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Mutations associated with amyotrophic lateral sclerosis convert superoxide dismutase from an antiapoptotic gene to a proapoptotic gene: Studies in yeast and neural cells

(*SOD1* gene/*Saccharomyces cerevisiae*)

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ABSTRACT **Familial amyotrophic lateral sclerosis (FALS) is associated with mutations in *SOD1*, the gene encoding copper/zinc superoxide dismutase (CuZnSOD). However, the mechanism by which these mutations lead to amyotrophic lateral sclerosis is unknown. We report that FALS mutant SODs expressed in yeast lacking CuZnSOD are enzymatically active and restore the yeast to the wild-type phenotype. In mammalian neural cells, the overexpression of wild-type *SOD1* inhibits apoptosis induced by serum and growth factor withdrawal or calcium ionophore. In contrast, FALS-associated *SOD1* mutants promote, rather than inhibit, neural apoptosis, in a dominant fashion, despite the fact that these mutants retain enzymatic SOD activity both in yeast and in mammalian neural cells. The results dissociate the SOD activity of FALS-associated mutants from the induction of neural cell death, suggesting that FALS associated with mutations in *SOD1* may not be simply the result of a decrease in the enzymatic function of CuZnSOD. Furthermore, the results provide an *in vitro* model that may help to define the mechanism by which FALS-associated *SOD1* mutations lead to neural cell death.**

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease with an incidence of ≈1 in 10,000. Approximately 10% of cases of ALS are familial (FALS), and in a subset of these cases, mutations in *SOD1*, which encodes copper/zinc superoxide dismutase (CuZnSOD), have been demonstrated (1). It has been hypothesized that the mutations in *SOD1* lead to FALS by decreasing the enzymatic activity of CuZnSOD (2). However, several findings have raised questions about this interpretation. (*i*) The determinations of low CuZnSOD activity were carried out *in vitro*, in some cases following isolation of the protein in nonphysiological conditions, without correlation of *in vivo* activity (2, 3). (*ii*) The FALS-associated *SOD1* mutations affect amino acid residues involved in enzyme dimerization or β-barrel turns, rather than those corresponding to the active site (1–4). The single exception, $His^{46} \rightarrow Arg$ (H46R) in which the active site is directly involved, is reported to lead to a very mild form of FALS (5); in contrast, severe forms result from mutations far removed from the active site. (*iii*) All mutations identified to date have been missense, rather than nonsense, mutations (1–4). (*iv*) It is unusual for a null or hypomorphic mutation to lead to a dominantly inherited disease when the mutated gene encodes an enzyme (6). (*v*) SOD activity measurements from lymphoblasts of patients with the G37R mutation have demonstrated normally active heterodimeric CuZnSOD, arguing that, at least for the G37R mutation, the total SOD activity is not compromised by heterodimerization of the wild-type monomer with the mutant monomer (7). (*vi*) Transgenic mice expressing the G93A mutation demonstrate motor neuron degeneration despite an increased level of SOD activity (8).

We report results from two complementary systems that allow the evaluation of the *in vivo* effects of wild-type and mutant CuZnSOD activity: yeast mutants null for *SOD1*, and conditionally immortalized mammalian neural cells. In the yeast *sod1* mutants, expression of yeast or human wild-type CuZnSOD or of A4V, G39A, G93C, or L38V human FALS mutant CuZnSOD eliminated paraquat sensitivity and hyperoxia sensitivity; *in vitro* SOD activity, tested at physiological pH, was only moderately reduced for these mutant CuZnSODs. In conditionally immortalized mammalian neural cells, the overexpression of wild-type *SOD1* inhibited apoptosis in association with an increase in CuZnSOD activity. However, expression of the FALS *SOD1* mutants A4V and G37R enhanced, rather than inhibited, apoptosis in a dominant fashion, despite increasing SOD activity nearly as much as the wild type. This finding dissociates the mutants' SOD enzymatic activity from their effect on neural cell death, suggesting that FALS associated with *SOD1* mutations may not be simply the result of a decrease in CuZnSOD enzymatic function. Furthermore, the results provide a cell culture model that may help define the mechanism by which FALS-associated *SOD1* mutations bring about neural cell death.

