Analysis of the Cytosolic Proteome in a Cell Culture Model of Familial Amyotrophic Lateral Sclerosis Reveals Alterations to the Proteasome, Antioxidant Defenses, and Nitric Oxide Synthetic Pathways*

Received for publication, September 26, 2002, and in revised form, December 5, 2002
Published, JBC Papers in Press, December 9, 2002, DOI 10.1074/jbc.M209915200

Simon Allen‡‡ §§, Paul Roy Heath‡§, Janine Kirby‡§, Stephen Barrie Wharton¶, Mark Robert Cookson**, Fiona Mhairi Menzies‡§, Rosamonde Elizabeth Banks‡‡, and Pamela Jean Shaw‡§

From the ‡Academic Unit of Neurology, Division of Genomic Medicine, University of Sheffield, the §Academic Unit of Pathology, Division of Genomic Medicine, University of Sheffield, Sheffield S10 2RX, United Kingdom, **Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, Maryland 20892, and ¶¶Cancer Research UK, Clinical Cancer Centre, St. James’s University Hospital, Leeds LS9 7TF, United Kingdom

Injury to motor neurons associated with mutant Cu,Zn-superoxide dismutase (SOD1)-related familial amyotrophic lateral sclerosis (FALS) results from a toxic gain-of-function of the enzyme. The mechanisms by which alterations to SOD1 elicit neuronal death remain uncertain despite intensive research effort. Analysis of the cellular proteins that are differentially expressed in the presence of mutant SOD1 represents a novel approach to investigate further this toxic gain-of-function. By using the motor neuron-like cell line NSE34 stably transfected with wild-type, G93A, or G37R human SOD1, we investigated the effects of mutant human SOD1 on protein expression using proteomic approaches. Seven up-regulated proteins were identified as argininosuccinate synthase, argininosuccinate lyase, neuronal nitric-oxide synthase, RNA-binding motif protein 3, peroxiredoxin 1, proteasome subunit β5 (X), and glutathione S-transferase (GST) Alpha 2. Seven downregulated proteins were identified as GST Mu 1, GST Mu 2, GST Mu 5, a hypothetical GST Mu, GST Pi B, leukotriene B4 12-hydroxydehydrogenase, and proteasome subunit β5i (LMP7). GST assays demonstrated a significant reduction in the total GST activity of cells expressing mutant human SOD1. Proteasome assays demonstrated significant reductions in chymotrypsin-like, trypsin-like, and post-glutamylhydrolase proteasome activities. Laser capture microdissection of spinal cord motor neurons from human FALS cases, in conjunction with reverse transcriptase-PCR, demonstrated decreased levels of mRNA encoding GST Mu 1, leukotriene B4 12-hydroxydehydrogenase, and LMP7. These combined approaches provide further evidence for involvement of alterations in antioxidant defenses, proteasome function, and nitric oxide metabolism in the pathophysiology of FALS.

Amyotrophic lateral sclerosis (ALS),¹ the most common form of motor neuron disease, is a fatal, adult-onset neurodegenerative disorder, characterized by selective loss of lower and upper motor neurons from the spinal cord and brain. Approximately 10% of ALS cases are inherited, and 20% of these familial ALS (FALS) cases result from dominantly inherited missense mutations in the gene encoding Cu,Zn-superoxide dismutase (SOD1) (1). As most FALS SOD1 mutants retain dismutase activity close to that of the wild-type enzyme (2), the injury to motor neurons associated with mutant SOD1 may result from a toxic gain-of-function of the enzyme rather than loss of its ability to catalyze the conversion of superoxide to hydrogen peroxide. Furthermore, mice with targeted deletion of the sod1 gene do not develop an ALS phenotype (3) in contrast to transgenic mice expressing mutant human SOD1 (4–6).

Several non-mutually exclusive hypotheses have been proposed to describe the toxic gain-of-function of mutant SOD1 (7, 8). These include altered free radical handling, altered copper/zinc binding, and formation of high molecular weight protein aggregates. Evidence for altered free radical handling has come from numerous observations; for example, indices of free radical damage are increased in the transgenic mutant SOD1 mice (9) and human ALS cases (10, 11). Cultured cells expressing SOD1 mutants have been shown to exhibit increased oxygen radical production and sensitivity to exogenously produced free radicals (12, 13). Intracellular superoxide can react with nitric oxide to produce the oxidant peroxynitrite (14). Several studies have provided evidence for the role of peroxynitrite and nitric oxide in SOD1-related ALS. Increased nitrosylation of proteins by peroxynitrite has been suggested by elevated levels of 3-nitrotyrosine in transgenic mutant SOD1 mice (15, 16). Reduced zinc binding has been demonstrated for several SOD1 mutants (17). Interestingly, zinc-deficient wild-type SOD1, as well as mutant SOD1, generates superoxide, which in turn increases peroxynitrite production. Apoptosis of cultured primary neurons...

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by the Welcombe Trust.
‡ Supported by the Motor Neurone Disease Association.
§§ To whom correspondence should be addressed: Academic Unit of Neurology, Division of Genomic Medicine, Beech Hill Rd., University of Sheffield, Sheffield, S10 2RX, UK. Tel.: 114-2712473; Fax: 114-2261201; E-mail: simon.allen@sheffield.ac.uk.

¹ The abbreviations used are: ALS, amyotrophic lateral sclerosis; SOD1, Cu,Zn-superoxide dismutase; GST, glutathione S-transferase; hSOD, human SOD; mSOD, mouse SOD1; FALS, familial amyotrophic lateral sclerosis; RT, reverse transcriptase; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ASS, argininosuccinate synthase; NOS, nitric-oxide synthase; NOS1, neuronal NOS; AMC, 7-amino-4-methylcoumarin; Z, benzyloxycarbonyl; PBS, phosphate-buffered saline; DTT, dithiothreitol; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; IPG, immobilized pH gradient gel; IEF, isoelectric focusing; DAB, 3,3′-diaminobenzidine; LTB4, leukotriene B4; 12i, 12-hydroxydehydrogenase; cyph A, cyclophilin A; Ptx I, peroxiredoxin I; ASL, argininosuccinate lyase; SALS, sporadic ALS; HNE, 4-hydroxy-2-nonenal; CSF, cerebrospinal fluid.

This paper is available on line at http://www.jbc.org
Proteome Alterations in a Motor Neuron Disease Model

rions induced by zinc-deficient SOD1 can be reduced by treatment with inhibitors of nitric-oxide synthase (18).

Protein aggregation as a toxic gain-of-function was initially proposed after the demonstration of anti-SOD1-reactive cytoplasmic inclusions in motor neurons and surrounding astrocytes in mutant SOD1 transgenic mouse models (19) and cell culture models (20) of human FALS. Aggregation may lead to toxicity in a number of ways. SOD1 aggregates may have altered free radical and metal ion chemistry as described above. They may also challenge the protein folding and degradative machinery of the cell, compromising housekeeping protein functions essential for cell viability (8). Indeed only modest inhibition of the proteasome complex is required to generate mutant SOD1 aggregates in transfected cell lines (21). Whatever the relative contributions of these potentially inclusive modes of SOD1 toxicity are, the molecular pathways they trigger that ultimately lead to neuronal degeneration are poorly understood. Studies using cell models (13) or transgenic mutant SOD1 mice (22) have demonstrated biochemical markers suggesting an apoptotic mode of programmed cell death for degenerating neurons (reviewed in Ref. 23). Recent developments in gene expression profiling technology have provided new impetus in the identification of molecular pathways activated by mutant ALS SOD1. Two independent studies using microarray analysis of transgenic mice expressing the human FALS-associated G93A SOD1 mutant (24, 25) and microarray analysis of human ALS spinal cord (26) have detected some characteristic gene expression changes. Differential regulation of apoptosis-, inflammation-, and antioxidant-related genes were findings common to all of these studies. The use of spinal cord tissue in these studies also underlined the possible involvement of protein expression changes in non-neuronal cells including microglia and astrocytes in motor neuron degeneration. Attributing any of these changes specifically to the motor neurons that account for a very small proportion of spinal cord tissue may not be possible. Primary alterations within degenerating motor neurons in response to mutant ALS SOD1 may go undetected among the protein changes occurring within the complex mixture of more abundant cell types in the central nervous system. We have addressed this issue by developing a cell culture model of FALS based upon a murine motor neuron-like cell line, NSC34, stably transfected with human FALS associated SOD1 mutants G93A and G37R (27–29). The NSC34 cells are a hybrid motor neuron χ neuroblastoma cell line that exhibits several features of motor neurons including neurofilament expression, the ability to generate action potentials, and induction of twitching in co-cultured muscle cells (30). Alterations in gene expression in this model were analyzed previously using a microarray approach. Expression of genes involved in the regulation of axonal transport, vesicular trafficking, and apoptosis were found to be altered by expression of ALS mutant hSOD1 (28). Here we extend these studies using proteomic techniques to analyze the alterations in protein expression. We have demonstrated that expression of both G37R and G93A hSOD1 results in the differential expression and altered function of proteins that regulate nitric oxide metabolism, intracellular redox conditions, and protein degradation.

