Mutant Cu, Zn Superoxide Dismutase that Causes Motoneuron Degeneration Is Present in Mitochondria in the CNS

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Mutations in Cu, Zn superoxide dismutase (SOD1) cause a fraction of amyotrophic lateral sclerosis (ALS), which involves motoneuron degeneration, paralysis, and death. An acquired activity by mutant SOD1 is responsible for the cellular toxicity, but how mutant SOD1 kills motoneurons is unclear. In transgenic mouse models of ALS, mitochondrial degeneration occurs early, before disease onset, raising the question of how mutant SOD1 damages mitochondria. Here we investigate the intracellular localization of SOD1 in the CNS to determine whether SOD1 is present in mitochondria, where it could directly damage this organelle. We show that endogenous mouse SOD1, wild-type human, and mutant human SOD1 (G93A), when expressed as transgenes, are colocalized with mitochondria in spinal cord by immunofluorescence confocal microscopy. By immunoelectron microscopy, we show that SOD1 is present within mitochondria at similar concentrations as in the cytoplasm. Thus SOD1, in addition to being a cytosolic enzyme, is present inside mitochondria in the CNS.

Key words: motor neuron disease; neurodegenerative disease; neurodegeneration; spinal cord; ALS; aging

Superoxide dismutase (SOD1) is a 17 kDa protein, known to be present in cytoplasm as a homodimer (Fridovich, 1986). Its function is superoxide dismutation. Mutations in SOD1 cause a fraction of amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease in which motoneurons degenerate, resulting in paralysis and death (Brown, 1995). Several experiments have demonstrated that mutant SOD1 causes motoneuron degeneration by gaining a toxic property, rather than a loss of superoxide dismutation function (Dal Canto and Gurney, 1995; Ripps et al., 1995; Wong et al., 1995). There are two major hypotheses regarding the toxic properties gained by the mutant SOD1. The first suggests that mutant SOD1 damages cells by producing peroxynitrite (Estevez et al., 1999), whereas the second proposes that mutant SOD1 damages cells by its enhanced peroxidase activity (Wiedau-Pazos et al., 1996; Yim et al., 1996, 1997). The validity of either hypothesis remains to be proven (Xu, 2000).

An important question in understanding the mechanism of motoneuron degeneration is what the cellular target for this toxicity is, or what is the cellular pathway that leads eventually to cell death. Previous studies have observed mitochondrial vacuolation in transgenic mice expressing mutant SOD1 (Dal Canto and Gurney, 1995; Wong et al., 1995), and the peak of this vacuolation correlates with the onset of clinical disease (Kong and Xu, 1998). Furthermore, widespread mitochondrial abnormalities are present before the peak vacuolation and clinical disease (Kong and Xu, 1998). The evidence suggests that mitochondrial damage is an early step in the mutant SOD1-induced motoneuron degeneration pathway, raising the question of how mutant SOD1 damages mitochondria.

SOD1 has been thought to be a cytoplasmic protein (Crapo et al., 1992). Some studies, however, have suggested the presence of a small fraction of the total cellular SOD1 in the intermembrane space of liver mitochondria (Weisiger and Fridovich, 1973; Tyler, 1975), although this has been controversial (Geller and Winge, 1982). In light of the new evidence that mitochondria are damaged in mice expressing mutant SOD1 and develop ALS, we revisited this issue and investigated whether mutant SOD1 is associated with mitochondria in the CNS. We present double immunofluorescence and ultrastructural evidence that a fraction of endogenous mouse SOD1 and transgenic wild-type and mutant human SOD1 in the mouse is present within mitochondria in the CNS. Thus, mutant SOD1 could exert its toxicity directly on mitochondria, leading to mitochondrial damage and onset of motoneuron degeneration.

MATERIALS AND METHODS

Transgenic mice. Two transgenic mouse lines (The Jackson Laboratory, Bar Harbor, ME) were used. One expresses human SOD1 mutant G93A

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(C57BL/6J-TgN(SOD1-G93A)1Gur^{dl}) (G93A mice), and the other expresses wild-type human SOD1 (WS mice) (Gurney et al., 1994). Nontransgenic littermates of G93A transgenic mice were used as wild-type controls (WT mice). All transgenic mice were identified using PCR according to Gurney et al. (1994). Mice were maintained at the University of Massachusetts Medical School animal facility according to the guidelines set forth by the Institutional Animal Care and Use Committee.