MATERIALS AND METHODS

Yeast Methods. A strain of *Saccharomyces cerevisiae* lacking the CuZnSOD gene, EGy118(*MATα leu2-3,11 his3Δtrp1-239 ura3-52 SOD1ΔA::LEU2*) was derived by gene deletion replacement from the parent strain EGy103 (*MAT α leu2-3,11 his3-1, Δtrp1-289 ura3-52 SOD1*$^+$). Plasmids encoding the yeast or human wild-type CuZnSODs or the human ALS mutant CuZnSODs expressed under the control of the yeast CuZnSOD promoter were constructed as follows. A 1.1-kb DNA fragment containing the yeast *SOD1* gene and promoter, with an *Nco* I site at the translation start site, was cloned into YEP351 (9), a multicopy yeast shuttle vector containing a *LEU2* selectable marker, at the *Eco*RI and *Bam*HI restriction sites. The yeast *SOD1* coding region was removed by digestion with *Nco* I and *Xba* I, leaving the yeast *SOD1* promoter and the

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Abbreviations: SOD, superoxide dismutase; ALS, amyotrophic lateral sclerosis; FALS, familial ALS.

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rest of the vector intact. cDNA clones of the human wild-type and FALS-associated G93C, L38V, G93A, and A4V mutants with an *Nco* I site introduced at the start codon and an *Xba* I site 715 bases downstream were kindly provided by T. Usdin (National Institutes of Health). These plasmids were digested with *Nco* I and *Xba* I and the gene-containing fragments were ligated into the similarly digested vector.

For measurements of *in vivo* activity, these plasmids were transformed into EGy118 (lacking CuZnSOD), creating a family of strains expressing different mutants. The vector was also transformed into the parent yeast strain, EGy103. For measurements of oxygen and paraquat sensitivity, stationary-phase liquid cultures were diluted, and 10^5 cells were streaked on freshly prepared SD plates lacking leucine but with various concentrations of paraquat (or without paraquat) and incubated at 30°C for 3 days; for oxygen sensitivity, the plates were incubated in an atmosphere of 90–100% oxygen. Soluble protein extracts were prepared by glass-bead lysis in 50 mM Tris, pH 7.4/0.1 mM EDTA with 0.5 mM phenylmethanesulfonyl fluoride, 0.25 mM L-1 tosylamino-2-phenylethyl chloromethyl ketone, and 0.25 mM 7-amino-1-chloro-3-tosylamido-2-hepatonone ("N^{α}-*p*-tosyl-L-lysine chloromethyl ketone") added to inhibit proteases. Total protein was determined with a Coomassie G250 binding assay (Bio-Rad). SOD was assayed by inhibition of the autoxidation of 6-hydroxydopamine at pH 7.4 (10). Western blots were performed with the Vectastain Kit (Vector Laboratories); anti-human SOD antibody was purchased from The Binding Site (San Diego).

Cell Culture and Expression Constructs. CSM14.1 temperature-sensitive immortalized rat nigral neural cells (11) were cotransfected with pGSOD-SVneo [which includes the genomic clone for human wild-type *SOD1* (12)], pGSOD(A4V)-SVneo (7), or pGSOD(G37R)-SVneo (7) and pBabe-puro (13) at a 20:1 ratio. After selection with puromycin (7 μg/ml), populations containing >100 colonies were compared, in order to prevent the bias inherent in comparing single colonies (11, 14). The activity of SOD was assessed by inhibition of 6-hydroxydopamine autoxidation (10). Apoptosis in cultured neural cells was induced and assessed as described (15).

