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Toxicity of Familial ALS-Linked SOD1 Mutants from Selective Recruitment to Spinal Mitochondria

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Summary

One cause of amyotrophic lateral sclerosis (ALS) is mutation in ubiquitously expressed copper/zinc superoxide dismutase (SOD1), but the mechanism of toxicity to motor neurons is unknown. Multiple disease-causing mutants, but not wild-type SOD1, are now demonstrated to be recruited to mitochondria, but only in affected tissues. This is independent of the copper chaperone for SOD1 and dismutase activity. Highly preferential association with spinal cord mitochondria is seen in human ALS for a mutant SOD1 that accumulates only to trace cytoplasmic levels. Despite variable proportions that are successfully imported, nearly constant amounts of SOD1 mutants and covalently damaged adducts of them accumulate as apparent import intermediates and/or are tightly aggregated or crosslinked onto integral membrane components on the cytoplasmic face of those mitochondria. These findings implicate damage from action of spinal cord-specific factors that recruit mutant SOD1 to spinal mitochondria as the basis for their selective toxicity in ALS.

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Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset fatal neurodegenerative disease characterized by selective loss of motor neurons in the spinal cord, brainstem, and motor cortex. Ten percent of disease instances are dominantly inherited, with ~20% of these arising from mutation in the ubiquitously expressed enzyme copper/zinc superoxide dismutase (SOD1) (Rosen et al., 1993). More than 100 ALS-causing mutations scattered throughout this 153 amino acid protein are known (Cleveland and Rothstein, 2001; Andersen et al., 2003). Studies in humans (Andersen et al., 1995), transgenic mice (Gurney et al., 1994; Wong et al., 1995; Bruijn et al., 1997; Ripps et al., 1995; Wang et al., 2002, 2003), and rats (Nagai et al., 2001; Howland et al., 2002) expressing ALS-linked SOD1 mutants have demonstrated that the mutants provoke selective, age-dependent killing of motor neurons through acquisition of one or more as yet unidentified toxicities, not through loss of dismutase activity (Reaume et al., 1996; Borchelt et al., 1994; Bruijn et al., 1998). For each mutant, higher levels of accumulation in these models correlate with earlier disease onset. Nevertheless, the level of accumulated mutant protein required for toxicity in mice differs markedly: to cause disease with similar onset and survival times, some mutants (e.g., $SOD1^{G93A}$ [Gurney et al., 1994] and $SOD1^{G37R}$ [Wong et al., 1995]) require ~10 times the level needed for others (e.g., $SOD1^{G85R}$ [Bruijn et al., 1997]).

SOD1 is a homodimer, and for its dismutase activity each subunit binds a copper atom at the catalytic site. The ALS-linked SOD1 mutations retain highly varied biochemical properties including levels of dismutase activity, binding of the catalytic copper, and subunit half-life (Borchelt et al., 1994; Ratovitski et al., 1999; Hayward et al., 2002; Rodriguez et al., 2002; Tiwari et al., 2002). Acquisition of most copper by SOD1 is facilitated by a Copper Chaperone for SOD1 (CCS) (Culotta et al., 1997), a member of the family of metallochaperones that are responsible for intracellular trafficking of metal ions (O'Halloran and Culotta, 2000). Disruption of the *CCS* gene in each of the three most frequently used ALS mouse models has shown that disease is unaffected by elimination of CCS-mediated copper loading (Subramaniam et al., 2002). This finding, and the absence of detectable copper loading onto SOD1 after injection of radiolabeled copper into CCS-deleted, mutant SOD1-expressing mice (Subramaniam et al., 2002), have been interpreted (Orr, 2002) to weigh against proposals (Beckman et al., 1993; Estevez et al., 1999) that the primary toxicity arises from aberrant, copper-mediated oxidative chemistry by the mutant enzymes. This is complicated, however, by an apparent ~20% residual dismutase activity measured in vitro in samples from CCS-depleted animals (Subramaniam et al., 2002).

Although the selectivity of motor neuron killing pro-

duced by the many ALS-linked mutants implies that they share one (or more) disease-provoking toxic properties, the mechanism(s) of such toxicity remains elusive. Mitochondrial abnormalities within motor neurons were observed as one of the early pathological features in both $SOD1^{G93A}$ and $SOD1^{G37R}$ mice (Dal Canto and Gurney, 1994; Wong et al., 1995; Kong and Xu, 1998), although similar pathology has not been found in other transgenic lines that develop ALS-like disease from expressing mutants $SOD1^{G85R}$ (Bruijn et al., 1997; Ripps et al., 1995) or $SOD1^{H46R/H48Q}$ (Wang et al., 2002). A ~25% decrement in mitochondrial function has been reported in $SOD1^{G93A}$ mice that accumulate an enzymatically active SOD1 mutant to >10 times the endogenous mouse SOD1 ($mSOD1^{WT}$) level, albeit this was neither seen before disease onset nor selective to mitochondria from spinal cord (Mattiazzi et al., 2002). Mutant $SOD1^{G93A}$ has also been detected within spinal cord mitochondria using electron microscopy (Jaarsma et al., 2001; Higgins et al., 2002) and in intact mitochondria from brain by its apparent resistance to protease digestion (Mattiazzi et al., 2002).

As to the mechanism underlying selective killing of motor neurons in ALS, we now identify preferential accumulation of mutant SOD1s with mitochondria to be: (1) common to a series of ALS-linked SOD1 mutants including one mutant that accumulates only to trace levels in the cytoplasm in human disease, (2) selective for spinal cord mitochondria, (3) independent of dismutase activity and CCS, (4) accompanied by covalent damage to mitochondrial bound proteins, including mutant SOD1s, and (5) mediated by very tight association and/or crosslinking to integral membrane proteins of the outer mitochondrial membrane. These findings implicate damage from action of spinal cord-specific factors that recruit mutant SOD1 to spinal mitochondria as the basis for their selective toxicity in ALS.

Results

Disease-Inducing Mutant SOD1s, but not Wild-Type SOD1, Associate with Spinal Cord Mitochondria

Mitochondria were isolated by differential sedimentation of central nervous tissue extracts from both nontransgenic and symptomatic mice expressing ALS-linked $SOD1^{G37R}$ mutant, followed by further purification through a density gradient (Figure 1A). Immunoblotting of equal proportions of aliquots from each step revealed a nearly quantitative recovery of mitochondria in the final purified fraction, as judged by retention of inner membrane and matrix markers (COX4 and Hsp60, respectively) (Figure 1B, lanes 1–4), while nonmitochondrial membranous organelles were essentially eliminated, as seen by absence of syntaxin 6 and synaptotagmin (Figure 1B, lane 4). Moreover, relative to other mitochondrial markers, the retention of a high level of cytochrome c, a component in the mitochondrial intermembrane space (IMS), indicated that mitochondria remained structurally intact during purification (Figure 1B, lane 4). Despite its high abundance in the initial extracts, the endogenous $mSOD1^{WT}$ was almost quantitatively removed during mitochondrial purification, confirming the absence of significant cytoplasmic contamination in the final purified mitochondria and demonstrating that little, if any, mouse $SOD1^{WT}$ associates with spinal cord mitochondria even in the presence of a high concentration of the disease-causing $SOD1^{G37R}$ mutant. In contrast, although it retains full specific activity as a superoxide dismutase and efficiently heterodimerizes with $mSOD1^{WT}$ (Wong et al., 1995; Borchelt et al., 1994), a proportion of the $SOD1^{G37R}$ mutant, in the absence of detectable endogenous $mSOD1^{WT}$, also remained associated with purified mitochondria (Figure 1B, lane 4).