Double immunofluorescence. Mice were killed by decapitation under anesthesia, and spinal cords were dissected quickly. Lumbar and cervical spinal cords were cut out, frozen immediately in OCT freezing media (Sakura, Torrance, CA) on dry ice, and stored at −80°C. Eight micrometer sections were cut using a cryostat and mounted on slides treated with Vectabond (Vector Laboratories, Burlingame, CA). The slides were incubated in PBS at room temperature for 30 min, followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.5, for 10 min. After washing, the sections were doubly stained with anti-SOD1 (Pardo et al., 1995) and anti-cytochrome C oxidase subunit 1 (COX1) antibodies (Molecular Probes, Eugene, OR) according the protocol described previously (Kong et al., 1998; Levine et al., 1999). The stained sections were examined and digitized using a confocal microscope (TCS-SP; Leica, Mannheim, Germany). Imaging analysis and three-dimensional reconstruction were conducted using MetaMorph (Universal Imaging Corporation, West Chester, PA).

Immunoelectron microscopy. Mice (60- to 80-d-old) were perfused under anesthesia with fixative (4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.5). Lumbar spinal cords from fixed mice were dissected out and dehydrated through an ethanol series to 100% ethanol and embedded in hard grade LR White resin (Electron Microscopy Sciences, Fort Washington, PA) by polymerization overnight at 60°C. Sixty-five nanometer sections were cut from the embedded tissue using a Reichert-Jung Ultracut E microtome and collected onto gold grids (200 mesh) (SPI Inc., Westchester, PA). Sections on grids were processed for immunogold electron microscopy according to recommendations by the manufacturer of the gold-conjugated antibodies (Amersham Biosciences, Piscataway, NJ) with some modification. Sections were etched in 0.1N HCl for 5 min, rinsed three times for 5 min each in TBS (in mM: 25 Tris, 140 NaCl, and 2.7 KCl, pH 8.0), placed in blocking buffer for 30 min (0.1% gelatin, 1% normal goat serum, and 0.3% Triton-X-100 in TBS), placed in primary antibody for 2 hr at room temperature, rinsed three times for 5 min each in TBS, placed in gold-conjugated secondary antibodies (10 nm gold anti-mouse and 5 nm gold anti-rabbit) for 1 hr, rinsed three times in TBS, fixed in 2% glutaraldehyde, rinsed three times in TBS, rinsed in water, stained with Reynold's lead citrate followed by aqueous 2% uranyl acetate, and dried on filter paper. The following antibodies and sera were used: normal rabbit serum (Vector Laboratories), rabbit polyclonal anti-SOD1 (Pardo et al., 1995), a second rabbit polyclonal anti-SOD1 (Biodesign, Saco, ME), mouse monoclonals against cytochrome oxidase subunit 1 (COX1) and against COX subunit 4 (Molecular Probes), and rabbit polyclonal anti-neurofilament light chain (Xu et al., 1993). All antibodies and serum were used at a 1:10 dilution.

The stained sections were viewed and photographed using a Philips CM10 transmission electron microscope. The negatives were digitized, and immunogold density was determined using MetaMorph software as follows. The number of gold particles were counted inside and outside mitochondria and then normalized to the areas of mitochondria and nonmitochondria, respectively. Myelin and extracellular areas are excluded from the measurement. However, it is not practical to exclude all other cytoplasmic organelles because the identity and the boundary of these organelles are often not unequivocal. Therefore, organelles other than mitochondria are included as nonmitochondrial area. Results were summarized as the average per mitochondrion and average per extramitochondrial space in a micrograph.

RESULTS

To determine whether SOD1 is associated with mitochondria, we examined whether SOD1 was present in mitochondrial fractions from mouse brain homogenates. SOD1 was detected in the mitochondrial fractions from WT mice and in increasing amounts from mice expressing wild-type human SOD1 (WS) and mice expressing mutant SOD1 (G93A) (data not shown). This result gave us preliminary evidence that SOD1 was associated with mitochondria in the CNS as was shown for liver (Weisiger and Fridovich, 1973; Tyler, 1975). As in the previous reports using liver, it was impossible to purify mitochondria using biochemical fractionation to the extent that other organelles could be eliminated as sources of SOD1 (Geller and Winge, 1982). Therefore, we decided to resolve this issue using morphological means.