RESULTS

The ability of the FALS-associated mutant CuZnSODs to substitute for wild-type CuZnSOD was examined by expressing the mutants and wild type in *S. cerevisiae* lacking the yeast CuZnSOD gene. Unlike wild type, the *sod1*$^-$ yeast grow poorly in air (21% oxygen), do not grow in 100% oxygen, and are exquisitely sensitive (≈3 orders of magnitude more sensitive than wild type) to redox cycling drugs such as paraquat. In every case, expression of either the wild-type *SOD1* gene or the FALS mutant genes, but not the control vector, returned the yeast to the wild-type phenotype, as defined by resistance to 1.25 mM paraquat and normal growth in an atmosphere of 100% oxygen (Table 1). *In vitro* assay of SOD enzymatic activity of soluble protein extracts of these yeast strains demonstrated that there was a moderate, 20–40% decrease in SOD activity associated with the FALS mutant SODs in comparison to wild-type SOD (Table 1 and Fig. 1*B*). Western blot analysis of the same extracts demonstrated expression of a corresponding amount of SOD protein (Fig. 1*A*). No SOD activity was detected (<0.05 unit/μg of protein) in yeast sod1$^-$ mutants that had not been transformed with *SOD1* expression constructs (under the growth conditions employed, yeast MnSOD activity is below the limit of detection).

Because of the retained enzymatic activity of the FALS-associated CuZnSOD mutants and the rescue of the *sod1*$^-$ mutant yeast, we considered the possibility that the FALS mutants might have an effect on neural survival independent of their effects on CuZnSOD activity. The expression of *BCL2* in yeast *sod1*$^-$ mutants has been shown to enhance their survival (16); *BCL2* expression has also been demonstrated to decrease the net cellular generation of reactive oxygen species in mammalian neural cells (16) and to inhibit lipid peroxidation in both hematopoietic and neural cells (16, 17). Therefore, we tested the hypothesis that the overexpression of *SOD1* would inhibit neural apoptosis. The transfected CSM14.1 cells had a 3-fold higher level of SOD activity than the control transfectants. This increase in SOD activity was associated with an inhibition of apoptosis, whether induced by serum withdrawal or by the calcium ionophore A23187 (Fig. 2). Inhibition of the activity of CuZnSOD with diethyldithiocarbamate (2 mM) inhibited the antiapoptotic effect of *SOD1* overexpression in parallel: survival of CSM14.1 cells overexpressing CuZnSOD in serum-free medium for 6 days was reduced from 60 ± 3% to 6 ± 4% by 2 mM diethyldithiocarbamate ($P < 0.01$ by t test, $n = 4$). The viability of control transfectants was 29 ± 4% in the absence, and 0% in the presence of 2 mM diethyldithiocarbamate. Paraquat (100 μM) reduced the viability of control transfectants in serum-containing medium from 94 ± 5% to 43 ± 8%, but not that of cells overexpressing *SOD1* (95 ± 4% vs. 87 ± 2%; $P > 0.05$ by t test, $n = 4$).

Table 1. Sensitivity to oxidative stress, SOD activity, and CuZnSOD levels of *sod1*$^-$ yeast expressing human FALS-associated or wild-type *SOD1*

Host strain	Plasmid	Growth in O_2	Paraquat sensitivity,* μM	SOD activity,† unit(s)/μg of protein	Antibody-reactive protein	
					Yeast	Human
y118 (*sod1*$^-$)	YEp351 (vector)	−	10	<0.05‡	−	−
y103 (*SOD1*$^+$)	YEp351 (vector)	++	1250	0.39–0.57‡	+	−
y118 (*sod1*$^-$)	YEp600 (yWT)	++	1250	1.47 ± 0.19†	++	−
y118 (*sod1*$^-$)	YEp-hWT	++	1250	1.39 ± 0.13	−	+
y118 (*sod1*$^-$)	YEp-hG93C	++	1250	0.96 ± 0.09	−	+
y118 (*sod1*$^-$)	YEp-hL38V	++	1250	0.99 ± 0.09	−	+
y118 (*sod1*$^-$)	YEp-hG93A	++	1250	1.21 ± 0.10	−	+
y118 (*sod1*$^-$)	YEp-hA4V	++	1250	0.84 ± 0.11	−	+