This mitochondrial association was a general property of ALS-linked SOD1 mutants with very different biochemical characteristics, as seen in spinal cord mitochondria prepared from lines that develop motor neuron disease from expression of three additional ALS-linked SOD1 mutants. These included mice expressing (1) mutant $SOD1^{G93A}$ that retains significant activity as a dismutase (Gurney et al., 1994; Howland et al., 2002), but which may also catalyze aberrant oxidative chemistry (Wiedau-Pazos et al., 1996; Andrus et al., 1998), (2) mutant $SOD1^{G85R}$ that retains little, if any, dismutase activity (Corson et al., 1998), and (3) mutant $SOD1^{H46R/H48Q}$ that carries two ALS-linked mutations that eliminate dismutase activity through loss of two of the four catalytic copper-coordinating histidines (Wang et al., 2002). Unlike $SOD1^{G37R}$ and $SOD1^{G93A}$, both inactive mutants also fail to form stable heterodimers with the wild-type SOD1 (Bruijn et al., 1997; Wang et al., 2002).

Mitochondria were isolated from spinal cords from symptomatic mice expressing each of the four ALS-linked SOD1 mutants, as well as from nontransgenic mice or aged mice expressing a high level of human wild-type SOD1 ($hSOD1^{WT}$) that never develop motor neuron disease. All four mutant SOD1s (Figure 2A, lanes 4, 6, 8, and 10) were present in the spinal cord mitochondrial fractions. In contrast, despite chronic expression of high levels of $hSOD1^{WT}$, none was detected with mitochondria (Figure 2A, lane 12). Similarly, $mSOD1^{WT}$ was also below detectable levels (except after very long exposures; quantified in Table 1) in these purified mitochondria, despite its known efficient heterodimerization in the cytoplasm with the $SOD1^{G37R}$ and $SOD1^{G93A}$ mutants.

The preferential association of mutant SOD1 with spinal cord mitochondria was most striking for $SOD1^{G85R}$ (Figure 2A, lanes 5 versus 6; Table 1), the mutant that accumulates to the lowest level and does not exhibit detectable dismutase activity. Furthermore, despite strikingly different cytoplasmic levels of the various SOD1 mutants (Figure 2A, lanes 3, 5, 7, 9) (and levels required to cause disease [Bruijn et al., 1997]), these four ALS-linked SOD1 mutants accumulated to levels that differed by no more than 2-fold in the mitochondrial fractions (e.g., compare signal intensities in Figure 2A, lanes 4, 6, 8, and 10 using an antibody that recognizes each mutant with equal affinity; Table 1).

Accumulation of mutant SOD1 with mitochondria was selective for affected tissues. Despite overall accumulation to levels comparable to that seen in spinal cords, neither the mutant SOD1s nor $hSOD1^{WT}$ was associated with mitochondria from skeletal muscle (compare Figure 2B, lanes 4, 6, 8, and 10). Despite showing the highest proportion associated with spinal cord mitochondria, no $SOD1^{G85R}$ was detectable in purified mitochondria from whole brain or liver (Figure 2C, lanes 8 and 10). Con-

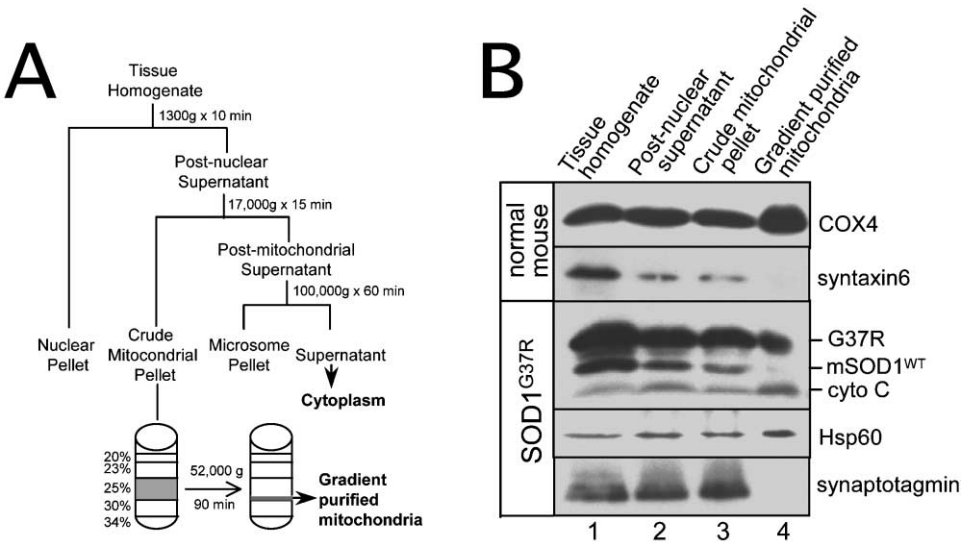

Figure 1. Isolation of Highly Purified Mitochondria

(A) Schematic of mitochondrial purification.

(B) Immunoblotting of equal proportions of each fraction from the purification. Mitochondrial markers COX4, cytochrome c, and Hsp60 were used to assess the recovery of mitochondrial fractions from nontransgenic or symptomatic SOD1^{G37R} transgenic mice, while immunoblotting for syntaxin 6 and synaptotagmin were used to assess the removal of other membranous components.

versely, although almost entirely absent from spinal cord mitochondria (Figure 2C, lanes 2 and 4), an easily detectable proportion of mSOD1^{WT} was associated with liver mitochondria (Figure 2C, lane 8), while mutant SOD1^{G85R} was almost completely excluded (quantified in Table 1).

Mutant SOD1 Association with Spinal Mitochondria Is not an Intrinsic Property of Motor Neurons, but Is Acquired with Age

Mitochondrial vacuolation has been documented to appear selectively within a few motor neurons at early ages (Dal Canto and Gurney, 1994; Wong et al., 1995; Kong and Xu, 1998) of some mice that later develop SOD1 mutant-mediated disease. To test whether association of mutant SOD1 with these mitochondria reflects an intrinsic property of motor neurons, mitochondria were isolated from mice that express mutant SOD1^{G85R} solely in neurons (Lino et al., 2002). These mice do not develop disease or pathology, with SOD1^{G85R} accumulating to $<$5% the level of endogenous mSOD1^{WT} (Figure 2D, lane 1). Although easily detectable in the cytoplasm using antibodies specific for human SOD1 (Figure 2D, lane 3), none of this neuronal SOD1^{G85R} was associated with spinal cord mitochondria (Figure 2D, lane 4). Similarly, when expressed ubiquitously, SOD1^{G85R} did not associate with spinal cord mitochondria by 5 weeks of age (Figure 2E, lane 2). However, a proportion was associated in 6-month-old, asymptomatic mice, with the amount escalating by more than 2-fold after disease onset (Figure 2C, lanes 2 and 4; Table 1). This age-dependent accumulation with spinal cord mitochondria was selective for mutant SOD1: no mitochondrial hSOD1^{WT} or mSOD1^{WT} was detected even in aged mice (Figure 2A, lane 12).

Mutant SOD1 in Mitochondria Is Independent of CCS and Dismutase Activity

By disruption in mice of the gene encoding CCS, mutant toxicity from SOD1^{G93A}, SOD1^{G37R}, and SOD1^{G85R} mice has been shown to be independent of CCS (Subramaniam et al., 2002). Spinal cord mitochondria from symptomatic SOD1^{G37R} mice with or without a functional *CCS* gene, as well as littermate nontransgenic controls, were generated. Unlike yeast where SOD1 import into the mitochondrial IMS is known to be mediated by CCS (Sturtz et al., 2001), the same level of SOD1^{G37R} was associated with mitochondria regardless of whether or not CCS was present (Figure 3A, lanes 2 and 4). Despite the constant association of SOD1^{G37R} with mitochondria, overall cytoplasmic dismutase activity in the absence of CCS was reduced to about 15%–20% of that in the presence of CCS (Figure 3A, lanes 5 and 6), as previously reported (Subramaniam et al., 2002), indicating that mitochondrial association is also independent of overall dismutase activity. Dismutase activity of SOD1^{G37R} associated with CCS-deficient mitochondria was reduced modestly compared with similar preparations from CCS-containing mice (Figure 3A, lane 7 versus 8), implying CCS-independent copper loading. Manganese SOD activity was unchanged.