Double immunofluorescence staining was performed on spinal cord sections from G93A mice using anti-SOD1 and anti-COX1 (mitochondrial marker) antibodies. Because of the high concentration of cytoplasmic SOD1 (which generates a high SOD1 staining background), it was difficult to demonstrate colocalization of SOD1 and COX1 signals in fixed mouse spinal cords. To reduce the level of extraorganelle cytosolic SOD1, unfixed frozen spinal cord sections from G93A mice were incubated in PBS for 30 min to allow unassociated SOD1 to diffuse away. Sections were then fixed with 4% paraformaldehyde and stained using routine double immunofluorescent staining method.

Widespread overlapping signals from mitochondria and SOD1 were observed (Fig. 1*A*) (notice the abundant yellow structures in the right panel; some examples are pointed out by *arrows*). All COX1-immunoreactive structures overlap with SOD1-positive structures, although many SOD1-positive structures are negative for COX1 (Fig. 1*A*). Because mitochondria are small, it was possible that overlaps seen in the XY dimensions were not real in the Z dimension. To rule out this possibility, serial sections were recorded using a confocal microscope and reconstructed in three dimensions. In all cases, the overlaps between the COX1 and SOD1 signals were confirmed (Fig. 1*B,C*) (also see supplemental videos at www.jneurosci.org). The same results were obtained in digitonin-permeablized cultured pheochromocytoma cell line and HeLa cells when their endogenous SOD1 was detected by double SOD1 and COX1 immunofluorescence (data not shown).

To determine whether the SOD1 is inside or attached to the outside of mitochondria, SOD1 was further localized at the ultrastructural level using immunoelectron microscopy (Fig. 2). Lumbar spinal cord sections from the three different mice (G93A, WS, and WT) were stained by immunogold for SOD1 (small particles) and mitochondrial enzyme cytochrome oxidase (large particles). Mitochondria were identified by their characteristic structure and by their positive staining for cytochrome oxidase (Fig. 2*A,C,E, large particles*). All mitochondria are positively stained for SOD1 (Fig. 2*A,C,E, small particles*). Far more small particles are present in G93A and WS spinal cords than those in the WT spinal cord, consistent with overexpression of SOD1 in the transgenic animals. Very few, if any, small particles are present in the matrix (Fig. 2*A,C, asterisks*), indicating that SOD1 is unlikely to be in the matrix compartment. Sections incubated with a second anti-SOD1 antibody (Biodesign) yielded the same staining pattern (data not shown). In control staining in which an anti-neurofilament subunit NF-L antibody was substituted for the anti-SOD1 antibody, small particles are largely confined to the cytoplasm and are segregated from the large particles that reside inside mitochondria (Fig. 2*B,D,F*). In two additional controls in which the sections were incubated with nonimmune rabbit serum followed by the secondary antibody or the sections were incubated with the secondary antibody alone, only sparse background particles were occasionally detected (data not shown).

To estimate the relative concentration of SOD1 inside mitochondria when compared with that in the intracellular space outside of mitochondria, we counted the small gold particles inside and outside mitochondria. These counts confirm that the gold particle densities representing SOD1 in WS and G93A mice are several fold higher than those in the WT mice (Fig. 3). The