Yeast EGy103 and EGy118 cells were transformed with the indicated plasmids. For measurements of oxygen and paraquat sensitivity, stationary-phase liquid cultures were diluted, and 10^5 cells were streaked on freshly prepared SD plates lacking leucine, with or without various concentrations of paraquat, and incubated at 30°C for 3 days; for oxygen sensitivity, the plates incubated in an atmosphere of 90–100% oxygen. The presence of SOD protein was determined by Western blot. yWT, yeast wild type.

*Highest paraquat concentration at which the strain would grow.

†Unless otherwise indicated, mean ± SD of triplicate samples from a single representative experiment.

‡Ranges from several typical experiments.

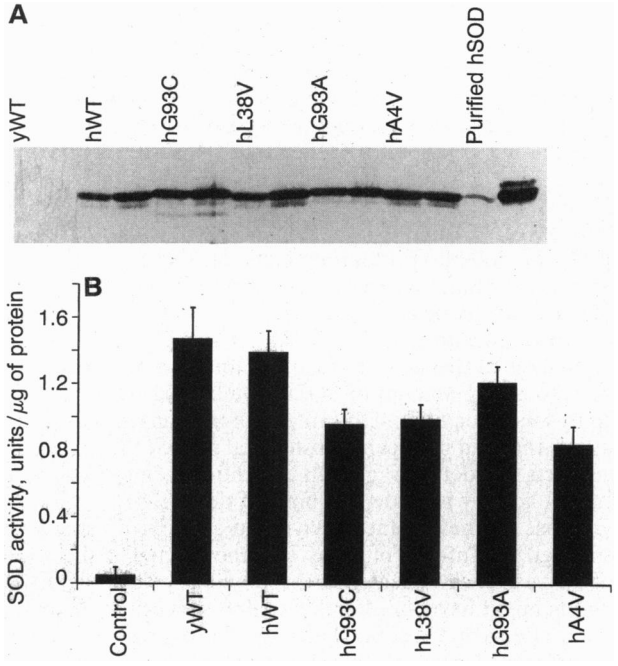

FIG. 1. (*A*) Expression in yeast of human wild-type or FALS-associated mutant human SOD as measured by immunoblot. Yeast strain EGy118 transformed with plasmids carrying the indicated genes was grown to late logarithmic phase in liquid medium. Soluble protein extracts were prepared and 10-μg samples were electrophoresed in an SDS/12.5% polyacrylamide gel, transferred to nitrocellulose, and detected with sheep anti-human CuZnSOD antibody (The Binding Site, San Diego) and the sheep Vectastain kit (Vector Laboratories). The labels are over the first (leftmost) of duplicate samples from different cultures and indicate the form of CuZnSOD being expressed. yWT, yeast wild type; hWT human wild type; human SOD1 mutants are shown in the next four lanes. Purified human SOD (Sigma) was loaded at 10 and 50 ng in the last two lanes as a marker. (*B*) SOD activity of FALS mutant genes expressed in yeast lacking the CuZn-SOD gene. Plasmids carrying the indicated form of the CuZnSOD gene were constructed and transformed into EGy118. Triplicate cultures were grown to late logarithmic phase and protein extracts were prepared as described in *Materials and Methods*. SOD activity was assayed by the 6-hydroxydopamine method (10). Values are averages of triplicate samples grown from different colonies.

