Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 8292–8296, August 1994
Neurobiology

Superoxide dismutase 1 with mutations linked to familial amyotrophic lateral sclerosis possesses significant activity

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Communicated by M. Daniel Lane, May 20, 1994

ABSTRACT **Familial amyotrophic lateral sclerosis (FALS) has been linked to mutations in the homodimeric enzyme Cu/Zn superoxide dismutase 1 (SOD1). Assay by transient expression in primate cells of six FALS mutant enzymes revealed a continuum of enzymatic activity bounded by the enzyme carrying the mutation Gly-85 → Arg, which was inactive, and mutant enzyme G37R carrying the Gly-37 → Arg change, which retained full specific activity but displayed a 2-fold reduction in polypeptide stability. The G37R mutant displayed similar properties in transformed lymphocytes from an individual heterozygous for the G37R and wild-type SOD1 genes; heterodimeric enzymes composed of mutant and wild-type subunits were detected, but there was no measurable diminution in the stability and activity of the wild-type subunits. Thus, for mutants such as G37R, either surprisingly modest losses in activity (involving only the mutant subunit) can yield motor neuron death, or alternatively, mutant SOD1 may acquire properties that injure motor neurons by one or more mechanisms unrelated to the metabolism of oxygen radicals.**

Familial amyotrophic lateral sclerosis (FALS) is an age-dependent autosomal dominant disorder (1–4) in which mutations in the homodimeric enzyme Cu/Zn superoxide dismutase 1 (SOD1) have been linked to disease in 20–50% of FALS families (3–7). The human SOD1 (HuSOD1) polypeptide is encoded by a single gene on chromosome 21 (8), producing an 153-amino acid metalloenzyme that catalyzes the conversion of O_2^- into O_2 and H_2O_2 (5, 8, 9). To date, 16 different SOD1 mutations have been reported in heterozygous individuals with FALS (3, 4, 10, 11). Initial studies of SOD1 activity in blood from six FALS cases reported 50–65% reductions in enzyme activity as a function of total protein (3, 12, 13), and it was suggested that FALS-linked mutations affect either enzyme activity or polypeptide stability, causing a nearly complete loss of function from the mutant allele. However, subsequent studies have reported that individuals heterozygous for a His-46 → Arg mutation, which lies in the active-site domain, possess blood enzyme activity levels that are 80% of normal (6). One problem in interpreting these data is that others have demonstrated 2- to 3-fold variability in the SOD1 levels of circulating erythrocytes in normal individuals (11, 12, 14), casting doubt on the significance of blood SOD1 levels. In the present study, we examined mutant SOD1 subunits in nonerythroid cells, using lymphoblasts derived from FALS individuals and DNA transfection into primate cells, to determine the intrinsic specific activity and stability of wild-type and mutant HuSOD1 polypeptides.

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MATERIALS AND METHODS

Cloning and Mutagenesis of Human *SOD1* Genes. A cDNA copy of the human *SOD1* gene was obtained by reverse transcription-coupled polymerase chain reaction (PCR) amplification using primers (sense primer, GTCGACAAGCATGGCCACGAAGGCCGTGTGC; antisense primer, CCGGTCGACAGGGAATGTTTATTGGGCGATCC) that contained terminal *Sal* I sites and converted sequences surrounding the start codon (underlined) from human GAGTTATGG to mouse CAAGCATGG, enhancing the consensus sequence for translation initiation (13). The PCR product was blunt-ligated into the *Sma* I site of Bluescript II KS(−) and was verified by sequencing. Mutations were engineered by oligonucleotide-directed mutagenesis by using a kit purchased from Amersham. The entire open reading frame of each mutant was confirmed by sequencing.