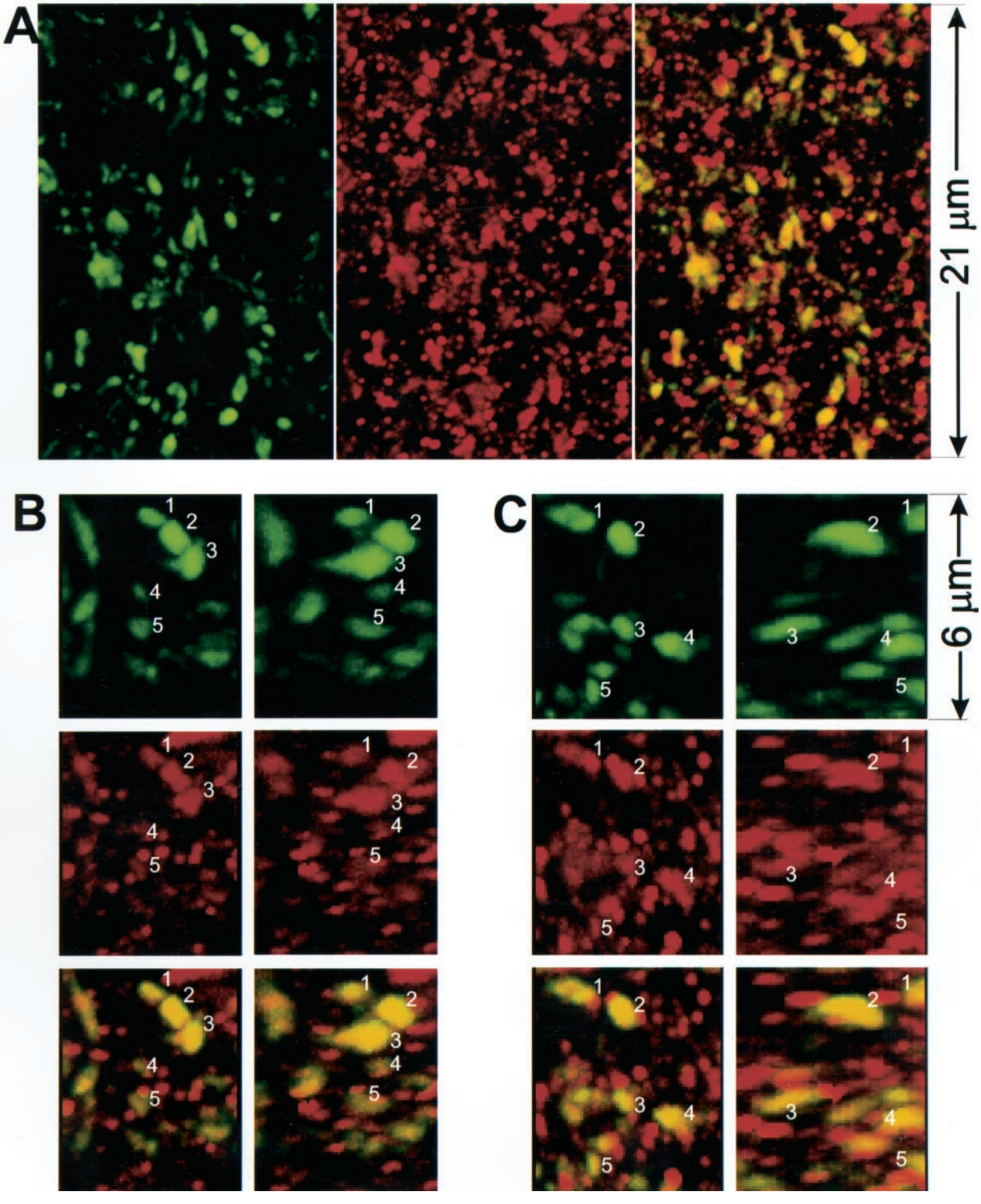

Figure 1. Double immunofluorescent staining of spinal cord sections from G93A mice using anti-COX1 (*green*) and SOD1 (*red*) antibodies. *A*, Low-magnification view of an area covering part of the ventral column motor axon exit zone. The *panel* on the *right* shows the superimposition of COX1 and SOD1 staining. *B, C*, High-magnification view of two small groups of mitochondria. The *three panels* on the *right* illustrate the same mitochondria as shown in the *left panels* except that they are rotated 40 and 90° in *B* and *C*, respectively. The *numbers* mark the same mitochondria viewed from the two different angles. The *bottom two panels* are superimposition of the *top two green* and *red panels* (for a complete three-dimensional view, see the supplemental videos at www.jneurosci.org).

density of SOD1 in mitochondria is slightly higher inside mitochondria than that in the extramitochondrial cytosolic space, although this difference is only significant in G93A mice (Fig. 3) ($p = 0.02$ using two-tailed Student's t test). In contrast to a high density of SOD1 both inside and outside of mitochondria, staining with a rabbit polyclonal antibody against neurofilament light chain shows a high density only outside of mitochondria and essentially no counts of gold particles within mitochondria (Fig. 3).