EXPERIMENTAL PROCEDURES

Reagents—All two-dimensional gel electrophoresis reagents were ultra-pure grade and purchased from Bio-Rad and Sigma. COMPLETE EDTA-free™ protease inhibitor mixture was purchased from Roche Molecular Biochemicals. Cell culture media and reagents were purchased from Invitrogen. Enhanced chemiluminescence (ECL) kits and glutathione-Sepharose were purchased from Amersham Biosciences. Reduced glutathione assay kit, reduced glutathione, 7-amino-4-methylcoumarin (AMC), Suc-L-LLVY-AMC, Z-ARR-AMC, and Z-LLE-AMC were purchased from Calbiochem. Owl silver stain and rabbit anti-rabbit glutathione transferase-Mu antibody were purchased from Autogen Bioimmune (Calne, UK). Sheep anti-bovine SOD1 antibody was purchased from The Binding Site (Birmingham, UK). Mouse anti-actin (AC-40), anti-mouse inductible nitric-oxide synthase monocular antibodies, and donkey anti-sheep IgG horseradish peroxidase conjugate were purchased from Sigma. Rabbit anti-rat neuronal nitric-oxide synthase antibody was a gift from Masataka Mori (University of Kumamoto, Japan). Rabbit anti-rat argininosuccinate lyase antibody was a gift from Richard Wiesinger (University of Tuebingen, Germany). Rabbit anti-mouse LMP7 antibody was a gift from John Monaco (University of Cincinnati). Rabbit anti-human proteasome subunit X antibody was a gift from Klavs Hendil (University of Copenhagen, Denmark). Rabbit anti-mouse LMP2 antibody was purchased from Affiniti Research Products Ltd. (Exter, UK). Rabbit anti-pericorne leukotriene B4 12-hydroxydehydrogenase antibody was a gift from Takehiko Yokomizo and Takao Shimizu (University of Tokyo, Japan). Swine anti-rabbit IgG horseradish peroxidase conjugate and goat anti-mouse IgG horseradish peroxidase conjugate were purchased from Dako (Ely, UK).

Cell Lines and Cytosol Preparation—NSC34 single cell clones stably expressing pCEP4 expression vector only, wild-type hSOD1, G93A hSOD1, and G37R hSOD1 have been described previously (27). Cytosol was prepared from NSC34 cells using a modified version of the method of Yang and co-workers (31). NSC34 cells were seeded into T175 flasks at a density of 9 × 10⁵ cells per flask and maintained in Dulbecco’s modified Eagle medium containing 10% v/v fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO₂. The culture was aspirated, and the cells were resuspended in 10 ml of ice-cold PBS, pH 7.4. Cells from two flasks were pooled for each cytosol preparation. The cells were centrifuged at 600 × g for 5 min. The pellets were washed twice with 30 ml of ice-cold PBS and pelleted as above. The pellets were resuspended in 200 μl of extract buffer (20 mM HEPES/KOH, pH 7.4, containing 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol (DTT), 10 mM sucrose, COMPLETE EDTA-free™ protease inhibitor mixture 1 tablet per 10 ml). The cells were homogenized with 30 passes of a mini homogenizer. The homogenates were centrifuged at 3000 rpm for 6 min at 4 °C; the post-nuclear S1 supernatant was harvested; the pellet was resuspended in 100 μl of extract buffer and homogenized as above; and the centrifugation step was repeated. The S1 supernatants were pooled and centrifuged at 13,000 × g for 10 min at 4 °C. The protein concentration of the resulting S2 supernatant was determined using a Coomassie G-250 assay (Pierce).

Two-dimensional Gel Electrophoresis—For analytical gels, cytosol containing 25 μg of protein was made up to 300 μl with 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM DTT, and 0.2% v/v ampholyte, pH 3–10 (Bio-Rad). For preparative gels, 100–500 μg of cytosolic protein was used. Each 300-μl sample was applied to a 17-cm, pH 3–10, immobilized pH gradient gel strip (IPG). The IPG strips were then rehydrated actively at 50 V for 16 h in a Bio-Rad Protein isoelectric focusing (IEF) cell. IEF consisted of 250 V for 15 min, linear ramping from 250 to 10,000 V over 3 h, followed by 10,000 for 60,000 V-h. The strips were then incubated at room temperature for 10 min in SDS-PAGE equilibration buffer (0.375 M Tris, pH 8.8, containing 6 M urea, 2% w/v SDS, 20% v/v glycerol) containing 2% w/v DTT followed by 10 min in SDS-PAGE equilibration buffer containing 2.5% w/v iodoacetamide. The strips were loaded onto 20 × 20 cm 14% SDS-polyacrylamide gels, and gel electrophoresis was performed in a Protean II xi electrophoresis cell (Bio-Rad). Analytical and preparative two-dimensional gels were stained with 0.1% silver stain and Biosafe Coomassie G-250 stain (Bio-Rad), respectively, according to the manufacturer’s instructions. Molecular weight and pI values of proteins were estimated with two-dimensional standards (Bio-Rad).

Two-dimensional Gel Image Analysis—Analytical two-dimensional gels were scanned using a Powerlook III scanner (UMax, Ascot, UK) and analyzed using Phoretix two-dimensional software (Non-linear Dynamics, Newcastle, UK). A previously described pairwise approach (32) was used to compare the intensities of protein spots between cell lines or cell culture models of ALS transfectants (27) expressing pCEP4 vector only with those expressing wild-type hSOD1 and NSC34 stable transfectants expressing pCEP4 vector only with those expressing G93A hSOD1. Comparisons of spot intensities were made between a pair of gels electrophoresed and
stained at the same time using samples prepared within the same experiment. Digests of in situ intensities were tested with Wilcoxon t test using no less than six pairs of gels.

**Glutathione-Sepharose Precipitation—**NSC34 cells grown in T75 flasks were resuspended in ice-cold PBS, pH 7.4. The cells were centrifuged at 600 × g for 5 min. The pellets were washed twice with ice-cold PBS and pelleted as above. The resulting pellets were resuspended in 0.5 ml per flask of GST extract buffer (20 mM potassium phosphate buffer, pH 7.0, containing 0.1% v/v Triton X-100 and protease inhibitors). The cells were homogenized with 30 passes of a mini homogenizer and then centrifuged at 13,000 × g for 10 min at 4 °C. The protein concentrations of the resulting supernatants were determined using the above Coomassie G-250 assay. Appropriate volumes containing 2.5 μg of protein were subjected to 1 ml with GST extract buffer and incubated with 100 μl of 25% v/v glutathione-Sepharose, equilibrated in 20 mM potassium phosphate buffer, pH 7.0, for 60 min at 4 °C with end-over rotation. The glutathione-Sepharose precipitates were pelleted by centrifugation at 13,000 × g for 30 s and then washed 6 times with 1 ml of 20 mM potassium phosphate buffer, pH 7.0, containing 50 mM NaCl followed by 3 washes with 1 ml of 20 mM potassium phosphate buffer, pH 7.0. The pellets were thoroughly aspirated and resuspended each in 300 μl of 7 % urea, 2 % thiourea, 4 % CHAPS, 30 mM DTT, and 0.2 % v/v ampholyte, pH 3–10. The resuspended pellets were incubated at room temperature for 10 min to denature and dissociate the glutathione-protein complexes and then centrifuged at 13,000 × g for 30 s. The resulting supernatants were subjected to two-dimensional electrophoresis as described above. The relative expression levels of glutathione-Sepharose binding proteins between cell lines were analyzed using silver staining and Wilcoxon t test as described above. All glutathione-Sepharose binding proteins detectable by Coomassie Blue staining were selected for identification by MALDI-TOF-MS.

**Western Blotting of One- and Two-dimensional SDS-PAGE—**Samples were Western-blotted as described previously (27) using antibodies diluted as indicated in the figure legends. The proteins were visualized using an ECL kit according to the manufacturer’s instructions. To determine the positions of spots corresponding to endogenous mouse SOD1 (mSOD1) and G93A hSOD1, two-dimensional gels were prepared using the method described above. Western blotting was performed using the following antibodies: for mouse IgG, 1:200 and that to LTB4 12HD was used at 1:50. Incubations were followed by 1:2000 secondary donkey anti-sheep IgG horseradish peroxidase conjugate. Antibody binding was visualized with 3,3-diaminobenzidine (DAB). Both membranes were stained with Coomassie R-250.

**Enzyme and Metabolite Assays—**Glutathione S-transferase assay reaction mixtures (33) consisted of 100 mM potassium phosphate, pH 6.5, containing 1 mM EDTA, 1 mM dithiothreitol, and 1 μM GSH reduced glutathione. Reactions were initiated by adding 100 μg of NSC34 post-nuclear S1 protein per ml assay reaction. The increase in absorbance at 340 nm was measured at 0–5 min at room temperature. Glutathione reductase assay reaction mixtures (34) consisted of 50 mM HEPES/KOH, pH 8.0, containing 0.1 mM EDTA, 0.1 mM oxidized glutathione, and 1 mM Na₂GSH. Two-dimensional gel electrophoresis was performed using 10 μl of NSC34 post-nuclear S1 protein per ml of assay reaction. The decrease in absorbance at 340 nm was measured at 0–5 min at room temperature.

