2000). Although aggresome formation may be a component of the cellular response to the accumulation of misfolded proteins when the capacity of the proteasome is exceeded (Kopito, 2000), the formation of such structures will also inevitably increase the local concentration of mutant SOD1 protein and associated proteins. As the proteasome has been implicated as a crucial regulator of multiple essential cellular processes, including gene expression, apoptosis, the cell cycle, development, and DNA repair (for review see Wolf and Hilt, 2004), aberrant interactions initiated by aggregate formation may result in the dysregulation of numerous pathways critical to cell survival.

The appearance of mutant SOD1 aggregates in mouse models of fALS (Brujin et al., 1997; Johnston et al., 2000; Watanabe et al., 2001; Wang et al., 2002) and in tissue samples from patients (Shibata et al., 1996; Kato et al., 2000) has suggested a role for aggregation in cellular toxicity. However, other observations with mutant SOD1 (A4V and V148G) have concluded that aggregate formation does not correlate with neuronal death in differentiated PC12 cells (Lee et al., 2002). In an effort to address this, we performed time-lapse experiments to follow the fate of individual differentiated PC12 cells expressing mutant SOD1 and showed that cell death correlates with an increased levels of mutant SOD1 protein and the formation of aggregates and occurs within 6 to 24 h after aggregates are visually detected. At any given time-point, only a small fraction (20–30%) of mutant SOD1-expressing cells have visible aggregates. Such variation may be due to the intrinsic heterogeneity of transient or stably expressing cell lines or perhaps due to differential levels of protein expression and cell-to-cell variation in protein homeostasis. If only these aggregate-containing cells are marked for cell death within the subsequent 24-h time period, the overall percentage of cells undergoing cell death at any single time point is much smaller, yet for the fraction of cells with aggregates, nearly all are destined for cell death. Moreover, as these aggregate-containing cells die, they are replaced by other cells that express aggregates. Using live-cell time-lapse microscopy, we are able to demonstrate the rapid cell death that ensues upon appearance of mutant SOD1 aggregates and clearly show that aggregate formation leads to cell death. Whereas aggregate-containing PC12 cells died rapidly, in contrast, 70% of cells with diffuse mutant SOD1 remained viable over the 120-h experimental period. A recent observation with cells containing huntingtin aggregates showed survival for more than 120 h and that aggregate-containing cells even exhibited a reduced risk for cell death (Arrasate et al., 2004). Even though some of these differences can be attributed to various technical differences (i.e., level of protein overexpression, cell type, method of detection/characterization), our studies on mutant SOD1 aggregates clearly indicate a direct link to neuronal cell death.

Materials and methods

Constructs

The pEYFP-N1-Hsp70 and the Hsp70 substrate binding domain deletion mutant (Hsp70/SBDx-YPFP) were previously described (Kim et al., 2002). The pEYFP-N1-LMP2, pTR2hyg/HtrGQ7–CFP, and pEYFP-N1-Ubi-YPFP constructs were previously described (Holmberg et al., 2004). The pTR-YFP or pTR-CFP vectors were generated by PCR amplification of YFP from pEYFP-N1 or CFP from pEF-CFP1 (CLONTECH Laboratories, Inc.) and subcloning into the pTR2hyg (CLONTECH Laboratories, Inc.), pTRE-SOD1-WT-CFP, pTRE-SOD1-WT-CFP, pTRE-SOD1-G85R-CFP, and pTRE-SOD1-G85R-CFP were generated by PCR amplification of WT SOD1 and G85R mutant SOD1 from plQ201 or plQ203 (gift from Dr. Q. Liu, Harvard Medical School, Boston, MA), respectively, and inserting into pTR-EYFP or pTR-CFP. pTRE-SOD1-G93A-CFP and pTRE-SOD1-G93A-CFP were generated by PCR-based site-directed mutagenesis at G93A of pTRE-SOD1-WT-CFP or pTRE-SOD1-WT-CFP. All constructs were verified by sequencing.