Most mutant SOD1 mouse models do not produce pathology in the brain (Wong et al., 1995; Bruijn et al., 1997; Wang et al., 2002), and no SOD1^{G85R} is associated with the corresponding brain mitochondria (Figure 2C). However, as initially reported (Wang et al., 1995), SOD1^{G37R} (line 42) is the SOD1^{G37R} line with the highest level of mutant expression and earliest disease onset, and it develops pathology in the brain in addition to the spinal cord. We took advantage of this to determine in this line that not only was mutant SOD1^{G37R} associated with brain mitochondria, but also that the absence of

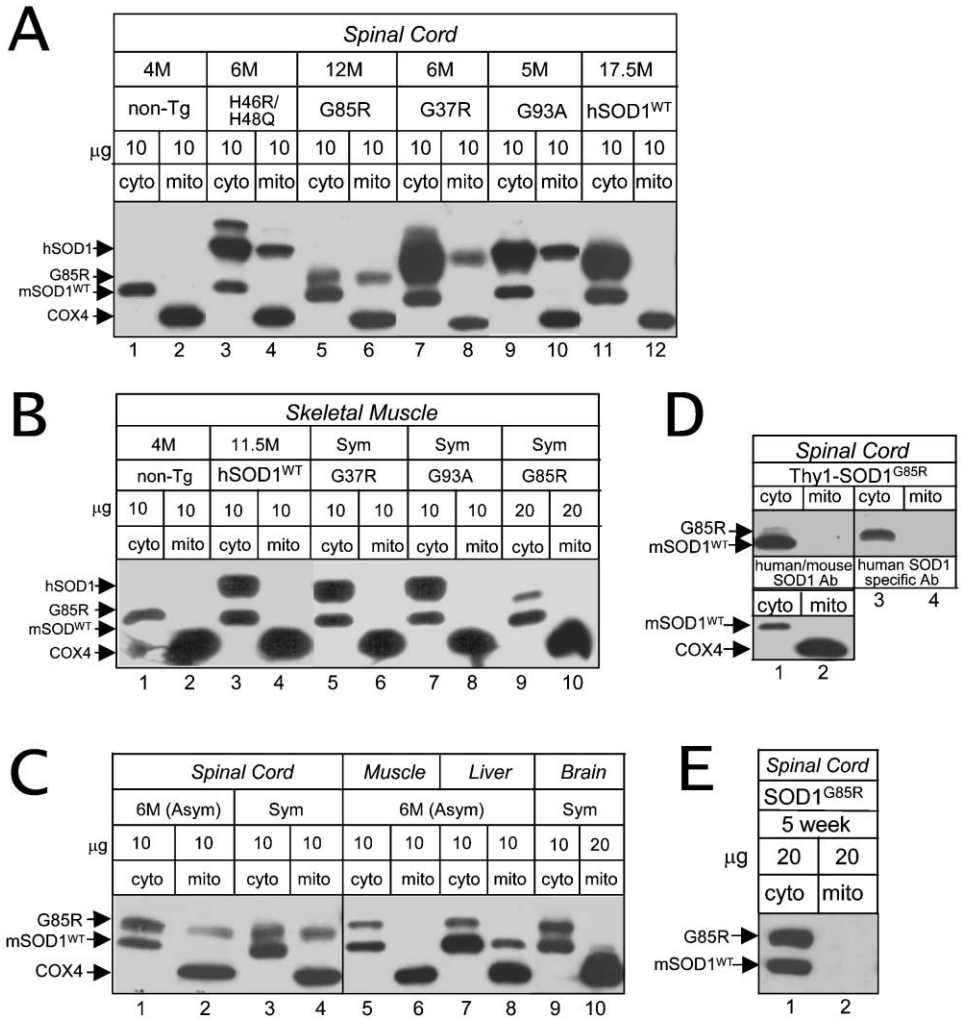

Figure 2. Selective Association of ALS-Linked Mutant SOD1s with Mitochondria of Affected Tissues

Mutant and wild-type SOD1 content in purified mitochondria (mito) and cytosolic (cyto) fractions were analyzed by immunoblotting samples from spinal cord (A, D, and E), hind limb skeletal muscle (B), or various tissues from SOD1 mutant, $hSOD1^{WT}$, or nontransgenic (non-Tg) mice (C), as indicated. Specific transgenic lines used in (A) and (B) included $hSOD1^{WT}$ (line 76), $SOD1^{G37R}$ (line 42), $SOD1^{G85R}$ (line 148), $SOD1^{G93A}$ (G1H line), and $SOD1^{H46R/H48Q}$ (line 139). Shown in (D) are Thy1-$SOD1^{G85R}$ mice, and in (E) $SOD1^{G85R}$ mice (line 164).

Table 1. Wild-Type and Mutant SOD1 Associated with Mitochondria

SOD1	Tissues	Relative Accumulated[a]	[SOD1]/[COX4][b]
$hSOD1^{WT}$ (17.5 mo)	spinal cord	8	0.7
$hSOD1^{G37R}$	spinal cord	10	21
$hSOD1^{H46R/H48Q}$	spinal cord	3.5	17
$hSOD1^{G93A}$	spinal cord	7	21
$hSOD1^{G85R}$ (presymptomatic)	spinal cord	0.5	6
$hSOD1^{G85R}$	spinal cord	1	13
$hSOD1^{G85R}$	skeletal muscle	0.5	<0.01[c]
$hSOD1^{G85R}$	liver	0.3	0.2
$mSOD1^{WT}$	liver	3	9

[a] The relative level of mutant or wild-type SOD1 to the level of endogenous mouse SOD1 in spinal cord cytosolic fraction, determined by immunoblotting. All SOD1 mutant mice were taken after clinical disease onset, except as noted.

[b] The relative level of SOD1s and COX4 in mitochondrial fractions determined by immunoblotting. Values are normalized to 1.0 for the $mSOD1^{WT}$/COX4 ratio in spinal cord fractions from 1-year-old normal mice.

[c] Undetectable even after very long exposures.

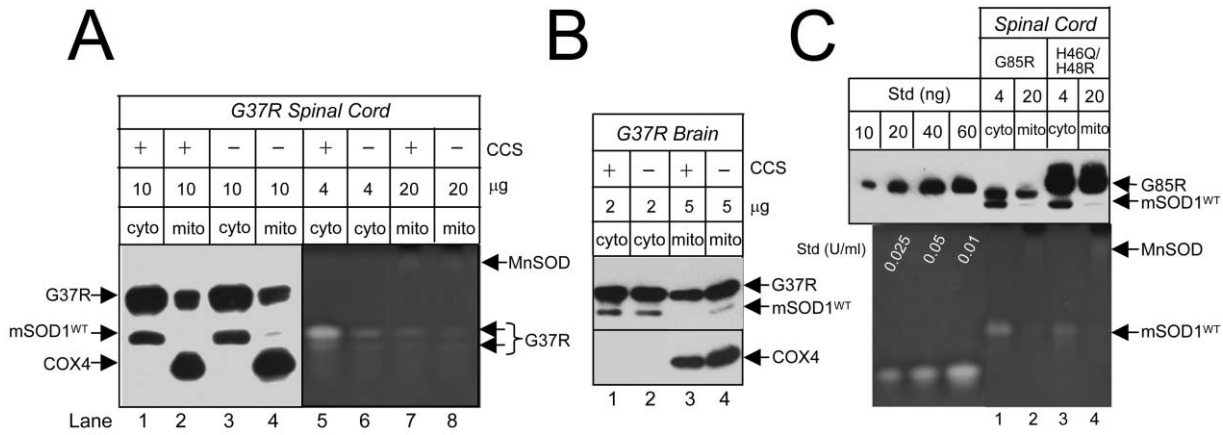

Figure 3. Association of Mutant SOD1s with Mitochondria Is Independent of CCS and Dismutase Activity

(A and B) Cytosolic and purified mitochondrial fractions from spinal cords (A) and brains (B) of symptomatic SOD1^{G37R} (line 42) transgenic mice with and without CCS were analyzed with SOD1 and COX4 antibodies or for dismutase activity (A, right).

