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Selective association of misfolded ALS-linked mutant SOD1 with the cytoplasmic face of mitochondria

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Mutations in copper/zinc superoxide dismutase (SOD1) are causative for dominantly inherited amyotrophic lateral sclerosis (ALS). Despite high variability in biochemical properties among the disease-causing mutants, a proportion of both dismutase-active and -inactive mutants are stably bound to spinal cord mitochondria. This mitochondrial proportion floats with mitochondria rather than sedimenting to the much higher density of protein, thus eliminating coincidental proportion floats with mitochondria rather than sedimenting to the buoyant density of mitochondria, thereby eliminating the possibility that association reflects cosedimentation with much more dense protein aggregates. Use of density gradient methods demonstrates that multiple mutant SOD1s float to the buoyant density of mitochondria, thereby eliminating the possibility that association reflects cosedimentation with much more dense protein aggregates. Furthermore, we uncover that the nature of both dismutase-active and -inactive mutant SOD1 association with spinal cord mitochondria involves very tight association with the outer mitochondrial membrane that is both alkali- and salt-resistant. The combination of sensitivity to proteolysis and immunoprecipitation with an antibody specific for misfolded SOD1 demonstrates that in all rodent models of mutant SOD1-mediated ALS, misfolded SOD1 is deposited onto the cytoplasmic face of the outer mitochondrial membrane, increasing antigenic accessibility of the normally structured electrostatic loop. Association of misfolded mutant SOD1 selectively onto spinal cord mitochondrial membranes documents that the nature of mutant SOD1 association with mitochondria is not the same in all tissues, implicating a unique tissue-selective composition of cytoplasmic chaperones, unique components on the cytoplasmic face of spinal mitochondria that have a selective affinity for mitochondrial membranes.

Results

SOD1 Association with Mitochondria Demonstrated by Buoyant Density Centrifugation. By using a single-step enrichment (a continuous iodixanol gradient) to fractionate initial tissue homogenates and in which both protein aggregates and mitochondria will sediment downward after sample loading at the top (19), mutant SOD1 association with mitochondria (especially for the dismutase-inactive mutant hSOD1G85R) has been proposed to represent coenrichment of SOD1 aggregates with mitochondria, rather than mutant association with mitochondria. To enrich for mitochondria while eliminating potential protein aggregates [which are known for all SOD1 mutants (3, 20, 21) but not wild-type (WT) SOD1 (12, 20)], differential sedimentation was initially used to enrich for mitochondria from tissue extracts. After this initial fractionation to remove most soluble components, samples were loaded at the bottom of a bound at the surface and imported into the intermembrane space (17). Alternative proposals include mutant import into the matrix of mouse brain mitochondria (18) or that apparent mitochondrial association of unfolded, dismutase-inactive mutants represents simple contamination of mitochondrially enriched fractions with protein aggregates (19). We now test these competing proposals. Use of density gradient methods demonstrates that multiple mutant SOD1s float to the buoyant density of mitochondria, thereby eliminating the possibility that association reflects cosedimentation with much more dense protein aggregates. Furthermore, we uncover that the nature of both dismutase-active and -inactive mutant SOD1 association with spinal cord mitochondria involves very tight association with the outer mitochondrial membrane that is both alkali- and salt-resistant. The combination of sensitivity to proteolysis and immunoprecipitation with an antibody specific for misfolded SOD1 demonstrates that in all rodent models of mutant SOD1-mediated ALS, misfolded SOD1 is deposited onto the cytoplasmic face of the outer mitochondrial membrane, increasing antigenic accessibility of the normally structured electrostatic loop. Association of misfolded mutant SOD1 selectively onto spinal cord mitochondrial membranes documents that the nature of mutant SOD1 association with mitochondria is not the same in all tissues, implicating a unique tissue-selective composition of cytoplasmic chaperones, unique components on the cytoplasmic face of spinal mitochondria, or misfolded SOD1 conformers unique to spinal cord that have a selective affinity for mitochondrial membranes.

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The authors declare no conflict of interest.