Analysis of SOD1 Enzyme Activity. Wild-type and mutant *SOD1* genes were cloned into the vector pEF-BOS (15) and transfected transiently into COS-1 cells (16). After 48 h, cells were lysed by a freeze/thaw cycle in water, which was then adjusted to contain 0.125 M Tris chloride (pH 6.8), 20% (vol/vol) glycerol, 0.025% bromophenol blue, and 0.1% Nonidet P-40. After centrifugation at 10,000 × *g* for 5 min, the supernatant was separated by electrophoresis on 7.5% polyacrylamide gels, and SOD1 activities were determined as described (17). The intensities of the SOD1 activity signals seen in our assay gels fall within a linear range bounded by 0.0625 μg and 2 μg of purified human erythrocyte SOD1.

Calculation of SOD1 Enzyme Specific Activity. Extracts of COS-1 cells were fractionated on SDS/polyacrylamide gels and immunoblotted with either of two polyclonal antisera raised against a synthetic HuSOD1 peptide (amino acids 125–137; Asp-Asp-Leu-Gly-Lys-Gly-Gly-Asn-Glu-Glu-Ser-Thr-Lys) or intact erythrocyte HuSOD1 polypeptide. Both antisera specifically recognized HuSOD1, with weak cross-reactivity for COS-1 SOD1. The immunoreactivity of these antisera was fully blocked by competition with excess peptide or whole protein (not shown).

SOD1 assay gels and immunoblots were quantified by using a Stratagene Eagle Eye II to digitize images. Pixel densities of SOD1-associated bands were quantified by using the program NIH IMAGE version 1.45 (public domain software by Wayne Rasband, National Institutes of Health). Specific activities were calculated by dividing measured activity by the amount of accumulated protein. All specific activity values were expressed as a percentage of wild-type activity.

Assessment of SOD1 Polypeptide Stability. Data were collected from two sets of measurements. In one set of experiments, repeated in triplicate, a single mixture of plasmid DNA encoding wild-type or mutant SOD1 was transfected

Abbreviations: SOD1, Cu/Zn superoxide dismutase; HuSOD1, human superoxide dismutase 1; FALS, familial amyotrophic lateral sclerosis.

into equivalent dishes of COS-1 cells as described (16). After 48 h, the cultures were metabolically radiolabeled for 15 min with 300 μCi (1 μCi = 37 kBq) of [^{35}S]cysteine per ml of culture medium. Cultures were harvested immediately or 24 h later, before labeled SOD1 was immunoprecipitated as described below. In the other set of experiments, repeated in duplicate, COS-1 cells in a 60-mm dish were transfected with 5 μg of either wild-type or mutant expression plasmid DNA with the reagent DOTAP (Boehringer Mannheim). Twenty-four hours after transfection, the cells were digested with trypsin and split into four wells of a 24-well dish. After an additional 24 h, the cells were labeled as described above.

Labeled cells were lysed in 50 mM Tris hydrochloride, pH 8.0/150 mM NaCl/5 mM EDTA/0.5% Nonidet P-40/0.5% deoxycholate at 4°C and then centrifuged at 10,000 × *g*. The supernatant was adjusted to 0.2% SDS, boiled for 10 min, and then centrifuged at 10,000 × *g* for 10 min. An equal amount of CCl_3COOH-precipitable radiolabel from each extract was then mixed with SOD1 antiserum, absorbed with protein A-agarose, and fractionated by SDS/PAGE. Each radiolabeled SOD1 polypeptide band was quantified with a Molecular Dynamics PhosphorImager. The percent of cellular radiolabeled SOD1 polypeptide remaining after a given period of chase was plotted on a logarithmic scale and fit to a curve with the assumption that degradation follows first-order kinetics (Cricket Graph III; Computer Associates, San Jose, CA).

Analysis of Human Lymphoblast Cell Lines. Human lymphoblast cell lines were maintained in Iscove's modified minimal essential medium supplemented with 10% (vol/vol) fetal calf serum. For analysis of SOD1 enzyme activity, cells were collected by centrifugation and washed twice in Dulbecco's phosphate-buffered saline (PBS) (Life Technologies, Grand Island, NY); extracts were analyzed by the activity gel assay described above.