Proteasomes assays were performed with modification to the method of Beyette and co-workers (35). NSC34 cells grown in T75 flasks were resuspended in 1 ml with ice-cold PBS, pH 7.4. The cells were centrifuged at 13,000 × g for 5 min. The pellets were washed twice with ice-cold PBS and pelleted as above. The resulting pellets were resuspended in 0.3 ml of proteasome extract buffer (20 mM Tris/HCl, pH 7.4, containing 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 5 mM ATP, 20% v/v glycerol, and 0.04% v/v Nonidet P-40). The resuspended cells were homogenized by 25 passes through a 21-gauge needle. The resulting homogenates were centrifuged at 13,000 × g for 15 min at 4 °C. The protein concentrations of the supernatants were determined using the Coomassie G-250 assay. Proteasome assay reaction mixtures consisted of 50 mM HEPES/KOH, pH 8.0, containing 5 mM EDTA, 100 μg of NSC34 extract protein per ml of assay reaction. The reactions were initiated by adding the appropriate concentration of the substrate and were incubated at the reaction temperature for 30 s. Succ-LVY-AMC was used at 50 μM, Z-ARR-AMC and Z-LLE-AMC were used at 100 μM. Hydrolysis of the peptides was measured at 340 nm excitation and 460 nm emission for AMC using a Denley spectrofluorometer. Standard curves were constructed using 0–50 μM AMC to convert fluorescence units to AMC concentration.

**Proteasome concentration** in NSC34 cells was determined using a commercial assay kit (Calbiochem) according to the manufacturer’s instructions.

**RT-PCR Analysis of Gene Expression in NSC34 Cells and Human Motor Neurons—**NSC34 cells expressing pCEP4 vector or G93A hSOD1 were harvested, washed in Hank’s buffered saline solution, and resuspended in TRIzol (Invitrogen). RNA was extracted according to the manufacturer’s protocol. Following treatment with DNase I (Invitrogen), the sample was divided in two, and cDNA synthesis was performed both with and without the addition of Superscript II reverse transcriptase (Invitrogen), according to the manufacturer’s instructions.

**Immunohistochemical Analysis of Motor Neurons—**Serial sections were cut from paraffin-embedded lumbar spinal cord from normal neurological controls and sporadic ALS cases. Following de-waxing, microwave retrieval (microwave for 10 min in 0.01M citrate buffer, pH 6.0) was used to convert fluorescence units to AMC concentration. The relative level of expression of each of the genes of interest was calculated relative to that of actin in the same sample.
Proteome Alterations in a Motor Neuron Disease Model

| Table I | Primer pairs used for RT-PCR analysis of gene expression in human motor neurones and mouse NSC34 cells |
|---------|-----------------------------------------------------------------------------------------------------|
|          | Product size |
| Human   | bp |
| ASS forward | CCGACGGCACCACCTCCTTTA | 67 |
| ASS reverse | CGCAGTTCCGGCTTCACTCA | 115 |
| GST Mu 1 forward | ACCTTCTCCCTGGTGATAGTGTTT | 74 |
| GST Mu 1 reverse | AACCCAGCTAAGTGCTGCTCTT | 74 |
| nNOS forward | AAGTTGTAAGACGACCAGCTGCAATT | 74 |
| nNOS reverse | TTGCAGAACAGATAGAAATATC | 74 |
| LMP7 forward | CTTAGCCTGGCCCAAAAGAGAGA | 78 |
| LMP7 reverse | AGGGAGTCGTCAACATGTTGACACA | 78 |
| LTB 12HD forward | TGCCGGGTCTCTTATATATGTA | 113 |
| LTB 12HD reverse | GAAGCTGCGTTGGATGCCACAG | 113 |
| Actin forward | GTAGTTTCCTGGAGTGGTCCAG | 113 |
| Actin reverse | CTAGAGATGCTTCTTGACCAATTC | 113 |

Mouse

| ASS forward | TGCCAAATAGACCTGGACAAAAGA | 86 |
| ASS reverse | CAAATTTATCACAAACATGCTGAA | 66 |
| GST Mu 1 forward | AGAGCTTCAATCCAGGACAGAAACG | 134 |
| GST Mu 1 reverse | TGCAGACCCATCGATAGATCATATC | 82 |
| nNOS forward | CAGCAGCTCTGATCTATTTGGAAT | 70 |
| nNOS reverse | TGCCAAATAGACCATGATGGAAT | 87 |
| LMP7 forward | AGGGAGTCGTCAACATGTTGACACA | 70 |
| LMP7 reverse | CTTAGCCTGGCCCAAAAGAGAGA | 87 |

nucleotide diphosphate kinase A by Western blotting (results not shown). Spots 3–6 (Fig. 1B) were all identified as cyclophilin A (cyph A) (Swiss-Prot accession number P17742) with 75, 68, 57, and 74% sequence coverage, respectively, by MALDI-TOF-MS analysis. Spot 7 (Fig. 1B) was identified as glyceraldehyde-3-phosphate dehydrogenase (Swiss-Prot accession number P16858) with 54% sequence coverage by MALDI-TOF-MS analysis.

To detect changes in cytosolic protein expression due to G93A hSOD1 expression, two-dimensional gels of cytosol from NSC34 cells transfected with pCEP4 vector only were compared with those of cytosol from NSC34 cells expressing G93A hSOD1 in a pairwise fashion. To control for changes resulting from hSOD1 expression and not the G93A amino acid change, two-dimensional gels of cytosol from NSC34 cells transfected with pCEP4 vector only were compared with those of cytosol from NSC34 cells expressing wild-type hSOD1. We detected 4 spots reduced (spots D1, D2, D3, and D4, Fig. 1A, Fig. 2, and Table II) and 4 spots increased (spots U1, U2, U3, and U4, Fig. 1B, Fig. 2, and Table II) in intensity in the G93A hSOD1 gels compared with pCEP4 vector only gels. Only one of these 8 spot changes (Fig. 2, spot D1) was found when pCEP4 vector only gels were compared with wild-type hSOD1 gels indicating that the other 7 spot changes were specific to G93A hSOD1 expression. This was the only change detected comparing pCEP4 vector only gels with wild-type hSOD1 gels; therefore, no spot changes were detected due to wild-type hSOD1 expression that were not seen due to G93A hSOD1 expression.

For identification of the differentially displayed spots by MALDI-TOF-MS, Coomassie Blue-stained two-dimensional preparative gels loaded with 100–500 µg of cytosolic protein were used. Only 6 of the 8 differentially displayed spots detected by silver staining were detected by Coomassie Blue staining even at the highest protein loads (spots D2, D3, D4, U2, U3, and U4). This prevented the detection and subsequent identification of the remaining 2 spots (spots D1 and U1) by MALDI-TOF-MS. The 6 Coomassie Blue-stained differentially displayed spots were identified by MALDI-TOF-MS analysis (Table III). Spot D2 that was reduced 2.3-fold in intensity in G93A hSOD1 cells (p < 0.02) (Fig. 2 and Table II) was positively identified as glutathione S-transferase Mu 1 (GST Mu 1) (Swiss-Prot accession number P17742) and 20 S proteasome subunit (LMP7) (Swiss-Prot accession number P28063), respectively (Table III).

The hypothetical 251000C21Rik protein sequence derived from the mouse RIKEN cDNA clone 251000C21Rik deposited in the Functional Annotation of Mouse (FANTOM) database (36), at www.gsc.riken.go.jp/e/FANTOM, was BLAST searched. The three highest scoring protein sequences were porcine, human, and rabbit NADP-dependent leukotriene B4 12-hydroxydehydrogenase (LTB 12HD) (Swiss-Prot accession number P35700), with 82.4, 79.7, and 76.9% sequence identity, respectively. This suggested that 251000C21Rik protein represented a mouse homologue of LTB 12HD.
Proteome Alterations in a Motor Neuron Disease Model

Fig. 1. Two-dimensional electrophoresis of NSC34 cytosol. Cytosol containing 25 μg of protein from NSC34 cells stably transfected with pCEP4 vector only (A) or expressing G93A hSOD1 (B) was separated by IEF using 17-cm, pH 3–10, IPG strips. Second dimension SDS-PAGE was performed on 14% polyacrylamide gels, and the proteins were visualized by silver staining. Horizontal arrows indicate the positions of spots corresponding to endogenous mouse SOD1 and recombinant G93A hSOD1. Arrowheads indicate the positions of spots corresponding to nucleotide diphosphate kinase A (A, spots 1 and 2), cyclophilin A (B, spots 3–6), glyceraldehyde-3-phosphate dehydrogenase (B, spot 7), and peroxiredoxin I (B, spot 8). Vertical arrows indicate spots decreased (A, spots D1–4) and increased (B, spots U1–4) in the presence of G93A hSOD1 expression. Molecular weight and pI calibration were performed using two-dimensional molecular weight and pI standards.