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In each of four mutant SOD1 models (hSOD1G93A and hSOD1H46R rats; hSOD1G85R and hSOD1G127X mice), significantly higher levels of each mutant SOD1 were present in floated mitochondria from affected spinal cord tissues compared with liver, a tissue unaffected by disease (Fig. 1D–F; compare lanes 3, 6, and 9; and SI Fig. 7). Furthermore, comparing spinal cord, brain, and cortical samples from the same animals, a higher proportion of dismutase-inactive mutants hSOD1G85R, hSOD1G127X, and hSOD1H46R floated with spinal cord mitochondria (Fig. 1E and F, compare lanes 3 and 6; and SI Fig. 7). Almost equivalent levels of the dismutase-active mutant hSOD1G93A floated with mitochondria from both spinal cord and cortex (Fig. 1D, lanes 3 and 6). Low electrophoretic mobility adducts of mutant SOD1 that were detergent- and reducing agent-resistant (reminiscent of oxidized multimers described in ref. 24) also floated with mitochondria, indicating that they are tightly bound to floated mitochondria (Fig. 1D–F, lanes 3). Thus, human SOD1 is a bona fide resident on or within spinal mitochondria, and there is preferential association of mutant SOD1 with mitochondria within affected tissues.

**Dismutase-Inactive SOD1 Is Deposited on the Cytoplasmic-Facing Surface of Spinal Cord, but Not Brain, Mitochondria.** Multiple prior efforts have disagreed on how mutant SOD1 is associated with mitochondria. Efforts had argued that association was onto the cytoplasmic mitochondrial face [for hSOD1G93A (17)], in the intermembrane space (IMS) and the cytoplasmic surface [for hSOD1G37R (17)], in the IMS [for hSOD1G93A (14, 25–27)], and in the matrix [for hSOD1G93A and hSOD1G85R (18)]. To resolve mutant location on floated mitochondria that were free from protein aggregates, protease sensitivity was used to determine the proportion of mutant SOD1 accessible to protease by virtue of location on the cytoplasmic face of intact mitochondria. Because WT SOD1 and WT-like mutants (e.g., hSOD1G93A and hSOD1G37R) are known to be tightly folded, hyperstable proteins that are resistant to proteinase K digestion (28, 29) (Fig. 2A), this could only be done for the dismutase-inactive mutants hSOD1H46R, hSOD1G85R, and hSOD1G127X, which are known to be susceptible to protease digestion (28, 29), presumably because of the adoption of less well folded or misfolded conformations. By using a saturating amount of proteinase K (as demonstrated in SI Fig. 8), the majority of each of the three dismutase-inactive mutant SOD1s associated with intact spinal cord mitochondria was protease-sensitive even in

*Fig. 1. A portion of SOD1 is localized to mitochondria enriched by buoyant density centrifugation. (A) Schematic outlining the enrichment of mitochondria according to buoyant density. (B) Demonstration of the selectivity of two SOD1 polyclonal antibodies used in this study. (C–F) SOD1 is enriched in floated mitochondria, compared with crude mitochondria and whole homogenates. SOD1 is enriched in spinal cord mitochondria and less so in cortical and liver mitochondria. VDAC, calnexin, and Hsp90 calnexin serve as markers for mitochondria, endoplasmic reticulum, and cytosol, respectively.*
the absence of detergent. Further, the remaining mutant protein (as well as Smac, a protein in the intermembrane space) was completely digested after detergent-mediated solubilization of mitochondrial membranes (Fig. 2A). In cortical mitochondria from the same animals, however, mutant SOD1 proteins remained protease-resistant when membranes were intact. These experiments indicate that these inactive mutant SOD1s are largely deposited onto the cytoplasmic face of the outer mitochondrial membrane in spinal cord as evidenced by sensitivity to proteolysis. In cortical mitochondria, the same mutants are completely insensitive to protease until addition of detergent, consistent with an intramitochondrial localization. Note that the absolute amounts of mutant SOD1 localized inside mitochondria is comparable in spinal cord and cortex. (C and D) The accumulation of mutant SOD1 protein on the surface correlates with disease because it is much less apparent in asymptomatic hSOD1G85R (C) and hSOD1H46R (D) animals. As expected, (A) hSOD1G85A and endogenous rat SOD1 (hSOD1) are not protease-sensitive. In all cases, the IMS protein Smac is only susceptible to proteolysis in the presence of detergent. Age is indicated in months. Data shown are representative of three independent experiments.