To determine the relative stability of mutant and wild-type SOD1 polypeptides in lymphoblasts, cells were metabolically radiolabeled for 15 min with [^{35}S]cysteine, and SOD1 was immunoprecipitated from Nonidet P-40-soluble proteins collected at 0 h and 24 h after labeling. The entirety of each immunoprecipitate was separated by high-resolution two-dimensional SDS/PAGE (18) with a pH gradient of 4–6.5, followed by fluorography and autoradiography.

RESULTS

Specific Activity of Mutant SOD1 Enzymes. To examine directly whether FALS-associated mutations affect the catalytic activity of HuSOD1 enzyme, we expressed transiently mutant polypeptides in monkey (COS-1) cells (Fig. 1). Human and monkey SOD1 homodimeric enzymes were distinguishable after electrophoretic separation in nondenaturing polyacrylamide gels and *in situ* evaluation of activity (17, 20, 21) (Fig. 1*A*). The transfection of COS-1 cells with wild-type HuSOD1 expression plasmids produced a new species of free-radical scavenging activity (Fig. 1*A*, lane 2), which comigrated with purified erythrocyte HuSOD1 (Fig. 1*A*, lane 9). In most experiments, expression of HuSOD1 was restricted to a relatively few cells, and the human subunits were found predominantly in homodimers. Cells transfected with all but one of the mutant SOD1 vectors produced novel activities, attributable to vector-encoded SOD1 enzyme (Fig. 1*A*, lanes 3–8). Further, enzymes carrying mutations that alter charge, Gly-37 → Arg and Gly-41 → Asp, exhibited electrophoretic migration different from that of wild-type HuSOD1 in these assay gels (Fig. 1*A*, lanes 4 and 5; mutant enzymes G37R and G41D). One mutation, Gly-85 → Arg, also caused the mutant polypeptide (G85R) to migrate anomalously on SDS/PAGE (Fig. 1*B*, lane 6). Of the six mutant enzymes—all named for the amino acid change in single-