(C) Cytosol and mitochondria purified from spinal cords of symptomatic SOD1^{G85R} (line 148) and SOD1$^{H46R/H48Q}$ (line 139) transgenic mice were assayed for accumulated SOD1 levels (top) and dismutase activity (bottom). MnSOD, manganese SOD activity.

CCS did not diminish this and reproducibly increased the proportion of mSOD1^{WT} within the mitochondrial fractions from both brain and spinal cord (Figures 3A and 3B, lane 4).

Analysis of cytosolic fractions from SOD1^{G85R} or SOD1$^{H46R/H48Q}$ confirmed that neither has detectable dismutase activity (Figure 3C, lanes 1 and 3), consistent with previous reports (Bruijn et al., 1997; Wang et al., 2002). Similarly, no dismutase activity corresponding to either SOD1 mutant was detectable in purified spinal cord mitochondria. Therefore, detectable dismutase activity is not required for the high level of mutant SOD1 accumulation with spinal cord mitochondria (Figure 3C, lanes 2 and 4).

Mutant SOD1s Result in Selective, Covalent Damage to Mitochondrial Bound Proteins Independent of Dismutase Activity and CCS

Disease-causing mutant SOD1s have previously been shown to generate prominent aggregates containing both the mutant and wild-type SOD1s (Bruijn et al., 1998), including the presence in spinal cord extracts of SDS-resistant forms of much reduced mobility on denaturing polyacrylamide gels (Johnston et al., 2000; Wang et al., 2002). Both cytoplasmic and purified mitochondrial fractions from phenotypic SOD1 mutant mice were examined for the presence of such aggregated forms. In samples from four mouse models of ALS-like motor neuron disease, immunoblotting with an SOD1 antibody revealed two slower mobility species at similar levels in the cytoplasmic compartment (Figure 4A, orange dots). Similarly, the corresponding mitochondrial fractions, including those from mice expressing either of the two dismutase-inactive mutants, contained a ~45 kDa doublet (Figure 4A, open and filled dots) along with a very slowly migrating SOD1 immunoreactive species that just entered the resolving gel (Figure 4A, blue dots). Each of these more slowly migrating species was mutant SOD1 dependent, as none of these species was found in either the cytoplasmic or mitochondrial fractions of nontransgenic mice or mice expressing high levels of hSOD1^{WT} (Figure 4A, lanes 1–4).

Comparison of SDS-resistant, SOD1 immunoreactive aggregates in SOD1^{G37R} mice that do or do not express the *CCS* gene further revealed that, like toxicity, the very slow mobility form and the lower band of the mitochondrial doublet were produced in the absence of CCS (Figure 4B, lane 4, blue and filled dots, respectively). The more slowly migrating species of the doublet (open dot) was completely absent in mice deleted of CCS. Although this and its mobility suggested that this species may represent a covalent adduct between SOD1^{G37R} (16 kDa) and CCS (32 kDa), immunoblotting with a CCS antibody failed to confirm this (not shown).

Age-Dependent Adducts of Mutant SOD1 Associated with Mitochondria Is Selective to Spinal Cord

Spinal cord mitochondria were also isolated from a transgenic rat (Howland et al., 2002) that develops progressive ALS-like motor neuron disease due to expression of SOD1^{G93A}. As in SOD1^{G93A} mice (data not shown), a significant proportion of the SOD1^{G93A} mutant is associated with spinal cord mitochondria purified from these rats beginning as early as 1.5 months of age (Figure 5A, lane 6), 1–2 months prior to either disease onset or the development of aggregates of mutant SOD1 and/or the protein folding chaperone Hsc70 (Howland et al., 2002). While no high molecular weight adducts of SOD1 were present in nontransgenic or SOD1^{G93A} rats at 1.5 months (Figure 5A, lanes 1–6), several slowly migrating species were found exclusively associated with the mitochondrial fraction (Figure 5A, lane 8) in 3.5-month-old, symptomatic SOD1^{G93A} rats. These slowly migrating species were comparable to those seen previously in the mouse models (Figure 4). Therefore, enrichment of mutant SOD1 and apparent covalent adducts of it with spinal cord mitochondria are common features of ALS-like disease in rodents.

Mitochondria were also purified from the brainstem, cerebellum, cortex, and the remaining brain regions

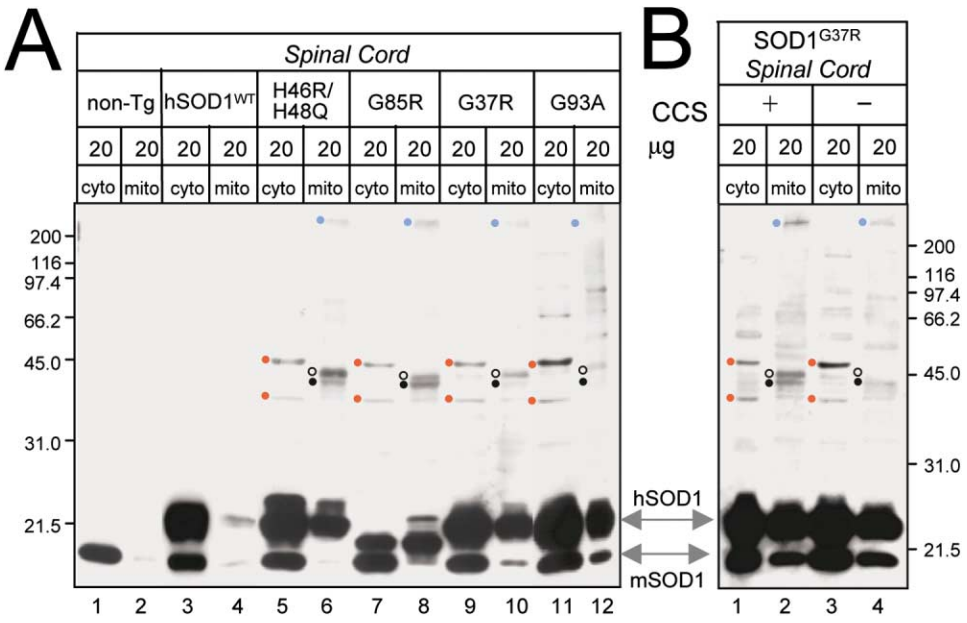

Figure 4. Mutant SOD1s Accumulating with Spinal Cord Mitochondria Result in Age-Dependent Adducts of Mutant SOD1 with Other Proteins Independent of Dismutase Activity and CCS

Cytosolic (cyto) and purified spinal mitochondrial (mito) fractions from a series of mouse lines (as indicated) were analyzed by immunoblotting with an antibody that binds with equal affinity to both human and mouse SOD1. Beyond transgene encoded SOD1 and endogenous mouse SOD1, a series of slower mobility SOD1 immunoreactive forms were detected including a very slowing migrating form (blue dots), two cytosolic forms (orange dots), and two mitochondrial-specific forms (open and black dots), one of which (open dot) requires the presence of CCS.