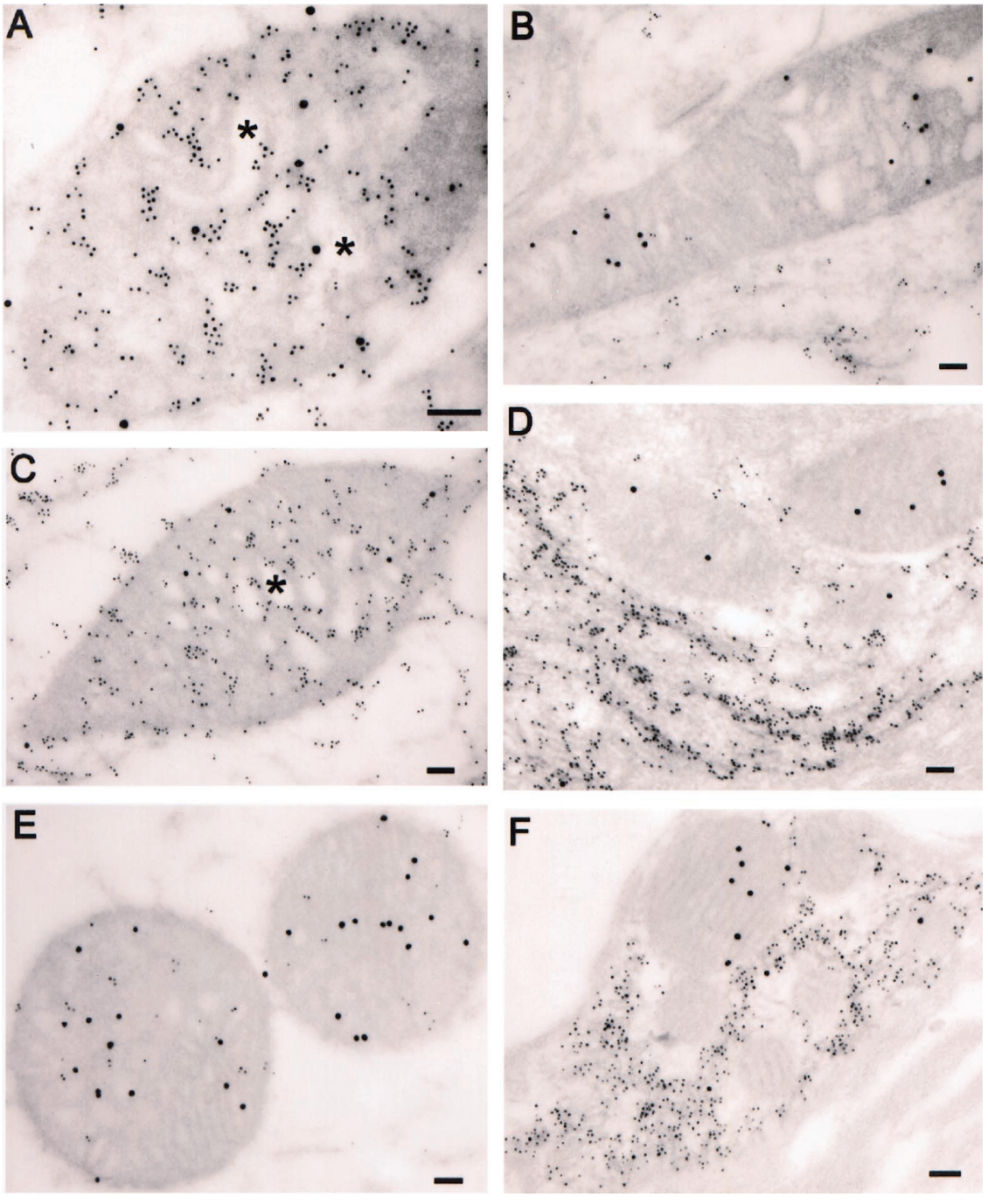

Figure 2. Immunogold localization of SOD1 in the spinal cord from G93A (*A, B*), WS (*C, D*), and WT (*E, F*) mice. *A, C, E,* Sections reacted with rabbit polyclonal anti-SOD1 (5 nm particles) and mouse monoclonals against COX subunit 1 and 4 (10 nm particles). *B, D, F*, Sections incubated with rabbit polyclonal anti-neurofilament light chain (5 nm particles) and mouse anti-COX1 and 4 (10 nm particles). Scale bars, 100 nm. Each *asterisk* indicates significantly less than the concentration within mitochondria ($p = 0.02$ using two-tailed Student's t test).