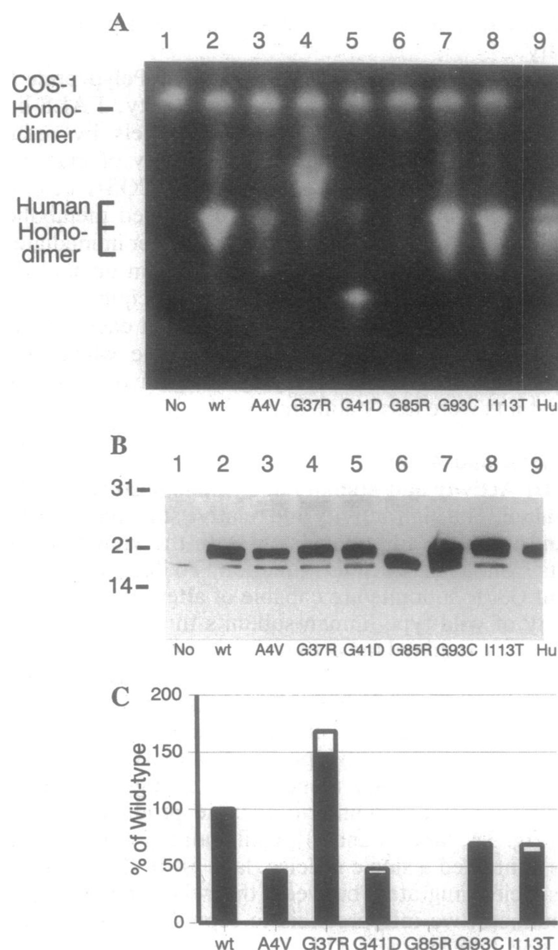

FIG. 1. Specific activity of SOD1 polypeptides encoded by FALS-linked mutations. (*A*) SOD1 activity assay gels. Mutant SOD1 polypeptides were expressed transiently in COS-1 cells as detailed in text. Lanes: 1, vector without HuSOD1 cDNA; 2, vector with wild-type (wt) HuSOD1 cDNA; 3–8, vectors encoding mutant HuSODs as follows: A4V, G37R, G41D, G85R, G93C, and I113T; 9, HuSOD1 (Hu) purified from erythrocytes (Sigma). (*B*) Immunoblot detection of SOD1 polypeptides; assessment of accumulated levels. Extracts analyzed in *A* were fractionated by SDS/PAGE on 15% gels (19) and immunoblotted with SOD1 antiserum as detailed in text. Lanes: 1, untransfected cells; 2–8, cells transfected with wild-type or mutant HuSOD1 as follows: A4V, G37R, G41D, G85R, G93C, and I113T; 9, purified human erythrocyte SOD1. (*C*) Specific activities of HuSOD1 mutants. SOD assay gels and SOD immunoblots were quantified, and specific activities were calculated as described in text. An average of two experiments is displayed with standard deviations as the open portion of bars.

letter code—only G85R lacked demonstrable evidence of activity (see lanes 6 of Fig. 1 *A* and *B*). Presumably, the G85R mutant is improperly folded and is inactive or fails to enter the activity assay gel.

The ratio of enzymatic activity to accumulated protein level [determined by quantitative immunoblotting with newly made HuSOD1 antibodies (Fig. 1*B*)] was used to calculate specific activity (Fig. 1*C*). Although the G85R mutant was inactive, the A4V (Ala-4 → Val mutation), G41D, G93C (Gly-93 → Cys mutation), and I113T (Ile-113 → Thr mutation) mutants retained 30–65% of wild-type specific activity. Notably, the specific activity of the G37R mutant was equal to or greater than that of the wild-type SOD1. Moreover, homodimeric, active, enzymes were identified for four of the six mutants after electrophoretic separation and assay in 8 M urea (20, 22)/polyacrylamide gels (not shown); the specific activity of the G37R mutant remained high. Thus, we con-

clude that five of the six mutants retain full or partial specific activity.

FALS-Linked Mutations Diminish SOD1 Polypeptide Half-Life. Apart from affecting intrinsic activity, FALS-linked SOD1 mutations could alter enzyme levels by reducing subunit half-life. To compare the longevity of mutant and wild-type HuSOD1 subunits, cultures of COS-1 cells were transfected transiently and then radiolabeled metabolically for 15–30 min. Cultures were harvested either immediately or after incubation for an additional interval in unlabeled medium. SOD1 polypeptides were immunoprecipitated, and the level of radioactive SOD1 was quantified at each time point (Fig. 2). Wild-type SOD1 was very stable when overexpressed in primate cells, displaying a half-life of ≈30 h. All of the mutants were less stable than wild-type SOD1, exhibiting half-lives of 20 (I113T), 16 (G93C), 13 (G37R), 10 (G41D), and 7.5 (G85R and A4V) h.

SOD1 Activity and Stability in Lymphoblasts from a FALS Individual. The high activity and relative stability of the G37R mutant are not readily consistent with the view that FALS results from a loss of SOD1 function. To determine whether mutant G37R subunits are capable of altering the activity or stability of wild-type human subunits through heterodimerization, we examined the activity, accumulation, and stability of mutant and wild-type SOD1 in a lymphoblast line established from a FALS individual heterozygous for the G37R SOD1 mutation (Fig. 3). Three active species were detected on activity gels of G37R lymphoblast extracts [the fastest and slowest migrating bands, respectively, comigrated with wild-type and G37R homodimeric enzyme produced in COS-1 cells (Fig. 3*A*, lanes 3 and 4)], while normal human lymphoblasts contained a single species (lane 5). In the mutant line, one species migrated between the wild-type and mutant homodimers; we interpret this to represent active mutant/wild-type heterodimers (20, 22). The level of mutant homodimer activity was ≈50% of either the heterodimer or the wild-type homodimer, which were of comparable activities. Overall, the level of total SOD1 activity was 60–80% of normal lymphoblasts, with the total (wild type plus mutant) amount of SOD1 polypeptide diminished by ≈40% (Fig. 3 *B* and *C*).