Western Blotting of NSC34 Cytosol with Antisera to Identified Proteins—To investigate whether the cytosolic steady-state levels of these proteins were altered in the presence of G37R hSOD1 as well as G93A hSOD1, Western blotting analysis was performed on NSC34 cytosol with antisera to identified proteins where available. Faint 46-kDa bands corresponding to cyph A and Rbm3, respectively (Fig. 3A). As none of the matched peptide masses were shared between the two proteins, it was concluded that spot U3 contained both cyph A and Rbm3. The number and intensities of peptides matched to Rbm3 were significantly increased in spot U3 from cells expressing G93A hSOD1 (Fig. 3B) compared with those expressing pCEP4 vector only (Fig. 3A). In contrast the number and intensities of peptides matched to cyph A were similar between both cell lines (Fig. 3, A and B). It was concluded that the increased intensity of spot U3 in cells expressing G93A hSOD1 was due to alteration of Rbm3 levels in the cytosol. We have also demonstrated that as well as spot U3, cyph A occupies spot positions 3–6 with pI values of 6.5, 6.7, 6.9, and 7.4 respectively (Fig. 1B and Table III).

Western Blotting of NSC34 Cytosol with Antisera to Identified Proteins—To investigate whether the cytosolic steady-state levels of these proteins were altered in the presence of G37R hSOD1 as well as G93A hSOD1, Western blotting analysis was performed on NSC34 cytosol with antisera to identified proteins where available. Faint 46-kDa bands corresponding to cyph A and Rbm3, respectively (Fig. 3A). As none of the matched peptide masses were shared between the two proteins, it was concluded that spot U3 contained both cyph A and Rbm3. The number and intensities of peptides matched to Rbm3 were significantly increased in spot U3 from cells expressing G93A hSOD1 (Fig. 3B) compared with those expressing pCEP4 vector only (Fig. 3A). In contrast the number and intensities of peptides matched to cyph A were similar between both cell lines (Fig. 3, A and B). It was concluded that the increased intensity of spot U3 in cells expressing G93A hSOD1 was due to alteration of Rbm3 levels in the cytosol. We have also demonstrated that as well as spot U3, cyph A occupies spot positions 3–6 with pI values of 6.5, 6.7, 6.9, and 7.4 respectively (Fig. 1B and Table III).
significant increases in the intensities of both ASL bands (Fig. 4 (nNOS), endothelial NOS, and inducible NOS. There were sig-
cytosol were probed with antisera to ASL, neuronal NOS

As steady-state
lyase (ASL) function to regenerate a pool of intracellular argi-
levels of ASS were increased in cytosol from cells expressing

mutant SOD1 expression resulted in either differential
post-transcriptional or post-translational processing of the ASL
mRNA or protein, respectively. The nature of this mutant
hSOD1-dependent alteration to ASL expression is currently
under investigation.

Alterations to Glutathione S-transferase Family Mem-
bers—As GST Mu 1 expression was reduced due to mutant
hSOD1 expression, we further investigated the expression and
function of other GST family members expressed in NSC34
cells. The broad range GST substrate 1-chloro-2,4-dinitroben-
zene was used to compare the overall GST activity of the
NSC34 cell lines. The GST activity within NSC34 cell extracts
was significantly reduced to ~60% of normal (p < 0.05) in cells
expressing G93A and G37R hSOD1 but not in those expressing
the wild-type enzyme (Fig. 5A). In contrast, glutathione reduc-
tase activity was not significantly different in any of the cell
lines (Fig. 5B). The levels of reduced glutathione in the NSC34
cells were also measured and found to be significantly reduced
to ~75% of normal (p < 0.05) in cells expressing G93A and
G37R hSOD1 but not in those expressing the wild-type enzyme
(Fig. 5C).

It was reasoned to be unlikely that reduced GST Mu 1
expression would be solely responsible for the dramatic reduc-
tion of glutathione conjugating capacity of the mutant hSOD1
expressing cell lines toward 1-chloro-2,4-dinitrobenezene. A
more global loss of GST family members may have accounted
for this markedly decreased activity. To investigate this fur-
ther, detergent extracts of NSC34 cells expressing either
pCEP4 vector only or G93A hSOD1 were prepared. These
extracts were precipitated with glutathione-Sepharose, and the
resulting precipitates were subjected to analytical two-dimen-
sional gel electrophoresis to compare the relative expression
levels of various GST sub-types between the two cell lines. Out
of 11 spots detectable by Coomassie Blue staining (Fig. 5, 6 and
7), 10 were positively identified by MALDI-TOF-MS (Table
III). Spot E that was significantly reduced (p < 0.05) in cells

| Spot | Vector only versus wild-type hSOD1 | Vector only versus G93A hSOD1 |
|------|----------------------------------|-------------------------------|
|      | Wilcoxon p value<sup>a</sup> | Average fold change | Wilcoxon p value<sup>a</sup> | Average fold change |
| D1   | 0.016                           | 16.7 (−)                 | 0.016                       | Absent in G93A     |
| D2   | 0.578                           | 1.1 (+)                  | 0.016                       | 2.3 (−)           |
| D3   | 0.156                           | 1.3 (−)                  | 0.016                       | Absent in G93A     |
| D4   | 0.813                           | 1.0 (+)                  | 0.016                       | Absent in G93A     |
| U1   | 0.375                           | 1.3 (+)                  | 0.031                       | 4.6 (+)           |
| U2   | 0.938                           | 1.1 (+)                  | 0.016                       | 1.9 (+)           |
| U3   | 0.469                           | 1.1 (+)                  | 0.031                       | 4.3 (+)           |
| U4   | 0.813                           | 1.2 (+)                  | 0.016                       | 1.8 (+)           |

<sup>a</sup> Where n = 7 pairs of gels.
<sup>b</sup> Indicates decrease due to hSOD1 expression; + indicates increase due to hSOD1 expression.

| Spot | Protein | Swiss-Prot accession no. | Sequence coverage % | Theoretical molecular mass | Molecular mass from gel<sup>a</sup> | Theoretical pl | pl from gel<sup>b</sup> |
|------|---------|-------------------------|---------------------|---------------------------|-----------------------------------|---------------|---------------------|
| D1   | ND<sup>b</sup> | ND | ND | ND | 29,000 | ND | 5.0 |
| D2   | Glutathione S-transferase Mu 1 | P10649 | 64 | 25,970 | 29,500 | 7.72 | 8.0 |
| D3   | 251000C21Rik protein 1 | Q9CP51 | 44 | 35,595 | 36,000 | 8.14 | 8.0 |
| D4   | Proteasome p5 subunit (LMP7) | P28063 | 43 | 23,047 | 25,000 | 6.89 | 6.9 |
| U1   | ND | ND | ND | ND | 38,000 | ND | 6.7 |
| U2   | Argininosuccinate synthase | P16460 | 25 | 46,585 | 43,000 | 8.36 | 8.3 |
| U3<sup>c</sup> | Cyclophilin A | P17742 | 44 | 17,972 | 19,000 | 7.73 | 6.5 |
| U4   | Peroxiredoxin I | P35700 | 47 | 22,390 | 25,500 | 8.26 | 6.9 |

<sup>a</sup> Determined by calibration with two-dimensional molecular weight and pl standards.
<sup>b</sup> ND, not determined.
<sup>c</sup> Two proteins present.

As GST Mu 1 was not available to us, we have observed similar increases in intensities, to those seen in
G93A hSOD1, of the corresponding spots (U3 and U4, respectively) in cytosol from G37R hSOD1 cells on silver-
stained two-dimensional gels when compared with control cytosol (results not shown).

Argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) function to regenerate a pool of intracellular argi-
nine that is dedicated to the synthesis of nitric oxide via the action of nitric-oxide synthase (NOS) (37, 38). As steady-state
levels of ASS were increased in cytosol from cells expressing
G93A and G37R hSOD1, the effects of mutant SOD1 expression
on the levels of other enzymes involved in arginine and
nitric oxide cycling were determined. Western blots of
NSC34 cytosol were probed with antisera to ASL, neuronal NOS
(nNOS), endothelial NOS, and inducible NOS. There were sig-
nificant increases in the intensities of both ASL bands (Fig. 4E)
and nNOS bands (Fig. 4F) in the cytosol from cells expressing
G93A and G37R hSOD1 (lanes 1 and 2) compared with those
in the cytosol from cells expressing pCEP4 vector only and wild-
type hSOD1 (lanes 1 and 3). Neither endothelial NOS nor
inducible NOS were detected in any of the cytosol samples by
Western blotting (results not shown). As well as an increase in the intensity of ASL bands in the cytosol from cells expressing
G93A and G37R hSOD1 compared with control cells (Fig. 4E),
we also observed an alteration in the pattern of ASL bands. An
ASL doublet was present in cytosol from cells expressing
G93A or G37R hSOD1 (lanes 2 and 4). This result indicated
that mutant SOD1 expression resulted in either differential
post-transcriptional or post-translational processing of the ASL
mRNA or protein, respectively. The nature of this mutant
hSOD1-dependent alteration to ASL expression is currently
under investigation.