DISCUSSION

This study presents immunofluorescence and ultrastructural evidence that a fraction of wild-type and mutated SOD1 is localized within mitochondria in the CNS. First, double immunofluorescence and three-dimensional reconstruction reveal that all mitochondria are colocalized with SOD1 signals in the spinal cord of mutant SOD1 transgenic mice (Fig. 1). Second, abundant SOD1 signals are detected within the mitochondria by immuno-EM (Fig. 2). In addition, quantification of immunogold staining against SOD1 reveals that the gold particle density inside mitochondria is similar to that in the cytosol outside of mitochondria, suggesting that SOD1 concentration inside mitochondria is at comparable levels with the cytosol.

The presence of SOD1 in mitochondria was reported by several previous studies, which suggested that SOD1 is in the intermembrane space (Weisiger and Fridovich, 1973; Peeters-Joris et al., 1975; Tyler, 1975; Henry et al., 1980). However, doubts were raised because a later study found that SOD1 in mitochondrial

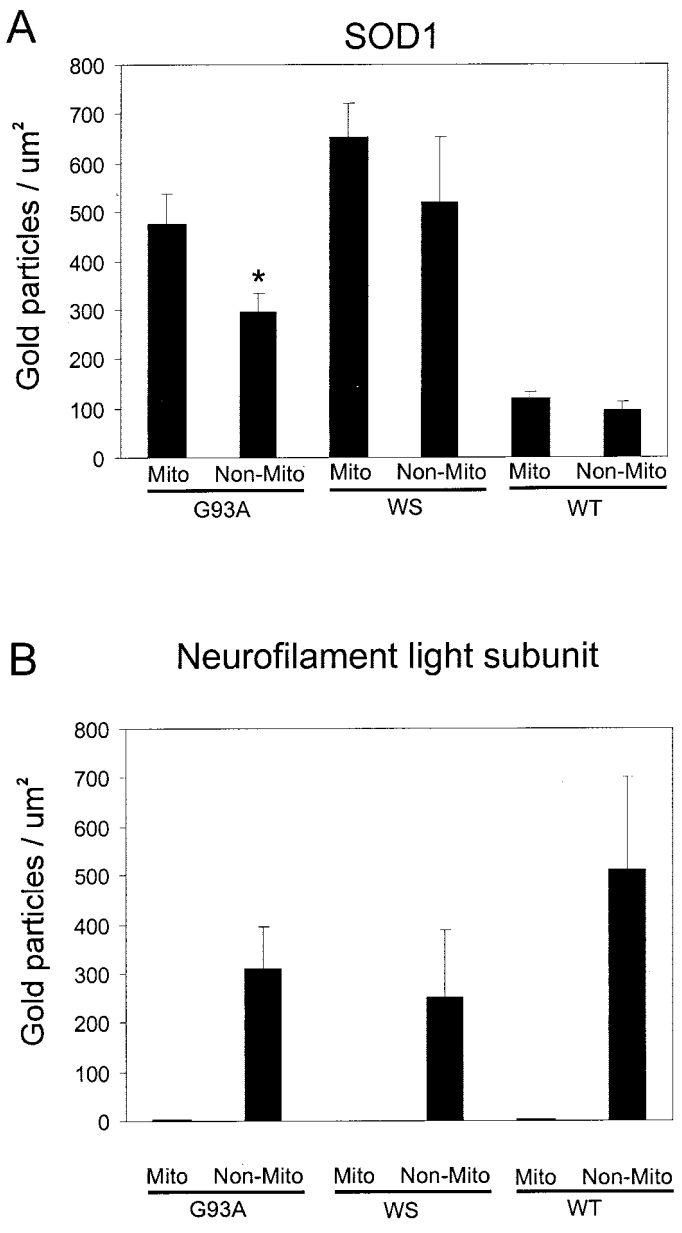

Figure 3. Density of immunogold particles staining for SOD1 (using the antibody from Biodesign) in mitochondrial and extramitochondrial cytosolic areas in the spinal cord of WT, WS, or G93A mice. The number of 5 nm gold spots within individual mitochondria (*mito*) and in the cytosolic area outside mitochondria (*non-mito*) in a micrograph were counted and divided by the mitochondrial area and cytosolic area outside mitochondria, respectively (see Materials and Methods). *A,* SOD1 density. Five electron micrographs were counted for each genotype. The *asterisk* indicates that the concentration of G93A SOD1 outside of mitochondria is significantly less than the concentration in mitochondria (two-tailed Student's *t* test). *B*, Neurofilament light chain density. Three electron micrographs were counted for each genotype.

fractions could be accounted for by contamination with lysosomes (Geller and Winge, 1982). Further doubts were raised when some laboratories reported the presence of SOD1 in peroxisomes (Keller et al., 1991; Dhaunsi et al., 1992; Wanders and Denis, 1992). These controversies are derived from the difficulty in making pure mitochondria completely devoid of contamination by other cellular organelles. Our current approach circumvented this difficulty by double immunofluorescence and immunogold staining on prefixed CNS spinal cords and cultured cells. The results demonstrate that a fraction of SOD1 is inside mitochondria in the CNS.