To determine whether the reduced abundance of G37R polypeptide results from diminished polypeptide stability in these cells, we examined the relative synthetic rate and stability of SOD1 polypeptides (Fig. 3*D*). Following pulse (15 min) labeling with [^{35}S]cysteine, two-dimensional SDS/PAGE analysis of immunoprecipitated SOD1 from wild-type lymphoblast extracts revealed two major SOD1 isoforms (Fig. 3*D*, filled arrowheads), as well as additional isoforms of lower abundance. The molecular basis for these isoforms is not known, but each must be rapidly generated from the initial translation product. Analysis of SOD1 immunoprecipitated from G37R lymphoblasts revealed a prominent more basic isoform (Fig. 3*D*, open arrowhead), as would be expected for the replacement of glycine with arginine. This more basic isoform (open arrowhead) was unambiguously identified as the mutant species on the basis of comigration with G37R polypeptides produced in COS cells (Fig. 3*C*). After 24 h of chase, the mutant polypeptide was selectively diminished with a half-life less than that of the wild-type polypeptide. However, there was no obvious reduction in the half-life of the wild-type subunit in G37R lymphoblasts.

DISCUSSION

Assay of the effect of six of the known FALS-linked mutations on HuSOD1 activity has revealed a continuum bounded by G85R, which has no activity, and G37R, which possesses 40–60% of wild-type subunit activity (diminished abundance with full specific activity). Although FALS is a dominant genetic disease (1–4), heterozygosity for any of the six mutants described here should only result in a net loss of 20–50% of the total expected activity (Fig. 4). Further reductions in SOD1 levels could occur if mutant polypeptides