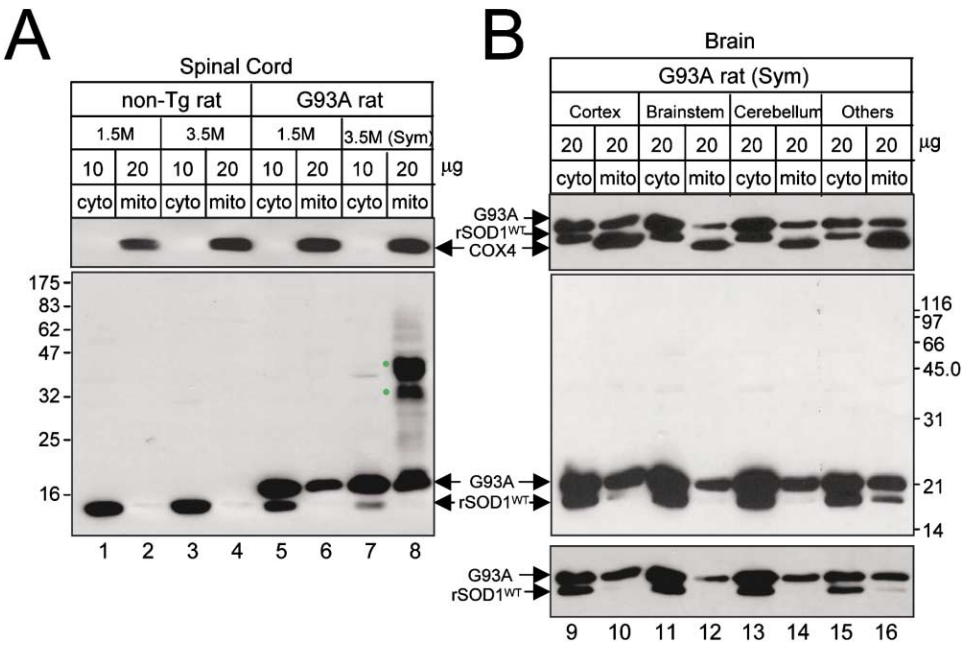

Figure 5. Age-Dependent Association of Mutant SOD1 Adducts Exclusively with Spinal Cord Mitochondria

Cytosolic (cyto) and purified spinal mitochondrial (mito) fractions from nontransgenic and SOD1^{G93A} transgenic rats were analyzed by immunoblotting with an antibody that binds with equal affinity to human and rat SOD1s. Spinal cord tissues (A) and different parts of the brain (B) including brainstem, cerebellum, cortex, and the rest of the brain (others) were immunoblotted with an antibody with equal affinity for rat SOD1 and SOD1^{G93A} and an antibody to COX4 (top) or the same rat/human SOD1 antibody without antibody to COX4 (middle). The bottom panel in (B) is a lower exposure of the middle panel. Green dots in (A) mark two slower mobility adducts in the purified spinal mitochondria from symptomatic rats.

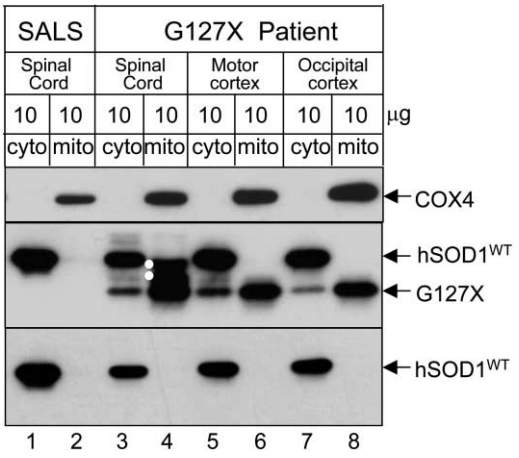

Figure 6. Selective Association of SOD1^{G127X} in Affected Tissues of a Familial ALS Patient

Cytosol and mitochondria were purified from postmortem human tissues from a sporadic ALS (SALS) patient and from a patient with a SOD1^{G127X} mutation. Immunoblots were probed with COX4 antibody (top), an antibody that preferentially identifies the SOD1^{G127X} mutant (middle) (see Experimental Procedures), and a SOD1 antibody to the C-terminal domain of hSOD1 truncated in the SOD1^{G127X} frameshift (bottom). White dots in lane 4 show positions of two SOD1^{G127X}-containing species that migrate slower than the major SOD1^{G127X} species.

(others) of the SOD1^{G93A} rat. Despite similar levels of mutant SOD1^{G93A} associated with these mitochondria and those from spinal cord (compare Figure 5A, lane 8, with Figure 5B, lanes 10, 12, 14, 16), covalently modified adducts of SOD1^{G93A} were found exclusively associated with mitochondria from spinal cord. Since brain mitochondria-associated mutants do not result in covalent modification of SOD1^{G93A} as they do in spinal cord (Figure 5A, indicated by green dots), mutant-mediated damage to mitochondrial bound proteins is spinal cord specific.

Preferential Association of Mutant SOD1 with Spinal Cord Mitochondria in Human Familial ALS

Spinal cord and brain mitochondria were isolated from an autopsy sample from an ALS patient whose disease was caused by a frameshift mutation at codon 127 (SOD1^{G127X}). This frameshift mutant accumulates to a level so low (~1%–2% of that of the remaining wild-type allele) that it is undetectable using typical SOD1 antibodies (Jonsson et al., 2004). To identify the mutant, an antibody was generated to a peptide including 17 residues (CIIGRTLVVHEKADDLG) of hSOD1 and 5 additional amino acids (GQRWK) representing the new carboxyl terminus of SOD1^{G127X} that are encoded by the wrong SOD1 reading frame following the frameshift mutation. This antibody preferentially recognized SOD1^{G127X}, although as expected it also reacted with the much more abundant hSOD1^{WT} (Figure 6, lane 3). Immunoblots with this antibody revealed a striking (more than 10-fold) enrichment of the SOD1^{G127X} mutant in gradient-purified mitochondria from spinal cord extracts (Figure 6, lane 4), as well as accumulation of two additional, more slowly migrating mutant species (white dots). All of these represent altered forms of SOD1^{G127X}, not hSOD1^{WT}, since none are detected with a SOD1 antibody against the C-terminal domain truncated in SOD1^{G127X} (Figure 6, bottom). SOD1^{G127X} accumulation was most pronounced for spinal cord mitochondria. Association of a lower level of mutant SOD1^{G127X}, including the absence of the slower mobility adducts, was also seen in mitochondria from motor or occipital cortex (Figure 6, lanes 6 and 8).

Association of Misfolded Mutant SOD1 with the Cytoplasmic Surface of Spinal Cord Mitochondria

The nature of the association of disease-causing SOD1 mutants with spinal cord mitochondria was initially identified by accessibility of the SOD1^{G85R} mutant to digestion by an added protease. Although hSOD1^{WT}, as well as the active mutants SOD1^{G37R} and SOD1^{G93A}, are strongly resistant to proteolysis in the presence or absence of detergents (Ratovitski et al., 1999), the inactive, more poorly folded SOD1^{G85R} is easily digested. Taking advantage of this, mitochondria were freshly purified from spinal cords of symptomatic SOD1^{G85R} mice, and aliquots were treated with proteinase K in the absence or presence of detergent to permeabilize mitochondrial membranes (Figures 7A and 7B). Consistent with intact outer membranes in the isolated mitochondria in the absence of added detergent, Tim23, a component of the mitochondrial inner membrane, was resistant to proteolysis (Figure 7B, lane 3) when detected with a Tim23 antibody recognizing an epitope within the IMS. At the same level of added protease, all Tim23 was degraded after detergent-mediated membrane disruption (Figure 7B, lane 4). Similarly, the integral inner membrane component, COX 4, was also resistant to proteinase K digestion only in the absence of membrane lysis. (Cytochrome c, a releasable IMS protein, was resistant to the mild proteolysis conditions whether or not the outer membrane was intact.) Both SOD1^{G85R} and its slower mobility adducts that were found only in association with spinal cord mitochondria (Figure 7B, lane 2) were quantitatively degraded in the absence of detergent (Figure 7B, compare lanes 2 and 3), consistent with binding of SOD1^{G85R} onto the cytoplasmic face of mitochondria.