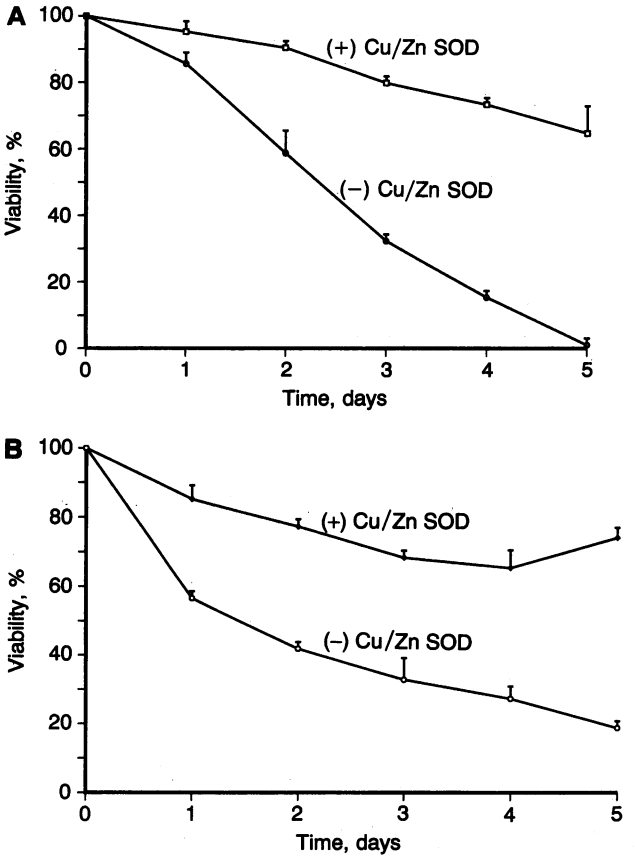

FIG. 2. Inhibition of apoptosis by the overexpression of CuZnSOD in conditionally immortalized neural cells. The plasmid pGSOD-SVneo (12), which includes human genomic DNA encoding wild-type CuZnSOD, was cotransfected at a 20:1 ratio with pBabe-puro (13), and transfectants were selected in medium containing puromycin (7 μg/ml). Control transfectants received pBabe-puro only. (*A*) Viability of transfected CSM14.1 cells treated with the calcium ionophore A23187 (1 μM), which induces apoptosis in these cells (11). (*B*) Viability of transfected CSM14.1 cells following the withdrawal of serum, which induces apoptosis in these cells (11). Differences between survival of cells transfected with pGSOD-SVneo and control transfectants were highly statistically significant in both *A* and *B* ($P < 0.001$ by two-way analysis of variance, $n = 4$, from two different stable transfections).

Two mutant human *SOD1* genes encoding the A4V and G37R amino acid substitutions were compared for their effects on SOD activity and on the inhibition of apoptosis. Both of these mutations are associated with FALS, with the former being the most common *SOD1* mutation observed in FALS (1). Expression of each of these led to an increase in SOD activity that was ≈70% of that seen with the expression of the wild-type *SOD1* (Fig. 3*A*). However, rather than leading to an inhibition of apoptosis commensurate with the increase in SOD activity, expression of each of the two mutants actually enhanced apoptosis in CSM14.1 cells (Fig. 3 *B* and *C*).

DISCUSSION

Unlike other systems utilized to assess FALS-associated CuZnSODs, the yeast system we employed for these studies allows the *in vivo* assessment of mutant SODs in the absence of wild-type CuZnSOD expression. Expression of human FALS-associated mutant SODs in yeast demonstrated that the four mutants tested were all able to substitute for the yeast CuZnSOD in strains lacking this enzyme and enable *sod1*$^-$ yeast to grow in medium with the redox cycling drug paraquat and in 100% oxygen (Table 1). In fact, we have yet to find any phenotypic difference between yeast strains expressing the human FALS mutant proteins and the strains expressing either yeast or human wild-type protein. The FALS mutant enzymes retained considerable (60–80%) SOD activity in this system, and SOD protein levels showed no discordance with activity levels, indicating that the high activity increase was not due to an abnormally high accumulation of the mutant proteins.