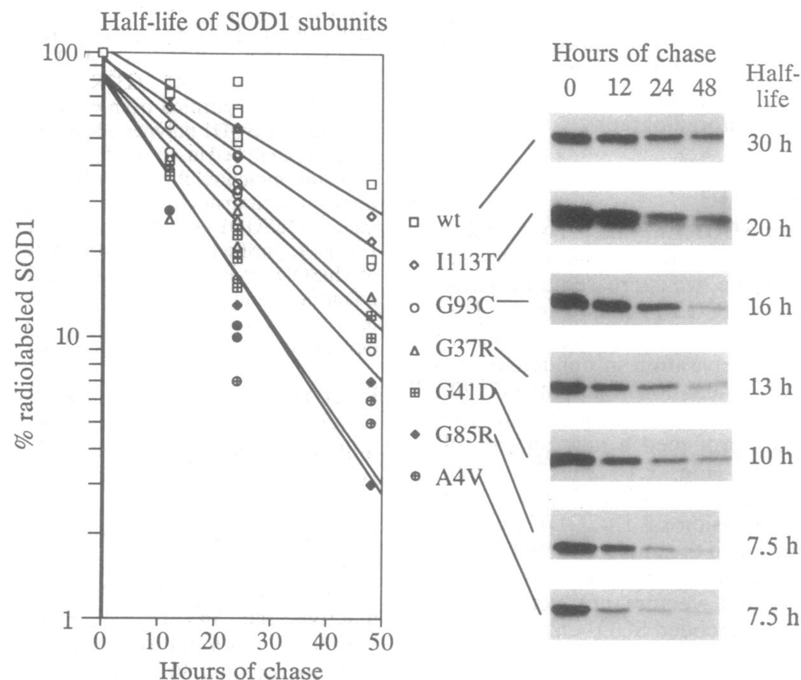

FIG. 2. Half-lives of wild-type (wt) and mutant HuSOD1 polypeptides. Replicate cultures of transfected COS-1 cells were metabolically radiolabeled as described in text. Radiolabeled SOD1 polypeptides were immunoprecipitated from detergent extracts of cells prepared either immediately after the labeling period or 12, 24, or 48 h later and analyzed by SDS/PAGE (19). The radiolabeled SOD1 polypeptide band was quantified on a Molecular Dynamics PhosphorImager. Half-lives of the wild-type HuSOD1 and each mutant were estimated as described in text. The graph displays data from three experiments in which cells were collected at 0 and 24 h of chase and two experiments in which cells were collected at 0, 12, 24, and 48 h. The order of plot lines is noted to the right of the graph; A4V and G85R overlap.