Cell culture and transfection

PC12 Tet-Off cells (CLONTECH Laboratories, Inc.) were maintained in DME supplemented with 10% heat-inactivated horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml G418 at 37°C in a 5% CO2-humidified atmosphere. PC12 Tet-Off cells were differentiated with 100 ng/ml nerve growth factor (Sigma-Aldrich) for 7 d and were continuously maintained in medium containing nerve growth factor for the remainder of all studies, as previously described (Vignali et al., 1996). For colocalization studies, cells were grown in Matrigel-coated BD Biosciences two-well glass slides (Lab-Tek). For live cell analysis, cells were grown in Matrigel-coated 35-mm glass-bottom dishes (MatTek Corp.). Transient transfections were performed using Lipofectamine Plus reagent using twice the manufacturer-recommended volume and 3 μg of SOD1 DNA constructs. Cotransfection of pEYFP-N1-Hsp70 with the SOD1 constructs was performed at a ratio of 1:10 (Hsp70/SOD1) and of pEYFP-N1-LMP2 with 1.3 μM/LMP2/CFP (PC12 cells were generated by cotransfecting PC12 Tet-Off cells with the pEYFP-N1-Ubi-YPFP construct and a puromycin-resistance pBacSpacp construct (gift from Dr. Y. Minami, Osaka University, Osaka, Japan), using Lipofectamine Plus (Invitrogen) and selecting with 2.5 μg/ml puromycin.

Western analysis

Transfected PC12 cells were harvested, resuspended in 20 mM Hepes, pH 7.9, with 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, and 1 mM PMSF and lysed using freeze–thaw buffer. Total protein amount was quantitated using a Bradford protein assay (Bio-Rad Laboratories). Protein lysates (10 μg) were resuspended in SDS sample buffer and loaded onto 12.5% SDS-PAGE gels. The gel was transferred to a nitrocellulose membrane and immunoblotted using an IRDye 800-conjugated anti–γ-tubulin antibody (600–132-215; Rockland Immunochemicals, Inc.) to detect CFP- or YFP-fused proteins. As a loading control, γ-tubulin was detected using an anti–γ-tubulin antibody (GTU-88; Sigma-Aldrich). Primary antibodies were detected with an anti–mouse Alexa Fluor 680-conjugated secondary antibody (Invitrogen). Blots were analyzed using an Odyssey infrared imaging system (LI-COR Bioscience).

SOD1 activity assay

The same protein lysates (15 μg) used for Western analysis, were loaded onto a 10% native PAGE gel. The gel was incubated in a riboflavin-NBT
[Sigma-Aldrich] solution at room temperature for 15 min (in the dark, as riboflavin is light sensitive). After removal of riboflavin-NBT, the gel was incubated with 0.1% TEMED for 15 min and exposed to light, to induce superoxide synthesis.

Filter-trap assay
The same protein lysates (100 μg) used for Western analysis, were resuspended in a 1% SDS solution in PBS, and loaded onto a cellulose acetate membrane (0.2 μm; Advantec MFS, Inc.). The membrane was washed with 1% SDS in PBS and protein was detected as per Western blotting protocol.

Visualization of fluorescently tagged proteins
Transfected PC12 cells were fixed in 4% formaldehyde in PBS for 10 min, quenched in 0.1 M Tris-HCl, pH 8.0, for 5 min, washed in PBS at room temperature, and mounted in Vectashield antifading solution (Vector Laboratories). Fixed samples were examined using a Leica TCS SP2/Leica DMIRE2 inverted confocal microscope equipped with 63× objective lens (Leica). Approximately 300 different transfected cells were scored, in three independent experiments, for aggregate formation. For measurement of LMP2 relocalization, a whole-cell Z stack (each slice in independent experiments, for aggregate formation. For measurement of LMP2-YFP measured against the intensity localized to the aggregate (quired, a maximum projection created, and whole-cell intensity of LMP2-LMP2 relocalization, a whole-cell Z stack (each slice