| Spot | Protein | Swiss-Prot accession no. | Sequence coverage % | Theoretical molecular mass | Molecular mass from gel<sup>a</sup> | Theoretical pl | pl from gel<sup>b</sup> |
|------|---------|-------------------------|---------------------|---------------------------|-----------------------------------|---------------|---------------------|
| D1   | ND<sup>b</sup> | ND | ND | ND | 29,000 | ND | 5.0 |
| D2   | Glutathione S-transferase Mu 1 | P10649 | 64 | 25,970 | 29,500 | 7.72 | 8.0 |
| D3   | 251000C21Rik protein 1 | Q9CP51 | 44 | 35,595 | 36,000 | 8.14 | 8.0 |
| D4   | Proteasome p5 subunit (LMP7) | P28063 | 43 | 23,047 | 25,000 | 6.89 | 6.9 |
| U1   | ND | ND | ND | ND | 38,000 | ND | 6.7 |
| U2   | Argininosuccinate synthase | P16460 | 25 | 46,585 | 43,000 | 8.36 | 8.3 |
| U3<sup>c</sup> | Cyclophilin A | P17742 | 44 | 17,972 | 19,000 | 7.73 | 6.5 |
| U4   | Peroxiredoxin I | P35700 | 47 | 22,390 | 25,500 | 8.26 | 6.9 |

<sup>a</sup> Determined by calibration with two-dimensional molecular weight and pl standards.
<sup>b</sup> ND, not determined.
<sup>c</sup> Two proteins present.

well as G93A hSOD1 when compared with control cells ex-
pressing wild-type hSOD1 or pCEP4 vector only. Although antisera raised to Rbm3 and Prx I were not available to us, we have observed similar increases in intensities, to those seen in
G93A hSOD1 cells, of the corresponding spots (U3 and U4, respectively) in cytosol from G37R hSOD1 cells on silver-
stained two-dimensional gels when compared with control cytosol (results not shown).
expressing G93A SOD1 was identified as hypothetical Rik061005A07 protein (Swiss-Prot accession number Q9DD25) (Fig. 5D and Table IV). The amino acid sequence of hypothetical Rik061005A07 protein, derived from the mouse RIKEN cDNA clone deposited in the FANTOM database (36), was BLAST searched. The five highest scoring protein sequences were rat GST Mu 3, human GST Mu 1, human GST Mu 2, rat GST Mu 2, and mouse GST Mu 2 with 95, 83, 81, 80, and 80% sequence identity, respectively, suggesting that hypothetical Rik061005A07 protein was a Mu class GST. In addition to hypothetical Rik061005A07 protein, we demonstrated significantly reduced expression (p < 0.05) of three other Mu class GST enzymes, namely GST Mu 1 (spot B) (Swiss-Prot accession number P10649), GST Mu 2 (spot D) (Swiss-Prot accession number P15626), and GST Mu 5 (spot C) (Swiss-Prot accession number P48774) in cells expressing G93A hSOD1 (Fig. 5 and Table IV). The most abundant GST precipitable by glutathione-Sepharose from NSC34 cells was GST Pi B that occupied 5 individual spot locations (spots F–J). Four of the GST Pi B spots were significantly less abundant (p < 0.05) in cells expressing G93A hSOD1 (Table IV and Fig. 5, D and E). In contrast to GST Mu and GST Pi, GST Alpha 2 (spot A) (Swiss-Prot accession number P10648) was found to be significantly increased (p < 0.05) in cells expressing G93A hSOD1 (Table IV and Fig. 5, D and E).

Alterations to Proteasome Activity and Proteasome Subunit Expression—Up-regulation of the LMP7 proteasome subunit is known to promote cleavage after hydrophobic (chymotrypsin-like activity) and basic (trypsin-like activity) residues and suppress cleavage after acidic residues (postglutamyl cleavages) (39). As LMP7 expression was significantly reduced due to G93A and G37R hSOD1 expression, the effects of these mutant forms of SOD1 on proteasomal chymotrypsin-like activity, trypsin-like activity, and the postglutamyl hydrolase activity were investigated in the NSC34 cell lines using the model fluorogenic peptides Suc-LLVY-AMC, Z-ARR-AMC, and Z-LLE-AMC, respectively (Fig. 6, A–C). All three activities were significantly reduced (p < 0.02) in the presence of G93A and G37R hSOD1 expression, with chymotrypsin-like activity showing the most marked reduction to ~70% of normal. There was no significant change in chymotrypsin-like activity (Fig. 6A), but there were small reductions in trypsin-like (Fig. 6B) and postglutamyl hydrolase activity (Fig. 6C) in the presence of wild-type hSOD1 expression.

As there was significant reduction in the chymotrypsin-like activity (Fig. 6A) and the cytosolic levels of the inducible immunoproteasome subunit LMP7 (β5i) due to mutant hSOD1 expression (Fig. 4), we investigated whether there were alterations in the level of expression of the constitutive β5 proteasome subunit, subunit X, and the other inducible immunoproteasome subunit LMP2 (β1i). Western blotting of the proteasome assay extracts with anti-LMP7 serum (Fig. 6D) confirmed the dramatic reduction of LMP7 expression (Fig. 4) in cytosol of cells expressing G93A and G37R hSOD1 (24 and 14%, respectively) and a less dramatic reduction in cells expressing wild-type hSOD1 (66%). In the case of the constitutive subunit (Fig. 6E), Western blotting detected a significant increase (p < 0.05) in expression of subunit X (β5) in cells expressing G93A and G37R hSOD1 (142 and 158%, respectively).
that mirrored the decrease in LMP7. Paradoxically, this coincided with decreased postglutamyl hydrolase activity (Fig. 6C).

We detected no significant change in the level of LMP2 expression (Fig. 6F) in cells expressing wild-type or G37R hSOD1, but we observed a small reduction ($p < 0.05$) in LMP2 expression in the cells expressing G93A hSOD1 (Fig. 6F). As there were substantial amounts of LMP2 remaining in the presence of hSOD1 expression (Fig. 6F), this probably accounts for the remaining chymotrypsin-like activity toward Suc-LLVY-AMC (Fig. 6A).

**Altersations to Expression of Antioxidant Enzymes, Proteasome, and Nitric Oxide-related Enzymes of Motor Neurons from ALS Cases**—To confirm whether the protein changes observed for GST Mu 1, LMP7, nNOS, ASS, and LTB$_4$ 12-HD were accompanied by alterations in the expression levels of the corresponding mRNAs, RT-PCR analysis was performed on NSC34 cells expressing pCEP4 vector or G93A hSOD1 (Fig. 7A). Expression levels of ASS, nNOS, LMP7, and LTB$_4$ 12-HD, but not GST Mu 1, were changed significantly ($p < 0.05$) due to G93A hSOD1 in NSC34 cells. To investigate whether the expression levels of these differentially regulated proteins were affected in motor neurons from human FALS cases, we performed RT-PCR analysis of gene expression in laser capture microdissected motor neurons from normal individuals or indi-


### Proteome Alterations in a Motor Neuron Disease Model

Relative expression levels and MALDI-TOF-MS analysis of glutathione-Sepharose binding proteins in vector only and G93A hSOD1-expressing NSC34 cells

| Spot | Average fold change | Wilcoxon p value | Swiss-Prot accession no. | Sequence coverage | Theoretical molecular mass | Molecular mass from gel | Theoretical pI | pI from gel |
|------|---------------------|-----------------|--------------------------|------------------|-----------------------------|------------------------|----------------|------------|
| A    | 2.1 (+)             | 0.031           | GST Alpha 2              | P10648           | 18                          | 25,533                 | 33,000         | 8.80       | 8.5        |
| B    | 4.0 (-)             | 0.031           | GST Mu 1                 | P10649           | 44                          | 25,970                 | 29,000         | 7.2         | 7.2        |
| C    | 1.4 (-)             | 0.031           | GST Mu 5                 | P48774           | 38                          | 26,635                 | 29,000         | 6.83       | 6.5        |
| D    | 2.6 (-)             | 0.031           | GST Mu 2                 | P15626           | 43                          | 25,717                 | 27,000         | 6.91       | 7.0        |
| E    | 2.9 (-)             | 0.031           | Rik061005A07             | Q0DD25           | 33                          | 25,680                 | 27,000         | 6.80       | 6.5        |
| F    | 1.3 (-)             | 0.313           | GST Pi B                 | P19157           | 25                          | 23,634                 | 26,000         | 8.13       | 6.0        |
| G    | 1.3 (-)             | 0.031           | GST Pi B                 | P19157           | 23                          | 23,634                 | 25,000         | 8.13       | 6.5        |
| H    | 1.6 (-)             | 0.031           | GST Pi B                 | P19157           | 32                          | 23,634                 | 25,000         | 8.13       | 6.5        |
| I    | 1.8 (-)             | 0.031           | GST Pi B                 | P19157           | 32                          | 23,634                 | 25,000         | 8.13       | 7.5        |
| J    | 1.2 (-)             | 0.031           | GST Pi B                 | P19157           | 32                          | 23,634                 | 25,000         | 8.13       | 8.0        |
| K    | 1.1 (-)             | 0.844           | ND                        | ND               | ND                          | ND                     | 25,000         | ND         | 6.5        |

* Where n = 6 pairs of gels.

+ indicates increase due to hSOD1 expression; - indicates decrease due to hSOD1 expression.

ND, not determined.