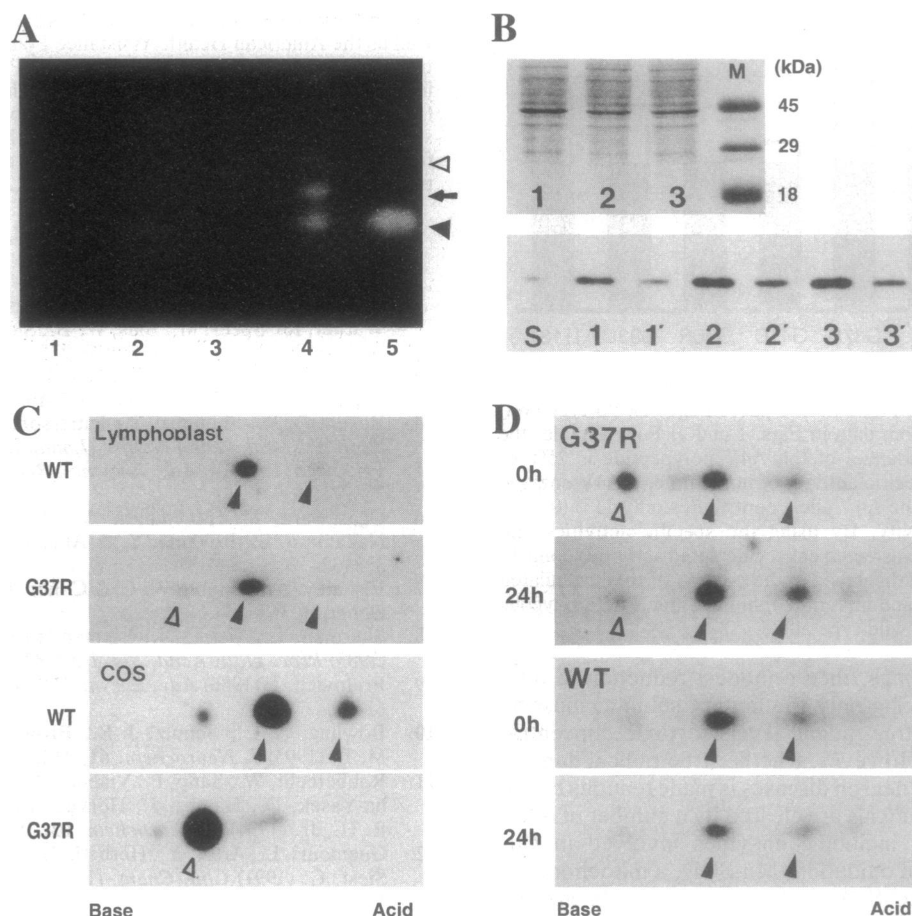

FIG. 3. Activity (*A*), abundance (*B* and *C*), and stability (*D*) of the G37R SOD1 mutant polypeptide in human lymphoblasts. (*A*) Activity of SOD1 in extracts from immortalized human lymphoblast lines. Lanes: 1, activity present in untreated COS-1 cells; 2 and 3, wild-type HuSOD1 homodimers (lane 2, filled arrowhead) and G37R homodimers (lane 3, open arrowhead) produced in transfected COS-1 cells; 4, extracts of G37R lymphoblasts (the arrow indicates the position of heterodimers of mutant and wild-type enzyme); 5, wild-type SOD1 present in normal human lymphoblasts. (*B*) Diminished steady-state accumulation of SOD1 in a human lymphoblast cell line derived from a G37R FALS patient. (*B Upper*) Lanes: 1, 2, and 3, 20 μg of Nonidet P-40-soluble proteins for Coomassie blue detection; M, molecular mass standards. (*B Lower*) Twenty (lanes 1, 2, and 3) and 10 (lanes 1′, 2′, and 3′) μg of Nonidet P-40-soluble protein for immunoblot detection of SOD1 polypeptide. Lanes: 1 and 1′, G37R lymphoblasts; 2, 2′ and 3, 3′, two normal lymphoblast cell lines (WT-1 and WT-2, respectively); S, 5 ng of purified human SOD1 standard. (*C*) Two-dimensional PAGE analysis of SOD1 polypeptides in G37R and normal lymphoblasts. Total SDS-soluble proteins from human lymphoblast cell lines [wild type (WT) and G37R] and COS cells expressing human SOD1 (wild-type and G37R) were separated by two-dimensional PAGE and immunoblotted, and SOD1 isoforms were detected with anti-HuSOD1 antibody. Open arrowheads, isoforms specific to the G37R; closed arrowheads, isoforms corresponding only to the wild-type SOD1. (*D*) Reduced half-life of G37R polypeptides in heterozygous, immortalized lymphoblasts from an FALS individual. Lymphoblast cell lines [the G37R and a wild-type (WT) line] were metabolically labeled with [^{35}S]cysteine for 15 min; SOD1 was immunoprecipitated from extracts taken at 0 and 24 h and analyzed by two-dimensional SDS/PAGE. Open arrowheads, G37R polypeptide; closed arrowheads, wild-type SOD1 polypeptide. The basis for the multiple charge isomers of both mutant and wild-type polypeptides seen in these experiments is unknown.

acted in a dominant manner to diminish the activity or stability of wild-type protein through heterodimerization (3, 23). Of the mutants we have analyzed here, the level of SOD1 activity in blood from an individual with the G85R mutation has been reported to be reduced ≈65%, whereas the level of activity in A4V cases was reduced ≈50% (3). Although these data from FALS blood are consistent with a partially dominant negative effect (3), it is unclear whether the values obtained from blood are sufficiently accurate to allow judgments regarding dominant effects, as blood SOD1 levels apparently vary considerably among individuals (11–13). Our results from the human G37R lymphoblasts demonstrate that heterodimerization with mutant subunits does not appreciably alter the activity or stability of wild-type subunits. When viewed together with previous studies, which have demonstrated that wild-type HuSOD1 activity is unaffected by heterodimeric interaction with chemically inactivated subunits (24), we believe that it is unlikely that the activity and the stability of the wild-type SOD1 subunits are affected by the properties of their dimeric partner. Thus, in FALS, any diminution in SOD1 activity will likely be restricted to the mutant subunit only, with the wild-type subunit continuing to provide 50% of the normal level of activity.

The linkage of FALS to mutations in SOD1 implicates free-radical damage as a mechanism of motor neuron degeneration (3, 4, 10, 11, 25, 26), and we have recently obtained immunocytochemical evidence of abundant SOD1 expression in motor neurons (C. A. Pardo, Z.-S.X., D.L.P., S.S.S., and D.W.C., unpublished data). However, we demonstrate in our present study that the potential activity of some mutants remains surprisingly robust. Unless there are cell-type-specific modifiers of SOD1 activity (for which there is no evidence), the enzyme activity values we measured in our cell cultures are likely to be applicable to all cells, including motor neurons. Moreover, in most heterozygous individuals, we predict that the activity and stability of the wild-type subunit are unaffected by heterodimerization. Therefore, if motor neuron degeneration in SOD1-linked FALS results