FRAP curve fitting and mathematical modeling
Two empirical formulas, one described by Ellenberg et al. (1997) and Lippincott-Schwartz et al. (2001),

\[ F_t = F_{\text{final}} \left[ 1 - \left( \frac{w^2}{w^2 + 4Dt} \right)^{1/2} \right] \]  

(1)  

and a second described by Axelrod et al. (1976),

\[ F_{(t)} = \left[ 1 - \exp \left( \frac{-\pi D t}{w^2} \right) \right] \]  

(2)

have been commonly used for fitting FRAP data and the determination of diffusion coefficients. However, neither of these equations yielded a completely satisfactory fit to our experimental data, suggesting that these proteins exhibit a behavior that cannot be explained solely by diffusion, rather also involves either the formation of higher-order complexes or binding interactions with one or more cytoplasmic macromolecules or structures. To incorporate such interactions into an optimal, biologically significant mathematical model of the mobility of WT and mutant SOD1-YFP proteins, we fit FRAP data using a recently described biphase recovery equation by Carrero et al. (2004; see equation S3 therein),

\[ F_t = \left[ 1 + \frac{1}{k} \exp \left( \frac{-2D t}{w^2} \right) - \left( \frac{k}{1 + \frac{1}{k}} \right) \exp \left( -k t \right) \right] \]  

(3)

classified by a fast recovery phase produced by a fraction 1/(1 + k) of the protein population diffusing rapidly with a diffusional transfer coefficient \( D_t \), and a slow recovery phase produced by the “turnover” of a fraction k/(1 + k) of the protein population bound with an unbinding (off) rate \( k \). All FRAP curves were fit with equation 3, using GraphPad Prism software [GraphPad Software], yielding satisfactory fits with residual mean squares \( \chi^2 < 0.0006 \) s−1. We then used the relationship by Carrero et al. (2004),

\[ D_t = \frac{(1 + 2h) + (1 + 2h)^2}{4h(1 - 2h)^2} \]  

(4)

to calculate the diffusion coefficient \( D_t \) from the experimentally derived diffusional transfer coefficient \( D_t \). From the diffusion coefficients, we have applied the Stokes-Einstein formula,

\[ D = \frac{kT}{6\pi\eta R_t} \]  

(5)

where the Boltzmann constant \( k \), the viscosity of the solution \( \eta \), and temperature \( T \) all remained constant throughout our studies, to yield a relationship between the diffusion coefficient and the Stokes’ radius of the molecule, \( D \sim R_t^{-1} \). Assuming that the molecule in question is a sphere with a Stokes’ radius \( R_t \), and a volume proportional to its molecular weight, we can relate the diffusion coefficient to molecular weight by \( D \sim M^{1/3} \). As WT SOD1 forms a highly stable homodimer, we expect WT-SOD1 fused to YFP to form a complex with a molecular weight of ~90 kD. Using \( D \sim M^{1/3} \) or where \( C \) is a constant to be calculated, and the experimentally derived diffusion coefficients (Table I), we approximated the average molecular weights resulting in the reduced diffusion for each protein population.

\[ D = (C) M^{1/3} \]  

(6)

Immunofluorescence analysis
The protocol for visualization of the 20-s endogenous proteasome was performed as previously described (Holmberg et al., 2004).

Online supplemental material
Fig. S1 shows mutant SOD1 aggregates retain similar properties in non-neuronal cells. Fig. S2 demonstrates WT-YFP also diffuses through mutant SOD1 aggregates. Fig. S3 shows that mutant SOD1 aggregates affect the localization of endogenous proteasome. Video S1 depicts how proteasome affected the localization of endogenous proteasome. Video S1 depicts how proteasome affected the localization of endogenous proteasome.
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