**Fig. 2.** SOD1 associates with the cytoplasmic face of the outer mitochondrial membrane and correlates with disease. (A) Mitochondria enriched via floatation from spinal cord, cortex, and liver were treated with 100 μg/ml proteinase K (PK) in the presence or absence of 0.5% Triton X-100 (Tx-100). The majority of mitochondrially associated hSOD1 mutants with a partially misfolded conformation including hSOD1G127X (A), hSOD1G85R (B), and hSOD1H46R (D), are found deposited on the cytoplasmic face of the outer mitochondrial membrane in spinal cord as evidenced by sensitivity to proteolysis. In cortical mitochondria, the same mutants are completely insensitive to protease until addition of detergent, consistent with an intramitochondrial localization. Note that the absolute amounts of mutant SOD1 localized inside mitochondria is comparable in spinal cord and cortex. (C and D) The accumulation of mutant SOD1 protein on the surface correlates with disease because it is much less apparent in asymptomatic hSOD1G85R (C) and hSOD1H46R (D) animals. As expected, (A) hSOD1G85A and endogenous rat SOD1 (hSOD1) are not protease-sensitive. In all cases, the IMS protein Smac is only susceptible to proteolysis in the presence of detergent. Age is indicated in months. Data shown are representative of three independent experiments.

**Dismutase-Inactive SOD1 Mutants Behave as Integral Membrane Proteins of Spinal Cord, but Not Brain, Mitochondria.** Divergent claims have been made concerning whether the association of mutant SOD1 with spinal cord mitochondria is as a loosely membrane-bound or soluble component (18) or is tightly associated with mitochondrial membranes whose linkage is resistant to alkali extraction (17). As an initial test of whether mutant SOD1 binding to the cytoplasmic face of spinal cord mitochondria was mediated by electrostatic interactions, floated mitochondria were repeatedly washed in a high-ionic strength buffer. However, just as was the case for the integral outer membrane protein VDAC, little to no mutant SOD1 was released (Fig. 3), demonstrating a salt-resistant linkage.

Next, although SOD1 protein does not contain any transmembrane segments nor a recognizable mitochondrial targeting signal (which we verified by using the bioinformatics tools DAS-TMfilter, TMPred, TMP, HMMPVal, SPLIT, PRED-TMR2, and ConPredII), alkali extraction of isolated floated mitochondria from either spinal cord or cortex from a variety of transgenic lines was used to separate peripherally bound membrane components from those more tightly associated (Fig. 4A). This revealed that for spinal cord mitochondria, endogenous SOD1 was mostly released, as expected for a soluble component of the intermembrane space (or matrix), or a peripheral membrane component (Fig. 4B, lanes 2, 5, 8, and 11). For mutant SOD1, both alkali-sensitive and alkali-resistant pools were found in spinal cord mitochondria (Fig. 4B, lanes 2 and 3). In contrast, in the alkali-resistant fraction of identically treated cortical mitochondria isolated from the same animal, only a very small proportion of hSOD1G85A was recovered.

**Fig. 3.** Mutant SOD1 is tightly bound to mitochondrial membranes in a salt-resistant manner. hSOD1G85A cannot be removed from isolated floated spinal cord mitochondria by extensive washing even with 1 M KCl. VDAC yields the expected pattern for an integral outer membrane protein. Data shown are representative of two experiments.
Mutant SOD1 is tightly bound to mitochondrial membranes in an alkali-resistant manner. (A) Schematic of alkali extraction. (B) Approximately one-third of hSOD1G93A, but almost no hSOD1WT protein, is tightly associated with spinal cord mitochondrial membranes in an alkali-resistant manner. In contrast, SOD1 is completely soluble in cortical mitochondria. (C) In contrast, hSOD1G93A, which adopts a partially unfolded conformation, is found in a higher proportion in spinal cord mitochondrial membranes compared with hSOD1G85R and hSOD1G127X. (D) Time-dependent and disease-related integration of hSOD1G127X and hSOD1G93A into spinal cord mitochondrial membranes. VDAC and cytochrome c are used to identify behaviors of well defined mitochondrial components that are integral and peripheral membrane-associated, respectively. Data are representative of three experiments.