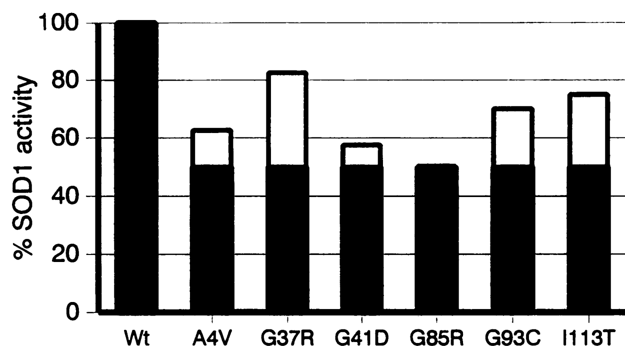

FIG. 4. Estimated SOD1 activity in heterozygous FALS individuals. The stability of each mutant as a percentage of wild-type (Wt) SOD1 was calculated from data in Figs. 1 and 2. For example, the half-life (and thus abundance) of the A4V polypeptide is 25% of normal, whereas the specific activity of homodimeric A4V enzyme is 45% of normal; thus the A4V allele contributes only 11% (0.25 × 0.45) of wild-type activity. By using the specific activities and half-lives measured in transfected cells, calculated activities contributed by each mutant are displayed in the context of total calculated activities [wild type (■) and mutant (□)] in individuals heterozygous for one mutant and one wild-type allele.

from free-radical damage, then a modest reduction in total SOD1 activity, involving only the mutant subunit, must be sufficient to elevate free-radical levels to toxic concentrations in some cases. However, whether free-radical damage alone underlies motor neuron diseases is made tenuous by the realization that free radicals are cleared by a number of other cellular components, including enzymes involved in glutathione reduction and oxidation; Mn-SOD, a mitochondrial matrix enzyme; and EC-SOD, a glycosylated Cu/Zn enzyme found in the extracellular space (9, 27). Furthermore, a recent study of FALS individuals with the A4V mutation found no evidence of increased free-radical damage (10). The absence of oxidative damage, combined with our finding that some SOD1 mutants are relatively stable and active, strongly supports the view that, in some SOD1-linked FALS cases, motor neuron degeneration may not result from a loss of SOD1 activity. Although it is possible that the degree of activity retained by mutant enzymes will correlate with disease severity or patient survival, a plausible alternative view is that at least some SOD1 mutants either affect processes unrelated to free-radical metabolism or acquire properties that injure neurons by mechanisms unrelated to O_2^- metabolism.

Note Added in Proof: Additional evidence that mutant SOD1 gains injurious properties arises from the recent report of motor neuron disease in a transgenic mouse line expressing a FALS-linked SOD1 mutation (Gly-93 → Arg) (28). Since bulk SOD1 activity levels were reported to be elevated in these transgenic animals, the mutant SOD1 must retain significant activity. Moreover, we have recently observed motor neuron disease in transgenic mice expressing G37R HuSOD1, which we have shown here to retain full specific activity and to be relatively stable.

We thank Drs. Douglas R. Spitz, Mark D. Scott, and John Eaton for helpful discussions; Dr. Shigekazu Nagata for the pEF-BOS expression plasmid; and Mr. Peter Sapp for help with lymphoblast cultures. This work was supported by grants from the U.S. Public Health Service (AG 05146, NS 20471, NS 27036, and NS 22849) as well as the American Health Assistance Foundation and the Amyotrophic Lateral Sclerosis Association. D.L.P. and D.R.B. are recipients of a Leadership and Excellence in Alzheimer's Disease award (AG 07914). D.L.P. and D.W.C. are recipients of Javits Neuroscience Investigator Awards (NS 10580 and NS 27036). Fellowships from the Muscular Dystrophy Association and the National Institutes of Health support Z.-S.X. and M.K.L.

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