Location of mitochondrial SOD1 was tested further by immunogold electron microscopy using a human SOD1-specific peptide antibody (Clement et al., 2003) and mitochondria purified from symptomatic SOD1^{G85R} mice. This revealed that 77% (64/83) of spinal cord mitochondria had detectable SOD1^{G85R} associated with them. Similarly, despite little SOD1^{G85R} associated with mitochondria purified from whole brain (Figure 2C), 66% (32/48) of mitochondria from the cortex had bound mutant. Most SOD1^{G85R} was found at or near the cytoplasmic surface of spinal cord (Figures 7C, 7E, and 7F) or cortex (Figures 7D and 7G–7I) mitochondria. Counting of gold particles associated with the periphery of the mitochondrial outer membrane revealed a density of gold labeling four times that found in the central IMS space (Figure 7L). Analysis of mitochondria from cortex of a symptomatic SOD1^{G37R} mouse (Figures 7J and 7K) revealed comparable proportions of this fully active mutant to be associated with the periphery and the IMS (Figure 7L), consistent with successful import of a significant fraction of this mutant after initial association with mitochondrial components on the cytoplasmic face.

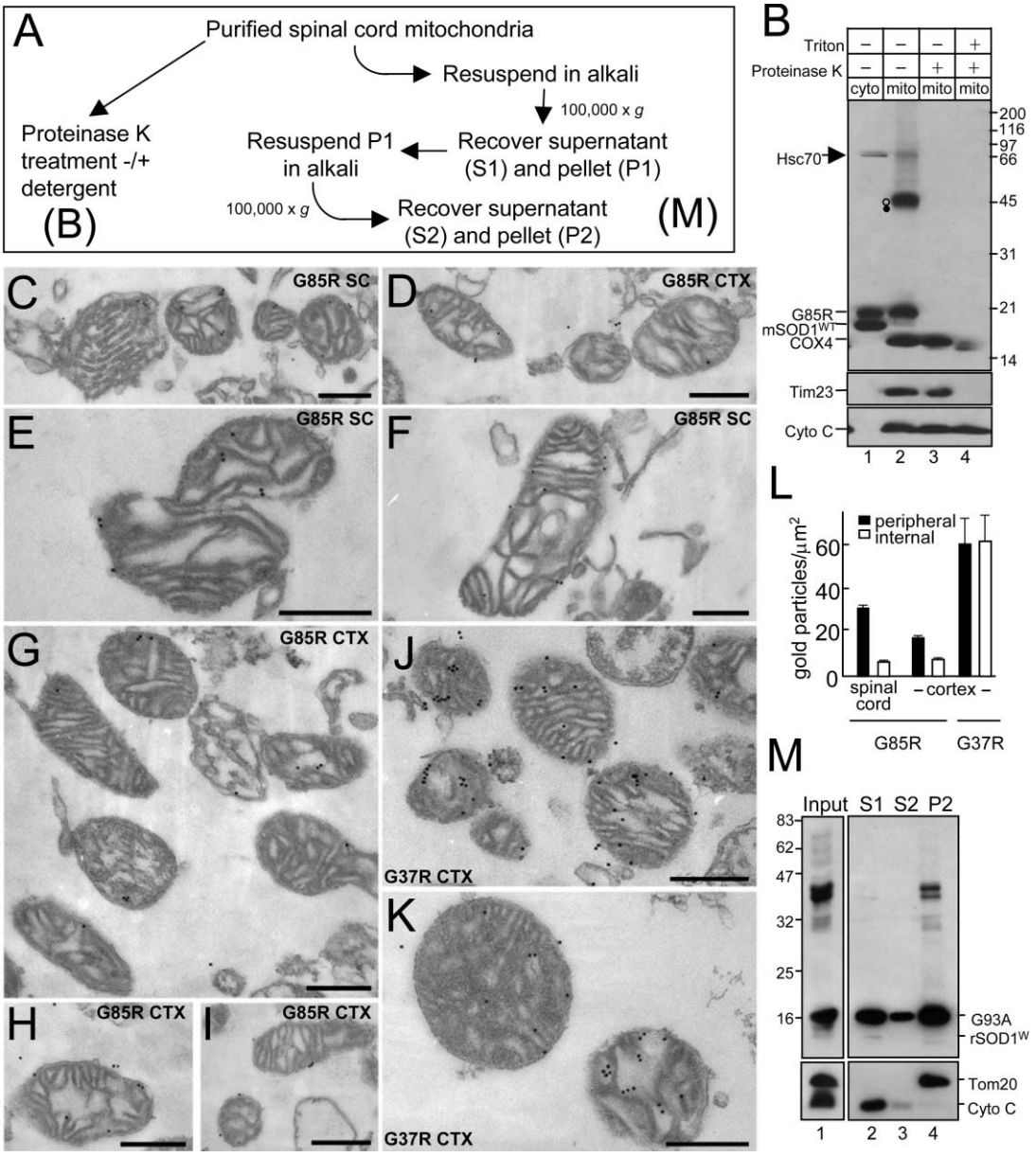

Figure 7. Mutant SOD1 Is Aggregated onto the Cytoplasmic Side of Spinal Cord Mitochondrial Outer Membranes and Imported into the IMS of Mitochondria

(A) Schematic of fractionations of spinal mitochondria in parts (B) and (M).

(B) Quantitative degradation of mitochondrially associated SOD1^{G85R} and its slower mobility adducts after addition of proteinase K in the absence of detergent. Proteinase K was added to 100 μg/ml to the cytosol or purified mitochondria from SOD1^{G85R} mice and the fractions then analyzed by immunoblotting for remaining SOD1 (mSOD1^{WT} and SOD1^{G85R}), COX4, Tim23, Hsc70, or cytochrome c. Analyses were performed in parallel with or without addition of Triton X-100, as indicated, to a final concentration of 0.2%.

(C–K) Immunogold electron microscopy following postembedment labeling of purified mitochondria from spinal cords (SC) or cortex (CTX) of SOD1^{G85R} (C–I) or SOD1^{G37R} (J and K) mice using an antibody specific for human SOD1. Scale bars equal 300 nm.

(L) Densities of peripheral or internal gold particles from images such as (B)–(K). Background densities of gold particles for CTX and SC are 0.001 and 0.0005 particle/μm^2, respectively.

(M) Mitochondria purified from symptomatic SOD1^{G93A} rats were treated with alkali as in (A), and supernatants (S1 and S2) and the final membrane-associated pellet (P2) were immunoblotted for mutant and rSOD1^{WT} (top), as well as the intermembrane component cytochrome C (bottom) or the mitocondrial import component TOM20 (middle), an integral membrane protein.