Two independent monoclonal antibodies were generated against the DSE2 region (amino acids 125–142). In detergent lysates of floated spinal cord mitochondria, both robustly detected misfolded SOD1 and covalently associated forms that migrated at positions consistent with cross-linked dimeric SOD1 (Fig. 5C, Upper, lanes 2 and 4). No similarly misfolded mutant SOD1 was associated with cortical mitochondria (Fig. 5C, Lower, lanes 2 and 4). Approximately 20% of hSOD1G93A and 50% of hSOD1G85R associated with floated spinal cord mitochondria were in a misfolded conformation that permitted DSE2 antibody binding (Fig. 5D). Protease sensitivity of the usually protease-resistant hSOD1G93A (28, 29) confirmed its misfolded conformation (Fig. 5D, Upper, compare lanes 6 and 8). Neither DSE2 antibody immunoprecipitated any misfolded SOD1 for either SOD1 mutant in the cytosol from either tissue (data not shown). Neither of the antibodies to DSE2 recognized hSOD1WT that had been imported into spinal mitochondria, even after detergent solubilization to liberate internal mitochondrial contents (Fig. 5D).

Recovery of intact, floated mitochondria (in the absence of detergent) by binding to a DSE2 antibody further demonstrated that for both dismutase-active and -inactive mutants, essentially all misfolded SOD1 was bound to spinal cord mitochondria that had antibody-accessible, misfolded SOD1 bound to the cytoplasmic face (Fig. 5E). This misfolded SOD1 was highly selectively enriched onto the outer mitochondria membrane, with a much smaller proportion (~10%) associated with microsomal fractions (100,000 × g pellet; Fig. 5D, Lower, lanes 6 and 10, and E, Lower, lanes 2 and 6). Thus, mutant hSOD1 is tightly and selectively bound to the cytoplasmic-facing surface of spinal cord mitochondria in a misfolded conformation.

**Discussion**

Floatation of mutant SOD1 protein with spinal cord, but not cortical or liver, mitochondria unambiguously demonstrates that...
the presence of these mutant subunits cannot reflect contamination of protein aggregates with mitochondria, as has been proposed (19). Misfolded SOD1 is bound in a very tight, alkali-resistant linkage to the cytoplasmic face of the outer mitochondrial membrane (as shown by protease sensitivity of misfolded, dismutase-inactive mutants). These findings are further supported by immunoelectron microscopy of spinal cord motor neurons in situ in which hSOD1G93A is found both within mitochondria and in close proximity to the mitochondrial surface (25). Furthermore, similar studies in hSOD1G85R spinal motor neurons also demonstrate a preferential distribution at the mitochondrial surface (C.V.V. and D.W.C., unpublished data). This is common to multiple dismutase-active and -inactive mutants and is found only for mitochondria isolated from tissues at highest risk during disease, findings that support mutant-derived damage to spinal cord mitochondria as a central feature of pathogenesis from ubiquitously expressed SOD1 (36) but not yet confirmed (37).

A pool of mutant SOD1 is also found within mitochondria, most likely in the intermembrane space, as is known for the WT SOD1 protein. Both the physical properties and the dual localization of SOD1 (cytosol and mitochondria) are reminiscent of those known for yeast adenylate kinase, which is also known to fold rapidly and spontaneously into a thermal- and proteolytic-resistant hyperstable conformation (38). For adenylate kinase, partitioning between the protein folds, its cryptic mitochondrial targeting signal is spontaneously into a thermal- and proteolytic-resistant hyperstable conformation (38). For adenylate kinase, partitioning between the protein folds, its cryptic mitochondrial targeting signal is

Misfolded SOD1 accessible to DSE2 antibody after detergent solubilization (B) [Panels C & D]

Elution (% SDS) Wash 3x Unbound (U)

Fig. 5. Mutant SOD1 associated with floated spinal cord mitochondria is misfolded. (A) The DSE2 epitope mapped onto hSOD1G37R crystal structure (Protein Data Bank ID code 1AZV), as generated by PyMol. (B) Schematic of immunoprecipitation experiments. (C) Floated isolated mitochondria from hSOD1H46R spinal cord and cortex were immunoprecipitated with two independent antibodies to the DSE2 region. Each uniquely identified a pool of misfolded SOD1 associated with spinal cord but not cortical mitochondria. (D and E) DSE2 recognizes a protease-sensitive pool of hSOD1G93A and hSOD1H46R but not hSOD1WT in solubilized (D) or intact floated spinal cord mitochondria (E), but not microsomal membranes.

through exposure of a normally buried hydrophobic surface (40, 41). Although an inherently misfolded mutant SOD1 would be predicted to bind other intracellular membranes, which would be consistent with previous reports (29, 42), our data clearly demonstrate that misfolded SOD1 conformers are preferentially associated with mitochondria, with only small amounts detectable in microsomal fractions.