Table IV

Several recent studies (24–26) employing genomic profiling technologies to identify gene expression changes in ALS have concentrated on whole spinal cord tissue from human ALS cases and transgenic mouse models of FALS. However, due to the low proportion of motor neurons compared with other cell types in spinal cord, the primary responses of motor neurons to mutant SOD1 toxicity that trigger cell death pathways may go undetected among the overall tissue transcriptional changes responding to motor neuron degeneration. To this end we have exploited a well characterized cell culture model of mutant SOD1-expressing NSC34 cells. Surprisingly, only one protein change (spot D1) was detected due to normal hSOD1 expression rather than the G93A mutant SOD1 protein. Further changes may have been detected using narrower pH ranges for the first dimension IEF along with a staining technique with a wider range of linearity than silver. However, it is of interest that our results using the Affymetrix oligonucleotide array also reveal only minor effects of wild-type hSOD1 on the mRNA expression profile of NSC34 cells compared with those of G93A hSOD1.

The differentially regulated proteins fall into four categories: (i) proteins involved in regulation of mRNA processing (Rbm3); (ii) proteins involved in NO metabolism (ASS, ASL, and nNOS); (iii) proteins involved in anti-oxidant defense (Alpha, Mu, and Pi class GSTs, LTB4 12-HD, and Prx I); and (iv) proteins involved in protein degradation (LMP7, subunit X). Our results provide further evidence for mutant SOD1-mediated alterations in the intracellular redox state and protein degradation machinery, which in turn supports the hypothesis that both altered free radical handling and abnormal protein aggregation are likely to be mechanisms contributing to motor neuron injury.

Rbm3 was shown to have elevated cytosolic levels in the presence of G93A hSOD1 expression. To date the exact function of this protein is unclear. It is a heterogeneous nuclear ribonucleoprotein that contains an N-terminal consensus sequence RNA binding domain and a C-terminal glycine-rich domain. Proteins with such domains have been shown to regulate mRNA stability and translation, mRNA splicing, and export of mRNA from the nucleus to the cytoplasm (41). The raised levels of this heterogeneous nuclear ribonucleoprotein in the cytosol in the presence of G93A hSOD1 expression may indicate alterations to protein biogenesis at the level of post-transcriptional mRNA processing and/or mRNA translation.

ASS acts in conjunction with ASL to regenerate arginine from citrulline for the purpose of nitric oxide production by NOS (37, 38). As with ASS, we found that both ASL and nNOS were up-regulated by G93A and G37R hSOD1. Additionally, ASL was shown to undergo mutant hSOD1-specific alterations.

**DISCUSSION**

Several recent studies (24–26) employing genomic profiling technologies to identify gene expression changes in ALS have concentrated on whole spinal cord tissue from human ALS cases and transgenic mouse models of FALS. However, due to the low proportion of motor neurons compared with other cell types in spinal cord, the primary responses of motor neurons to mutant SOD1 toxicity that trigger cell death pathways may go undetected among the overall tissue transcriptional changes responding to motor neuron degeneration. To this end we have exploited a well characterized cell culture model of mutant SOD1-expressing FALS (27–29) to enable us to analyze the proteome changes that occur as a direct result of SOD1 toxicity in cells with a motor neuron phenotype.

By using a combination of two-dimensional electrophoresis, mass spectrometry, and Western blotting, we identified seven up-regulated proteins as ASS, ASL, nNOS, Rbm3, Prx I, subunit X, and GST Alpha 2. Seven down-regulated proteins were identified as GST Mu 1, GST Mu 2, GST Mu 5, a hypothetical GST Mu homologue, GST Pi B, LTB4 12-HD, and LMP7. We also demonstrated that the mRNA expression levels of GST Mu 1, LTB4 12-HD, and LMP7 were similarly changed in motor neurons isolated from FALS cases. In the case of GST Mu 1 in the cell culture model, and nNOS in the model and isolated motor neurons, the protein changes were not reflected by the mRNA levels as determined by RT-PCR. This was probably due to the semi-quantitative nature of the RT-PCR technique, as recent studies in our laboratory employing the Affymetrix Murine Genome Oligonucleotide array have demonstrated 2.3-fold up-regulation and 7.6-fold down-regulation of nNOS and GST Mu 1 mRNA, respectively, due to expression of G93A hSOD1 in NSC34 cells. Alternatively, only one protein change (spot D1) was detected due to normal hSOD1 expression rather than the G93A mutant SOD1 protein. Further changes may have been detected using narrower pH ranges for the first dimension IEF along with a staining technique with a wider range of linearity than silver. However, it is of interest that our results using the Affymetrix oligonucleotide array also reveal only minor effects of wild-type hSOD1 on the mRNA expression profile of NSC34 cells compared with those of G93A hSOD1.

The differentially regulated proteins fall into four categories: (i) proteins involved in regulation of mRNA processing (Rbm3); (ii) proteins involved in NO metabolism (ASS, ASL, and nNOS); (iii) proteins involved in anti-oxidant defense (Alpha, Mu, and Pi class GSTs, LTB4 12-HD, and Prx I); and (iv) proteins involved in protein degradation (LMP7, subunit X). Our results provide further evidence for mutant SOD1-mediated alterations in the intracellular redox state and protein degradation machinery, which in turn supports the hypothesis that both altered free radical handling and abnormal protein aggregation are likely to be mechanisms contributing to motor neuron injury.

Rbm3 was shown to have elevated cytosolic levels in the presence of G93A hSOD1 expression. To date the exact function of this protein is unclear. It is a heterogeneous nuclear ribonucleoprotein that contains an N-terminal consensus sequence RNA binding domain and a C-terminal glycine-rich domain. Proteins with such domains have been shown to regulate mRNA stability and translation, mRNA splicing, and export of mRNA from the nucleus to the cytoplasm (41). The raised levels of this heterogeneous nuclear ribonucleoprotein in the cytosol in the presence of G93A hSOD1 expression may indicate alterations to protein biogenesis at the level of post-transcriptional mRNA processing and/or mRNA translation.

ASS acts in conjunction with ASL to regenerate arginine from citrulline for the purpose of nitric oxide production by NOS (37, 38). As with ASS, we found that both ASL and nNOS were up-regulated by G93A and G37R hSOD1. Additionally, ASL was shown to undergo mutant hSOD1-specific alterations.

* J. Kirby, P. R. Heath, and P. J. Shaw, unpublished results.
to either its post-transcriptional or post-translational processing. The human ASL gene product has been shown previously to undergo highly variable splicing (42). The nature of the alteration here to the mouse ASL gene product is currently under investigation.

By using microelectrode biosensor measurements, we have determined previously that NSC34 cells expressing wild-type hSOD1 exhibit enhanced NO release, whereas those expressing ALS mutant hSOD1 exhibit reduced NO release following cell stress induced by serum withdrawal (29). Both groups of cells expressing wild-type or mutant hSOD1 show decreased superoxide release in the same experimental paradigm. In addition, the mutant SOD1-expressing NSC34 cells are more sensitive to apoptosis stimulated by NO-releasing compounds (29). The mechanism by which NO release is reduced in the mutant SOD1-expressing NSC34 cells has yet to be determined. The reduction may result in a compensatory response by the cells in the form of up-regulation of the arginine/NO recycling pathway. The role of NO in ALS pathogenesis remains controversial due to its dual role as a neuroprotective and neurotoxic agent. At low concentrations NO can protect cells against oxidative stress, presumably by induction of adaptive responses in the

---

**Fig. 6.** Alterations to proteasome activity and subunit expression due to ALS mutant SOD1 in NSC34 cells. A–C, assays. Lysates prepared from NSC34 cells stably transfected with pCEP4 vector only (white bars), expressing wild-type hSOD1 (black bars), expressing G37R hSOD1 (light gray bars), or expressing G93A hSOD1 (dark gray bars) were assayed for chymotrypsin-like activity using 50 μM Suc-LLVY-AMC (A), trypsin-like activity using 100 μM Z-ARR-AMC (B), and post-glutamyl hydrolase activity using 100 μM Z-LLE-AMC (C). Six fluorescence measurements were taken from lysates extracted on 4 separate occasions. Specific activities are expressed as percentage of pCEP4 vector control. D–F, Western blots and densitometry. Lysates prepared from NSC34 cells stably transfected with pCEP4 vector only (lane 1 of blot, white bars on histogram), expressing wild-type hSOD1 (lane 2, black bars), expressing G37R hSOD1 (lane 3, light gray bars), or expressing G93A hSOD1 (lane 4, dark gray bars) were Western-blotted using antisera to LMP7 (D), subunit X (E), and LMP2 (F) all diluted 1:1000. Proteins were visualized using enhanced chemiluminescence. The relevant bands, from lysates extracted on 4 separate occasions, were quantified by densitometry, and their intensities are expressed as percentages of pCEP4 vector control.
form of up-regulation of antioxidant proteins (43, 44). In contrast, inhibition of nNOS has been shown to protect rat motor neurons from cell death induced by oxidative stress (45). The findings that inhibition of nNOS in a cell culture model of FALS did not confer resistance to mutant SOD1 toxicity (46) and that blockade of NOS either by chemical means (47) or targeted deletion of the nNOS gene (48) had little effect on disease progression in a transgenic mouse model of human ALS have raised uncertainties regarding the importance of NO production in ALS pathogenesis. However, the relevance of our findings here to ALS is reinforced by studies reporting nNOS up-regulation in human ALS cases (49, 50) and inducible NO up-regulation in transgenic mice expressing ALS hSOD1 mutants (50). Furthermore, increased levels of nitrotyrosine have been shown in human cerebrospinal fluid in sporadic ALS (SALS) (51) as well as the spinal cord of a transgenic mouse model (51). We anticipate that exploitation of recently developed “nitroproteomic” techniques (52, 53) will eventually clarify the contribution of altered nitric oxide metabolism to ALS.