Furthermore, treatment with alkali (which releases soluble proteins and those bound peripherally to membranes, but not integral membrane proteins [Fujiki et al., 1982]) removed less than half of the SOD1^{G93A} associated with membranes of purified spinal cord mitochondria. While the alkali-soluble proportion of SOD1^{G93A} was consistent with a previous report of a significant level of this mutant accumulated in the IMS (Higgins et al., 2002), the majority of the SOD1^{G93A} was very tightly membrane associated, remaining membrane bound even after re-

peated alkali treatment (Figures 7A and 7M, lane 4). Cytochrome c, which is tethered in the IMS, was quantitatively released by alkali as expected (Figure 7M), while integral membrane proteins, such as Cox 4 or those of the mitochondrial import machinery (e.g., TOM 20; Figure 7M) were quantitatively retained in the alkali-resistant, membrane-associated pellets.

Discussion

One of the great puzzles in the study of inherited neurodegenerative diseases is why the nervous system, and particular subsets of neurons, is selectively targeted for toxicity by widely or ubiquitously expressed mutant proteins. This study offers one of the first glimpses into how cells from the spinal cord may be selectively targeted for degeneration in models of ALS: SOD1 mutants with highly divergent biochemical characteristics and a 20-fold disparity among cytoplasmic levels are recognized as import substrates by components on the cytoplasmic face of spinal cord mitochondria, with similar amounts of each very tightly associated with mitochondrial import components and/or other integral mitochondrial membrane proteins. Mutant association and import does not occur in unaffected tissues such as muscle or liver. This universal presence of mutant SOD1 on and in spinal cord mitochondria begins contemporaneous with the earliest pathology and well prior to disease onset, is found for both catalytically active and inactive mutants, is unaffected by CCS-dependent loading of the catalytic copper, and is most strikingly found in human ALS caused by an unstable SOD1 mutant that accumulates only to trace cytoplasmic levels. Because a high fraction of spinal mitochondria have mutant SOD1 associated, it is highly likely that many of these must be generated in cell types beyond motor neurons, consistent with the non-cell-autonomous toxicity of these mutants (Clement et al., 2003). Collectively, these findings support mitochondrial association to be directly related to disease toxicity.

Since essentially no wild-type SOD1 is found in spinal cord mitochondrial fractions, mutant association cannot simply reflect a general pathway of mitochondrial import of SOD1, residual cytoplasmic contamination of the purified mitochondria, or leaky, mutant-damaged outer mitochondrial membranes. The absence of wild-type SOD1 from spinal cord mitochondria coupled with a high proportion of dismutase active mutant SOD1s apparently imported into the IMS, while inactive mutants are primarily aggregated onto the cytoplasmic surface, firmly indicates that misfolded mutant SOD1s are recognized by spinal mitochondria as substrates for selective import. Conversely, the appearance of a significant proportion of wild-type SOD1, but not SOD1 mutants, in purified mitochondria from liver clearly reveals the presence of one or more heretofore unrecognized, cell type-specific mechanisms for such association and presumed import. The involvement in ALS of factors that mediate tissue-specific mitochondrial import also offers a simple explanation for the association with ALS of a variant in the import signal of the mitochondrial SOD2 (Van Landeghem et al., 1999). Further work is now critical to identify the tissue-specific factors that modify mitochondrial import and how this pathway relates to the mechanisms and components previously described (Pfanner and Wiedemann, 2002).

We propose that the universal association of SOD1 mutant with mitochondria exclusively within affected tissues represents the common property of these mutants that initiates a cascade of damage (Figure 8). In this view, the mutants initially damage mitochondria by aggregation onto integral membrane components (including those directly involved in import) and/or within the IMS, impeding the normal functioning of those components in either compartment. Active mutants that bind the catalytic copper either in the cytoplasm and/or IMS may damage themselves by their own (aberrant) catalysis, thereby exacerbating kinetically or thermodynamically unstable protein folding. Inactive, inherently misfolded mutants like $SOD1^{G85R}$ are probably recipients of damage after aggregation, perhaps as a consequence of proximity to reactive species released from or within mitochondria. Such damage further enhances mutant misfolding, perhaps driving mutant oligomerization as has been seen in structural analyses of two such mutants (Elam et al., 2003) and/or formation of SDS-resistant adducts to other mitochondrial components. Such a "feed forward" amplification mechanism could explain the precipitous, rapid loss of motor neurons after disease initiation.

The tissue selectivity of mitochondrial association strongly implies that spinal cord-specific mitochondrial and/or cytoplasmic factors are necessary to facilitate association. The known mitochondrial import pathway requires the cytoplasmic chaperones Hsp90/70 for delivery of unfolded proteins (Young et al., 2003) and strongly favors metal-free apo SOD1 proteins for delivery of wild-type SOD1 into mitochondria (Okado-Matsumoto and Fridovich, 2002; Field et al., 2003). Indeed, toxicity can be delayed by co-induction of a stress response including Hsp70 and Hsp90 (Kiernan et al., 2004). Thus, one conserved aspect of toxicity may be impairment of mitochondrial import pores with poorly folded, covalently crosslinked mutant SOD1 independent of either dismutase activity or a requirement for the catalytic copper. Selective mitochondrial damage is consistent with other examples of neurodegenerative disease, including mutations in Hsp60, a mitochondrial matrix protein, and frataxin, an inner membrane protein, which result in hereditary spastic paraplegia and Friedreich ataxia, respectively (Koehler et al., 1999; Roesch et al., 2002; Puccio and Koenig, 2002; Hansen et al., 2002; Hörtnagel et al., 2003). Moreover, mutation in a component of mitochondrial import, TIMM8a, causes human deafness dystonia (Roesch et al., 2002).

Finally, selective SOD1 mutant-mediated damage to spinal cord mitochondria as a primary toxicity in ALS is especially attractive since mitochondria are the gatekeepers of caspase-directed cell death (Newmeyer and Ferguson-Miller, 2003). Indeed, death of motor neurons, but not other neurons, from activation of an intrinsic cell death pathway involving both motor neuron-specific and a classic mitochondrially mediated FADD/caspase 8 pathway can be enhanced by SOD1 mutants (Raoul et al., 2002). Accumulation of mutant SOD1 has also been associated with cytochrome c release from mitochondria, translocation of Bax, truncation of Bid, and

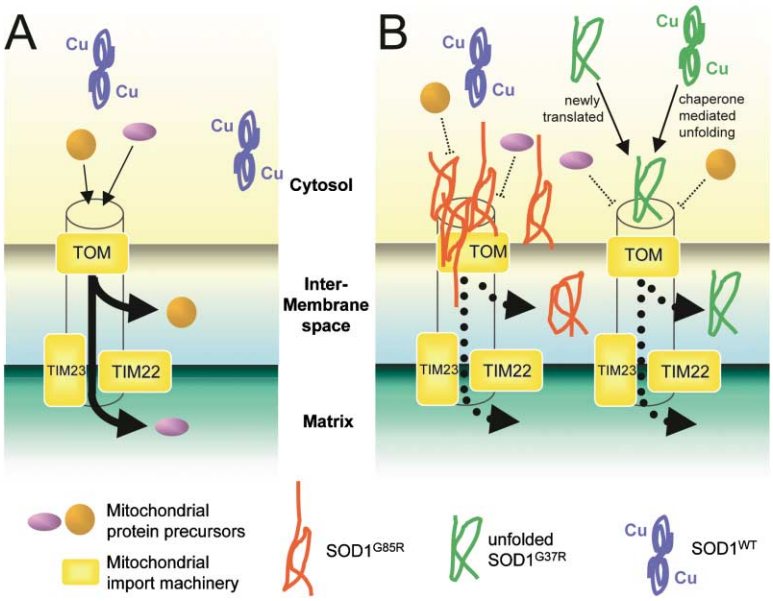

Figure 8. Model for Selective ALS-Linked SOD1 Mutant-Mediated Damage Selectively to Spinal Mitochondria by Impairing Import Channels