In conjunction with previous data showing that mitochondria are an early target for damage by mutant SOD1 in transgenic mice (Dal Canto and Gurney, 1995; Wong et al., 1995; Kong and Xu, 1998), these results raise the possibility that mutant SOD1 might damage mitochondria directly. This is supported by morphological observation in human ALS, which showed abundant abnormal mitochondria (Hirano et al., 1984; Nakano and Hirano, 1987). Although not all transgenic mouse models for ALS (e.g., G85R) (Bruijn et al., 1997) exhibit morphologically abnormal mitochondria, functional abnormalities cannot be ruled out. Indeed, mutant SOD1, when introduced into cells, causes mitochondrial dysfunction (Carri et al., 1997; Kruman et al., 1999). Using cybrid technology, Swerdlow et al. (1998) demonstrated mitochondrial dysfunction in platelet cells isolated from humans with sporadic ALS. The efficacy of creatine treatment in slowing the disease progression in mice expressing mutant SOD1 further supports that mitochondrial damage plays a role in mutant SOD1-induced motoneuron degeneration (Klivenyi et al., 1999).

Our results agree with recently published data demonstrating that SOD1 is in the intermembrane space of yeast mitochondria (Sturtz et al. 2001) and a report showing that mutant SOD1 was associated with vacuolated mitochondria in the CNS of ALS transgenic mice (Jaarsma et al., 2001). Our data show that endogenous SOD1 in wild-type mice is in morphologically normal mitochondria, and that both normal and ALS-causing mutant human SOD1 in transgenic mice are in mitochondria, probably at concentrations similar to that in the cytoplasm. Although our antibody does not distinguish between mouse and human SOD1 (it detects both), it is unlikely that normal and mutant SOD1 are segregated. This is because when either is overexpressed, signal intensity is proportionally increased in both the mitochondrial and cytoplasmic compartments (Fig. 3).

Mitochondria are known to be a major, and probably the most predominant, source of oxidative free radicals in cells, and thus, may provide a fertile environment for the effects of the mutant SOD1 toxicity. For instance, if either of the two major hypotheses regarding the nature of the mutant SOD1 toxicity (see introductory remarks) is correct, mitochondria could provide abundant substrates for the mutant SOD1 to further generate toxic free radicals, which in turn damage mitochondria from within. Some possible downstream effects have been reported in literature, including impairment in energy metabolism (Hatazawa et al., 1988), elevated oxidative stress (Beal, 1998; Hall et al., 1998), increased sensitivity to excitotoxicity (Ikonomidou and Turski, 1996; Rothstein, 1996; Bittigau and Ikonomidou, 1997), deficient axonal transport (Cleveland, 1999), and deregulation of apoptosis (Kostic et al., 1997; Li et al., 2000). Of particular interest is a recent observation that the vulnerability to excitotoxicity in motoneurons appears to be selectively enhanced when mitochondrial function is impaired (Kaal et al., 2000). Taken together, our results indicate that mitochondrial damage is an important area of investigation in the causes of ALS.

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