Expression of mutant hSOD1 resulted in a complete loss of the LTB4 12HD protein from NSC34 cells. LTB4 12HD may have a wider role in antioxidant defense as well as in lipid messenger metabolism. A recent study (54) demonstrated that LTB4 12HD was effective in reducing a wide variety of cytotoxic α,β-unsaturated aldehydes and ketones including products of lipid peroxidation such as 4-hydroxy-2-nonenal (HNE). HNE has been shown to be elevated in the cerebrospinal fluid of SALS cases (55) and SALS spinal cord (56, 57). A potential chemoprotective role for the enzyme was suggested by its ability to confer resistance of LTB4 12HD-transfected cell lines to HNE-induced apoptosis (54). The association of a significant amount of LTB4 12HD with membranes (54) and the nuclear associated staining of motor neurons in this study supports the notion that the enzyme may help to protect membranes from oxidative damage.

Other antioxidant enzymes that were down-regulated were GST Mu 1, GST Mu 2, GST Mu 5, GST Pi B, and a hypothetical Mu class GST Rik061005A07. The GST family catalyzes the conjugation of reduced glutathione to toxic compounds to allow their elimination from cells by glutathione conjugate transporters (58). Here we demonstrated this function of GST to be significantly impaired in G93A and G37R hSOD1-expressing NSC34 cells. Previously, human neuroblastoma cells overexpressing mouse GST Mu showed increased resistance to cell death induced by hydrogen peroxide, peroxynitrite, and HNE (59). Human GST Mu when added to rat neuronal cultures protected against HNE cytotoxicity (60). Additional to their detoxification role, GST family members have also been shown to regulate apoptotic pathways. Mouse GST Mu has been shown recently to interact physically with and inhibit apoptosis signal-regulating kinase 1 (ASK1) that is known to activate p38 pathways (61). The GST family also contains enzymes that have a wider role in antioxidant defense as well as in lipid messenger metabolism. A recent study (54) demonstrated that LTB4 12HD was effective in reducing a wide variety of cytotoxic α,β-unsaturated aldehydes and ketones including products of lipid peroxidation such as 4-hydroxy-2-nonenal (HNE). HNE has been shown to be elevated in the cerebrospinal fluid of SALS cases (55) and SALS spinal cord (56, 57). A potential chemoprotective role for the enzyme was suggested by its ability to confer resistance of LTB4 12HD-transfected cell lines to HNE-induced apoptosis (54). The association of a significant amount of LTB4 12HD with membranes (54) and the nuclear associated staining of motor neurons in this study supports the notion that the enzyme may help to protect membranes from oxidative damage.

Prox I was shown to occupy at least two spot positions on our two-dimensional gels. The most abundant of the two forms, which was unchanged on our gels, had an apparent pl (8.2) in close agreement with the theoretical value (8.26) for Prox I. The less abundant form, which was up-regulated in the presence of mutant SOD1 expression, had a more acidic pl of 6.9. Prox I, II, and III (62, 63) have all been shown to undergo acidic shifts when cultured cells are subjected to hydroperoxide-mediated oxidative stress. A recent study (64) has demonstrated that the acidic shift of Prox II produced under conditions of oxidative stress was due to conversion of an essential active site cysteine to cysteic acid. The possibility that Prox I undergoes over-oxidation of its cysteine residues due to mutant SOD1 expression

![Figure 7](http://www.jbc.org/)

**Fig. 7.** RT-PCR and immunohistochemical analysis of human motor neurons. RT-PCR (A), relative expression levels of ASS, nNOS, GST Mu 1, LMP7, and LTB4 12HD in NSC34 cells expressing pCEP4 vector (white bars) or G93A hSOD1 (black bars), and in laser captured human motor neurons from normal controls (white bars) or SALS cases (black bars) were measured by densitometry of RT-PCR products generated on at least 5 separate occasions. The levels are expressed relative to that of actin. B, immunohistochemistry. Human lumbar spinal cord sections from normal neurological controls and sporadic ALS cases were stained using antisera raised to ASS, GST Mu, LMP7, and LTB4 12HD. Cytoplasmic staining in anterior horn motor neurons is indicated with an asterisk. Focal granular brown staining in the cytoplasm of neurons from SALS cases represents lipofuscin, indicated with a chevron. Nuclear staining of LTB4 12HD, in anterior horn motor neurons and glia, is indicated with large and small arrows, respectively. Black bar indicates 100 μm.
therefore warrants further investigation.

All of the antioxidant proteins shown here to be down-regulated (GST family and LTβ, 12HD) or post-translationally modified (Ptx1) have been shown previously to either contain the antioxidant-response element in their genes, undergo nuclear factor E2 p45-related factor (Nrf2)-dependent transcriptional regulation via antioxidant-response element binding, and/or undergo up-regulation by chemical agents known to activate Nrf2 (65, 66). It is tempting to speculate that this down-regulation is due to alterations in Nrf2 status. The down-regulation of genes encoding antioxidant proteins is likely to contribute significantly to the increased sensitivity of the mutant SOD1-expressing NSC34 cells to oxidative stress (27). Identification of the primary alteration to SOD1 biochemistry that drives this transcriptional down-regulation of antioxidant proteins may prove to be insightful in determining how the mutant enzyme triggers cell death in motor neurons.

The expression of both G93A and G37R hSOD1 resulted in a significant reduction in amount of the LMP7 proteasome subunit (β5i) that coincided with a reduction in chymotrypsin-like activity and an increase in the amount of subunit X (β5). The 20 S proteasome can contain three interferon-γ-inducible β subunits LMP7, LMP2, and MECL that are homologous and interchangeable with three constitutive β subunits X, Y, and Z, respectively (39-69). It is likely that interferon-γ-inducible β subunits favor the production of peptides with basic or hydrophobic C termini that are most suited to transport into the endoplasmic reticulum via the transporter associated with antigen presentation prior to major histocompatibility complex class I presentation. Several studies have reported proteasome activity and subunit content alteration in response to disease-associated protein aggregation and/or oxidative stress (70-73). SOD1-containing aggregates have been observed in both transgenic mouse (19) and cell culture models of FALS (20, 21). Similarly ubiquitinated protein aggregates have been observed in human ALS (74). The down-regulation of LMP7 and up-regulation of its displacing partner, subunit X, may suggest an adaptive response to a challenge to the proteasome resulting from ALS mutant SOD1 expression. The observed reduction in proteasome activity may contribute over time to the abnormal intracellular aggregation of intracellular proteins observed in motor neurons, in cellular and animal models as well as human disease.

In conclusion we have used proteomic approaches to identify protein alterations in a cell culture model of SOD1-related FALS. The novel approach in this study is that we have applied proteomic technology to motor neuronal cells expressing mutant SOD1 at levels approximating those seen in human FALS. Several of the protein changes were corroborated at the transcriptional level in motor neurons isolated from human FALS cases. The proteome alterations we have identified are accompanied by functional consequences and may contribute to or represent responses to cellular changes that ultimately trigger cell death pathways resulting in neurodegeneration. These protein changes provide further evidence for the altered free radical handling and protein aggregation hypotheses to explain the toxic gain-of-function of FALS-associated mutant SOD1.

Acknowledgments—We thank Liz Stewart for performing the MALDI-TOF-MS analysis and data base searching; Gillian Forster, Lynne Baxter, and Catherine Gelilethorpe for performing the immunohistochemical analysis of spinal cord; Neil Cashman for providing the parent NSC34 cell line; Denise Figlewicz for providing the hSOD1 expression constructs; Michael Blackburn and Alan Spivey (Department of Chemistry, University of Sheffield) for use of the fluorimeter; and all of our colleagues who provided antibodies.