Two mechanisms, which are not mutually exclusive, are consistent with age-dependent binding of mutant SOD1 selectively to mitochondrial membranes only on spinal cord mitochondria. First, an enhanced level of a misfolded mutant SOD1 conformer in which a unique surface is exposed [perhaps because of fluctuations of the electrostatic loop (43–45)] may interact with unique, tissue-selective array of cytoplasmic chaperones to facilitate association/presentation to mitochondria. Second, there may be components unique to the cytoplasmic face of spinal cord mitochondria to which misfolded SOD1 binds. Indeed, it is recognized that mitochondria from different tissues (which perform different biological activities) obligatorily have different protein compositions (22, 23). Furthermore, the tissue type differences reported here call into question the pathological relevance of data collected on mitochondria from tissues that are not the primary target in disease. Combined with the very stable binding (resistance to alkali and high ionic strength), we propose that mutant SOD1 conformers bind to an integral mitochondrial component enriched in spinal mitochondria by exposure of one or more hydrophobic SOD1 surfaces.

In any case, a central determinant for mutant SOD1 association with mitochondria would be the steady-state proportion of misfolded SOD1. Thus, for mutants that adopt a well folded, native-like conformation (like hSOD1G37R or hSOD1G93A), high total accumulated levels would be required to drive mitochondrial membrane association because only a small proportion of the total protein is accumulated as a misfolded conformer. For dismutase-inactive mutants, accumulation at much lower levels would drive a comparable level of accumulation of misfolded mutant. For both mutant classes, SOD1 harboring ALS-linked mutations have significantly slower folding kinetics in vitro (38). Consistent with all of this, in mice, hSOD1G85R causes disease at levels up to 50 times lower than the dismutase-active mutants (3). In human disease as well, the least
stable mutants (accumulating to the lowest levels) correlate with the most rapid disease progression (46).

Finally, mitochondrial association is accompanied by accumulation of low-mobility species, the most prominent of which appears to be a transiently cross-linked dimer. A similar species has recently been shown to be a feature also common in sporadic disease (47), albeit in human disease it remains untested whether this dimer is selective for mitochondria. Determining this is now central to testing whether promiscuous mitochondrial–membrane association of SOD1 may contribute to pathogenesis in sporadic ALS.

Materials and Methods

Mice were deeply anesthetized by isoflurane inhalation and decapitated. Spinal cords were flushed out of the vertebral column with PBS and quickly placed in 5 volumes of ice-cold homogenization buffer (HB) [250 mM sucrose, 10 mM Hepes–MgCl₂, 1 mM EDTA, plus protease inhibitors). Cortices were quickly dissected from whole brain. Tissues were homogenized with seven strokes of a glass-pestle homogenizer at 4°C and on ice. Homogenates were centrifuged at 1,000 × g for 10 min. Supernatants were recovered, and pellets were washed with ½ volume HB. Supernatants were pooled and centrifuged at 17,000 × g for 15 min to yield S3 and a crude mitochondrial pellet. Crude mitochondria were gently resuspended in HB and then adjusted to 1.204 g/ml Optiprep (iodixanol) and loaded on the bottom of a polycarbonate tube. Mitochondria were overlaid with an equal volume of 1.175 g/ml and 1.078 g/ml Optiprep and centrifuged at 50,000 × g for 4 h (SW-55; Beckman). Mitochondria were collected at the 1.078/1.175 g/ml interface and washed once to remove the Optiprep. Optiprep stock solution (1.32 g/ml, Axis Bioshield) was diluted in 250 mM sucrose, 120 mM Hepes–NaOH (pH 7.4), 6 mM EDTA plus protease inhibitors.

Additional materials and methods are discussed in Supplemental Materials and Methods.