When expressed in neural cell lines, the two FALS mutant SODs tested stimulated the process of apoptosis, whereas the wild-type protein inhibited it. SOD activity was increased in all three cases. Our results, and the lack of correlation observed between the level of SOD activity and the onset and severity of *SOD1*-associated FALS, may reflect a discordance between the enzymatic (dismutase) activity of CuZnSOD and the property of *SOD1* mutants that leads to neural cell death in FALS. The results do not necessarily imply that the death of motor neurons in FALS is apoptotic, since different cell types may respond differently to similar insults (19). Furthermore, it is possible that the mechanism by which the expression of FALS-associated *SOD1* mutants leads to neural cell death in cultured neural cells is unrelated to motor-neuron death in FALS. However, the dominant character of the effect, the lack of correlation between SOD activity and FALS severity, the lack of identified nonsense mutations, and the reported motor neuron degeneration in transgenic mice expressing a FALS-

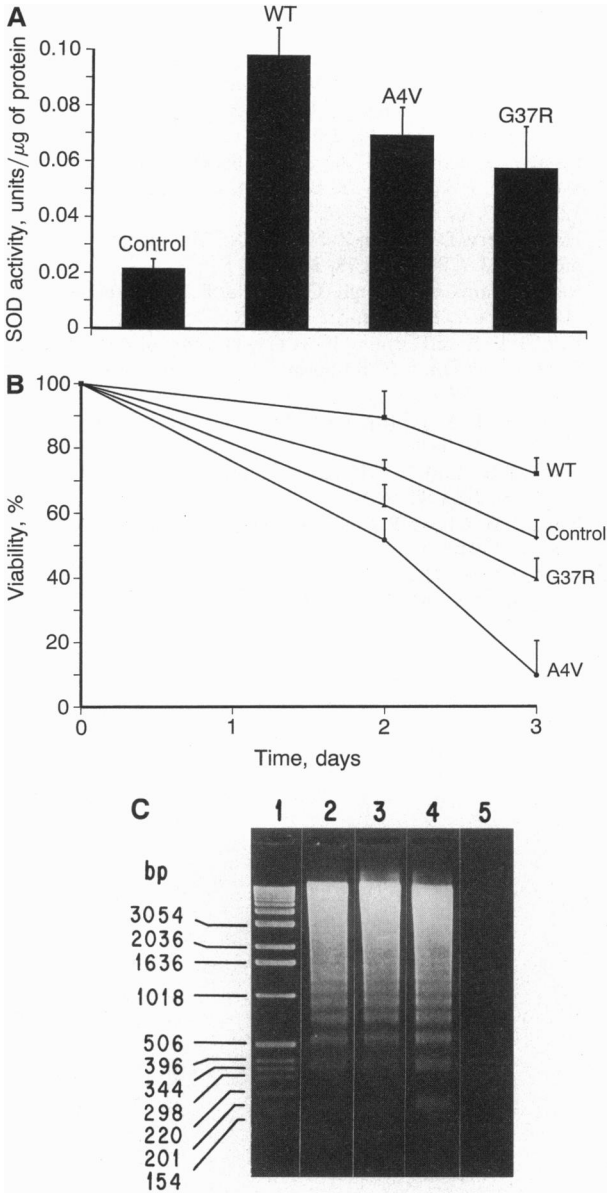

FIG. 3. FALS-associated CuZnSOD mutants retain SOD enzymatic activity but promote apoptosis rather than inhibit it. (*A*) SOD activity. Protein extracts were prepared by glass-bead lysis (18) and quantitated with a dye binding assay (Bio-Rad protein assay). SOD activity was assayed by measuring the effect of protein extracts on 6-hydroxydopamine autoxidation (10). WT, wild type. (*B*) Viability of CSM14.1 cells following the withdrawal of serum. Cells transfected with the wild-type *SOD1* survived to a greater extent ($P < 0.05$ by two-way analysis of variance, $n = 8$) than the control cells; in contrast, the cells transfected with the G37R construct survived significantly less ($P < 0.05$ by two-way analysis of variance, $n = 8$) than the control cells, and cells transfected with the A4V construct survived highly significantly less ($P < 0.01$ by two-way analysis of variance, $n = 8$) than the control cells. Each assay was repeated six times with different transfectants. Viability was measured as described in the legend to Fig. 2. (*C*) Internucleosomal fragmentation of DNA (characteristic of apoptosis) in CSM 14.1 neural cells transfected with the control vector (lane 2) or vector encoding the G37R (lane 3) or A4V (lane 4) mutant, but not in cells transfected with the vector expressing wild-type human *SOD1* (lane 5). In each case, DNA was extracted from 4×10^6 cells after 2 days in serum-free medium. Lane 1 contained molecular size markers (Boehringer Mannheim).

associated mutant SOD (8) are all compatible with the effect reported here.