REFERENCES
1. Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Gotto, J., Oregan, J. P., Deng, H. X., Rahmani, Z., Kruszyn, A., McKennawayes, D., Cayababay, A., Gaston, S. M., Berger, R., Tanzi, R. E., Halperin, J. J., Herzfeld, B., Van den Berg, H. W., Bird, T., Deng, G., Mulder, D. W., Smyth, C., Laing, N. G., Soriano, E., Pericak-Vance, M. A., Haines, J. J., Bouleau, A. G., Gusaella, J. S., Horvitz, H. R., and Brown, R. H. (1993) Nature 362, 692-696.
2. Borchelt, D. R., Lee, M. K., Stunt, H. S., Guarnieri, M., Xu, Z.-S., Wong, P. C., Brown, R. H., Price, D. L., Sisodia, S. S., and Cleveland, D. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8292-8296.
3. Reaume, A. G., Elliott, J. J., Hoffman, E. K., Kowal, N. W., Ferrante, R. J., Siekew, D. F., Wilcox, H. M., Flood, D. G., Beal, M. F., Brown, R. H., and Stuber, W. D. (1996) Nat. Genet. 13, 43-47.
4. Gurney, M. E. (1994) X-Exp. Neurol. 125, 172-173.
5. Wong, P. C., Pardo, B. A., Borchelt, D. R., Lee, M. K., Copeland, N. G., Jenkins, N. A., Sisodia, S. S., Cleveland, D. W., and Price, D. L. (1995) Neuron 14, 1109-1116.
6. Bruijn, L. I., Becher, M. W., Lee, M. K., Anderson, K. L., Anderson, S. D., Copeland, N. G., Sisodia, S. S., Rothstein, J. D., Borchelt, D. B., Price, D. L., and Cleveland, D. W. (1997) EMBO J. 16, 2577-2587.
7. Beckman, J. S., Estes, A. G., Crow, J. P., and Barbeito, L. (2001) Trends Neurosci. 24, S15-S20.
8. Cleveland, D. W., and Rothstein, J. D. (2001) Nat. Neurosci. 4, 806-819.
9. Williamson, S. A., Ramos, L. E., Xu, Z., and Beal, M. F. (1998) J. Neurochem. 71, 1321-1324.
10. Liu, R., Althaus, J. S., Ellerbrock, B. R., Becker, D. A., and Beal, M. E. (1998) Ann. Neurol. 43, 409-419.
11. Shaw, P. J., Ince, P. G., Falkous, G., and Mantle, M. (1995) Ann. Neurol. 38, 691-695.
12. Pardo, C. A., Copeland, N. G., Jenkins, N. A., and Cleveland, D. W. (1997) Science 279, 1257-1261.
13. Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Gotto, J., Oregan, J. P., Deng, H. X., Rahmani, Z., Kruszyn, A., McKennawayes, D., Cayababay, A., Gaston, S. M., Berger, R., Tanzi, R. E., Halperin, J. J., Herzfeld, B., Van den Berg, H. W., Bird, T., Deng, G., Mulder, D. W., Smyth, C., Laing, N. G., Soriano, E., Pericak-Vance, M. A., Haines, J. J., Bouleau, A. G., Gusaella, J. S., Horvitz, H. R., and Brown, R. H. (1993) Nature 362, 692-696.
Proteome Alterations in a Motor Neuron Disease Model

(2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11575–11580
44. Gonzalez-Zulueta, M., Ensz, L. M., Mukhina, G., Lebovitz, R. M., Zwacka, R. M., Engelhardt, J. F., Oberley, L. W., Dawson, V. L., and Dawson, T. M. (1998) J. Neurosci. 18, 2040–2055
45. Estevez, A. G., Spear, N., Manuel, S. M., Raddi, R., Henderson, C. E., Barbeito, L., and Beckman, J. S. (1998) J. Neurosci. 18, 923–931
46. Doroudchi, M. M., Minotti, S., Figlewicz, D. A., and Durham, M. D. (2001) Neuropathol. 12, 1239–1243
47. Upton-Rice, M. N., Cudkowicz, M. E., Mathew, R. K., Reif, D., and Brown, R. H. (1999) Ann. Neurol. 45, 413–414
48. Facchinetti, F., Sasaki, M., Cutting, F. B., Zhai, P., Macdonald, J. E., Reif, D., Feil, M. F., Huang, P. L., Dawson, T. M., Gurney, M. E., and Dawson, V. L. (1999) Neuroscience 109, 1483–1492
49. Phou, R. K., Shaw, P. J., Ince, P. G., and Smith, M. E. (2000) Amyotroph. Latera. Scler. Other Motor Neuron Disord. 1, 259–267
50. Almer, G., Vukosavic, S., Romero, N., and Przedborski, S. (1999) J. Neurochem. 72, 2415–2425
51. Tohgi, H., Abe, T., Yamazaki, K., Murata, T., Ishizaki, E., and Isobe, C. (1999) Ann. Neurol. 46, 129–131
52. Li, J., Lee, J., and Johnson, J. A. (2002) J. Biol. Chem. 277, 19396–19401
53. Nandi, D., Woodward, E., Ginsburg, D. B., and Monaco, J. J. (1997) EMBO J. 16, 5363–5375
54. Griffin, T. A., Nandi, D., Cruz, M., Fehling, H. J., Kaer, L. V., Monaco, J. J., and Colbert, R. A. (1998) J. Exp. Med. 187, 97–104
55. Dinger, Q., Lewis, J. J., Strum, K. M., Dimayuga, E., Bruce-Keller, A. J., Dunn, J. C., and Keller, J. N. (2002) J. Biol. Chem. 277, 13935–13942
56. Stefanis, L., Larsen, K. E., Rideout, H. J., Sulzer, D., and Greene, L. A. (2001) J. Neurosci. 21, 9549–9560
57. Eskinazi, A., Ratan, G., Benahmed, M., Louisot, P., and Lunardi, J. (2001) Proteomics 1, 1105–1110
58. Sheehan, D., Meade, G., Foley, V. M., and Dowd, C. A. (2001) Biochem. J. 360, 1–16
59. Xie, C., Lovell, M. A., Xiong, S., Kindly, M. S., Guo, J.-T., Xie, J., Amaranth, V., Montine, T. J., and Markesbery, W. R. (2001) Free Radic. Biol. Med. 31, 73–81
60. Xie, C., Lovell, M. A., and Markesbery, W. R. (1998) Free Radic. Biol. Med. 25, 979–988
61. Cho, S.-G., Lee, Y. H., Park, H.-S., Ryoo, K., Kang, K. W., Park, J., Eom, S.-J., Kim, M. J., Chang, T.-S., Choi, S.-Y., Shim, J., Kim, Y., Gong, M.-S., Lee, M.-J., Kim, S. G., Ichijo, H., and Choi, E.-J. (2001) J. Biol. Chem. 276, 12479–12485
62. Mitsumoto, A., Takegawa, Y., Okawa, K., Iwamatsu, A., and Nakagawa, Y. (2001) Free Radic. Biol. Med. 30, 625–635
63. Rabilloud, T., Heller, M., Rigobelli, M.-P., Bindoli, A., Aebersold, R., and Lunardi, J. (2001) Proteomics 1, 1105–1110
64. Rabilloud, T., Heller, M., Gassner, F., Luche, S., Rey, C., Aebersold, R., Benahmed, M., Louissot, P., and Lunardi, J. (2002) J. Biol. Chem. 277, 19396–19401
65. Li, J., Lee, J., and Johnson, J. A. (2002) J. Biol. Chem. 277, 388–394
66. Thimmulappa, R. K., Mai, K. H., Sriruma, S., Kessler, T. W., Yamamoto, M., and Biewal, S. (2002) Cancer Res. 62, 5196–5203
67. Nandi, D., Woodward, E., Ginsburg, D. B., and Monaco, J. J. (1997) EMBO J. 16, 5363–5375
68. Griffin, T. A., Nandi, D., Cruz, M., Fehling, H. J., Kaer, L. V., Monaco, J. J., and Colbert, R. A. (1998) J. Exp. Med. 187, 97–104
69. Kingsbury, D. J., Griffin, T. A., and Colbert, R. A. (2000) J. Biol. Chem. 275, 24156–24162
70. Ding, Q., Lewis, J. J., Strum, K. M., Dimayuga, E., Bruce-Keller, A. J., Dunn, J. C., and Keller, J. N. (2002) J. Biol. Chem. 277, 13935–13942
71. Stefanis, L., Larsen, K. E., Rideout, H. J., Sulzer, D., and Greene, L. A. (2001) J. Neurosci. 21, 9549–9560
72. Tanaka, T., Engelender, S., Igarashi, S., Raso, R. K., Wanner, T., Tanzi, R. E., Saw, A., Dawson, V. L., Dawson, T. M., and Ross, C. A. (2001) Hum. Mol. Genet. 10, 919–926
73. Engidawork, E., Juranville, J. F., Fountoulakis, M., Dierssen, M., and Lubez, G. (2001) J. Neural Transm. 61, (suppl.) 117–130
74. Mather, K., Martin, J. E., Swash, M., Woldes, G., Brown, A., and Leigh, P. N. (1993) Neuropathol. Appl. Neurobiol. 19, 141–145
Analysis of the Cytosolic Proteome in a Cell Culture Model of Familial Amyotrophic Lateral Sclerosis Reveals Alterations to the Proteasome, Antioxidant Defenses, and Nitric Oxide Synthetic Pathways

Simon Allen, Paul Roy Heath, Janine Kirby, Stephen Barrie Wharton, Mark Robert Cookson, Fiona Mhairi Menzies, Rosamonde Elizabeth Banks and Pamela Jean Shaw

J. Biol. Chem. 2003, 278:6371-6383.
doi: 10.1074/jbc.M209915200 originally published online December 9, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209915200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 74 references, 28 of which can be accessed free at
http://www.jbc.org/content/278/8/6371.full.html#ref-list-1