(A) In the normal spinal cord cells, little $SOD1^{WT}$ is either associated with spinal mitochondria or imported into the IMS despite uptake into mitochondria from liver (Figure 2C; Okado-Matsumoto and Fridovich, 2001). (B) As we have shown here, ALS-linked SOD1 mutants all selectively associate with spinal mitochondria, with a proportion of each aggregated on the cytoplasmic surface. An additional proportion is imported, presumably requiring the action of both the Hsp70 and Hsp90 chaperones for unfolding and presentation to the TOM import complex, as has been shown for other import substrates (Kiernan et al., 2004). The selectivity of association with spinal mitochondria suggests action of as yet unidentified cytoplasmic and/or mitochondrial factors that are unique to spinal cord and that facilitate association. Association is probably driven by mutant misfolding, accompanied by production of higher molecular weight adducts and SDS-resistant aggregates. These clog import channels or otherwise inhibit outer mitochondrial membrane components, thus functionally damaging mitochondria. Mutants that bind the catalytic copper may also catalyze aberrant chemistry on one or both sides of the mitochondrial outer membrane.

activation of the executioner caspase 3 (Vukosavic et al., 2000; Pasinelli et al., 2000; Li et al., 2000; Guegan et al., 2001, 2002). Survival can be extended by increased synthesis of the apoptosis inhibitor Bcl-2 (Kostic et al., 1997), infusion of a broad-spectrum caspase inhibitor (Li et al., 2000), or by administration of minocycline, which has been correlated with inhibition of cytochrome c release from mitochondria (Zhu et al., 2002). When combined with multiple lines of evidence that have demonstrated that toxicity is non-cell-autonomous, that is, does not arise solely from mutant damage directly within motor neurons (Lino et al., 2002; Gong et al., 2000; Pramatarova et al., 2001; Clement et al., 2003), the presence in one or more spinal cord cell types of components that mediate mitochondrial association and import of SOD1 mutants provides a mechanistic explanation for selective toxicity to motor neurons from ubiquitously expressed SOD1 mutants in familial ALS.

Experimental Procedures

Transgenic Mice

Transgenic mice expressing $hSOD1^{WT}$, $SOD1^{G37R}$, and $SOD1^{G85R}$ were as originally described (Wong et al., 1995; Bruijn et al., 1997). $SOD1^{G93A}$ transgenic mice (Gurney et al., 1994) expressing lower and higher levels were obtained from the Jackson Laboratory. Transgenic mice expressing $SOD1^{H46R/H48Q}$ were from Wang et al. (2002); tissues from transgenic mice expressing $SOD1^{G85R}$ under the Thy1 promoter (Lino et al., 2002) were provided by Dr. Pico Caroni. Transgenic rats expressing $SOD1^{G93A}$ were described by Howland et al. (2002).

Human Autopsy Samples

All ALS autopsy samples were obtained with informed consent and frozen at −80°C. Sporadic ALS samples were obtained from a collection at Johns Hopkins University School of Medicine. In conformance with the Helsinki Declaration approval by the ethical committees of the Universities of Copenhagen and Umeå, samples from a patient carrying the $SOD1^{G127X}$ mutation were obtained after a postmortem time of 20 hr.

Subcellular Fractionation

Mitochondria were purified by a modification of an earlier protocol (Okado-Matsumoto and Fridovich, 2001). Tissues (frozen or fresh) were homogenized at 1:5 (w/v) ratio (in a buffer containing 210 mM mannitol, 70 mM sucrose, 10 mM Tris [pH 7.5], and 1 mM EDTA [pH 7.5]) and centrifuged at 1300 × g for 10 min. The pellet was washed with a half volume of the same buffer and the supernatants were combined and centrifuged again. The subsequent supernatant was then centrifuged at 17,000 × g for 15 min to produce a crude mitochondrial pellet. The supernatant was further subjected to centrifugation at 100,000 × g for 1 hr and the final supernatant was designated the cytosolic fraction. The crude mitochondrial pellet was washed once with 50 mM KCl (in the extraction buffer) and sedimented at 52,000 × g for 1.5 hr on a discontinuous Nycodenz gradient (Okado-Matsumoto and Fridovich, 2001). Material at the 25%–30% interface was collected and designated as purified mitochondria. Protein concentrations were measured using the BCA method. In the case of $Thy1\text{-}SOD1^{G85R}$ mice, frozen spinal cords were combined with SOD1 null cortex materials (1:3, w/w) before subcellular fractionation so as to have enough overall starting tissue extract to purify mitochondria. To remove soluble IMS components and peripheral membrane proteins, purified spinal cord mitochondria were resuspended in alkali (0.1 M Na_2CO_3 [pH 11.5]), incubated on ice for 30 min, and then centrifuged at 100,000 × g for 30 min, as previously described (Fujiki et al., 1982).

Immunoblot Analysis

Samples were separated on either regular SDS-PAGE gel or SDS-PAGE containing 3 M urea and transferred to nitrocellulose and probed with various antibodies including a rabbit polyclonal raised against a peptide identical in human, rat, and mouse SOD1s and a peptide antibody recognizing human, but not mouse or rat, SOD1 (Clement et al., 2003; Howland et al., 2002). A polyclonal antibody to $SOD1^{G127X}$ mutant was generated to the 22 carboxy-terminal amino acids (CIIGRTLVVHEKADDLGGQRWK) as described (Jonsson et al., 2003). This was affinity purified against the peptide and preadsorbed with $hSOD1^{WT}$ that had been denatured by exposure to 6 M guanidinium chloride and 3 mM of the copper chelator DTPA. COX4 and cytochrome c antibodies were from CloneTech. Hsp60, syntaxin 6, and synaptotagmin antibodies were from StressGen. HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies were used and detected by ECL (Amersham).

Immuno-Electron Microscopy

Mitochondria were isolated from fresh tissues, collected in homogenization buffer, and fixed by addition of an equal volume of fixative (4% formaldehyde, 0.4% glutaraldehyde, 4 mM $CaCl_2$ in 0.1 M cacodylate buffer [pH 7.3]) and incubation for 30 min at 4°C. Mitochondrial pellets were then collected and washed with 0.1 M cacodylate/2 mM $CaCl_2$ and processed for embedment in LR White (EMS). Ultrathin sections (70–80 nm) were collected on 300 mesh nickel grids. Sections were etched with saturated sodium periodate (Sigma, St. Louis, MO) for 15 min and blocked with 4% bovine albumin (BSA) in Tris-buffered saline (TBS) for 45 min. Grids were incubated with a human SOD1-specific peptide antibody (1:100) in 1% BSA/TBS and incubated at 4°C overnight followed by goat anti-rabbit IgG conjugated to 10 nm gold (Amersham, Arlington Heights, IL, 1:30) for 1 hr at room temperature. The sections were postfixed in 2% glutaraldehyde for 20 min, rinsed in distilled water, and contrasted with 2% uranyl acetate for 15 min and lead citrate for 10 min. Sections were observed in a Philips (model 208) electron microscope. Gold particles in the central and peripheral mitochondrial areas were counted. The peripheral region was defined as that within 30 nm of either side of the outer mitochondrial membrane, a distance representing the theoretical maximum distance a gold particle could be from the antigen, based on the approximate length of the two ~15 nm long IgG molecules separating the gold and the antigen.

Dismutase Activity Assay

Superoxide dismutase assays were performed in situ (Borchelt et al., 1994) after separation on 7.5% nondenaturing acrylamide gels containing 25% glycerol. Gels were stained in 50 mM potassium phosphate buffer (pH 7.8) containing 82 μM riboflavin, 0.1 mM nitrobluetetrozolium, and 27.5 μM TEMED for 45 min on a shaker, and then briefly destained.

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