Two recent studies (7, 8) demonstrated reduced half-lives and enzymatic activity of the FALS-associated G93C, G93A, and A4V mutants. The current study documents moderate, 20–40% reductions in activity of the G93C, G93A, and A4V mutants in the yeast expression system and of the A4V and G37R mutants in the conditionally immortalized mammalian neural cells. The more modest reduction of SOD activity observed in the present study in comparison to the studies of Borchelt *et al.* (7) and Gurney *et al.* (8) may be due to differences in cell types utilized, expression systems used, levels of endogenous wild-type SOD in the different systems, efficiencies of expression in the various cells, biochemical mechanisms or rates of degradation of mutant SODs, expression of other genes involved in the metabolism of reactive oxygen species, or other variables.

The antiapoptotic effect of the wild-type CuZnSOD corroborates the theory that apoptotic signaling is mediated by reactive oxygen species (20). Expression of the antiapoptotic gene *BCL2* inhibits apoptosis by reducing the net generation of reactive oxygen species (16). As shown here, overexpression of wild-type CuZnSOD also inhibits neural apoptosis. Thus, our data confirm and enhance the theory that reactive oxygen species are involved in the induction of apoptosis (16, 17, 20).

However, the FALS mutants tested here, A4V and G37R, convert the wild-type CuZnSOD from an inhibitor to an inducer of apoptosis. This occurs in spite of an increase in SOD activity, indicating that another process is occurring when the FALS mutant SODs are expressed. Several possibilities have been suggested that could explain this phenomenon. Previous work by Yim *et al.* (21, 22) has demonstrated that under certain conditions, the physiological activity of CuZnSOD is associated with generation of hydroxyl radicals. Thus, one conceivable mechanism by which the FALS-associated *SOD1* mutants may enhance neural cell death is through the enhanced generation of hydroxyl radicals. A second possibility is that the mutants act through a decrease in metal binding, as has been observed for the G85R mutation made in yeast CuZnSOD (23). The generation of peroxynitrite from the superoxide anion and nitric oxide is yet another mechanism by which the FALS mutant proteins could conceivably induce motor-neuron degeneration (8, 20).

The dissociation of SOD activity from the induction of apoptosis is perhaps less surprising when one considers that the FALS-associated mutations map almost exclusively to positions that are involved in the structure of the β-barrel or in subunit dimerization (2), rather than to positions in the catalytic site. Only one mutant out of seven that we have tested so far in the yeast system has lost the ability to rescue *sod1*$^-$ yeast—the G85R mutant made in the yeast CuZnSOD (23).

Our results provide a cell culture system for examining the mechanism(s) by which the expression of *SOD1* mutants associated with FALS leads to neural cell death. This model may prove to be useful for evaluation of possible FALS mechanisms, as well as for the large-scale screening of potentially therapeutic agents for ALS.

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