Changes in the Spinal Cord Proteome of an Amyotrophic Lateral Sclerosis Murine Model Determined by Differential In-gel Electrophoresis*§

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Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by loss of motor neurons resulting in progressive paralysis. To date, more than 140 different mutations in the gene encoding CuZn-superoxide dismutase (SOD1) have been associated with ALS. Several transgenic murine models exist in which various mutant SOD1s are expressed. We used DIGE to analyze the changes in the spinal cord proteome induced by expression of the unstable SOD1 truncation mutant G127insTGGG (G127X) in mice. Unlike mutants used in most other models, G127X lacks SOD activity and is present at low levels, thus reducing the risk of overexpression artifacts. The mice were analyzed at their peak body weights just before onset of symptoms. Variable importance plot analysis showed that 420 of 1,800 detected protein spots contributed significantly to the differences between the groups. By MALDI-TOF MS analysis, 54 differentially regulated proteins were identified. One spot was found to be a covalently linked mutant SOD1 dimer, apparently analogous to SOD1-immunoreactive bands migrating at double the molecular weight of SOD1 monomers previously detected in humans and mice carrying mutant SOD1s and in sporadic ALS cases. Analyses of affected functional pathways and the subcellular representation of alterations suggest that the toxicity exerted by mutant SODs induces oxidative stress and affects mitochondrial, cellular assembly/organization, and protein degradation. "Molecular & Cellular Proteomics 8: 1306–1317, 2009."
lytic symptoms. Here we present the identity of and discuss the possible significance of 53 proteins found to be differentially regulated in ALS transgenic mice.

**EXPERIMENTAL PROCEDURES**

**Transgenic Mice**—The G127x hSOD1 mice (7) were developed in our laboratory and backcrossed with C57BL/6J BomTac mice for >25 generations. Mice expressing G85R mutant hSOD1 were obtained from Dr. Don Cleveland and were similarly backcrossed with C57BL/6J BomTac mice for >15 generations (6). The G127x mice attained their peak body weights at 195 ± 14 days of age, and onset of symptoms, as observed by changes in grip strength and stride pattern, occurred at day 208 ± 15. Mice were regarded as terminally ill (216 ± 15 days) when they were no longer able to reach the food. Five mice, 196 days old and without any symptoms, were chosen for the study. As controls, five non-transgenic mice of the C57BL/6J BomTac strain used for the backcrossing were used (Taconic Europe, Bornholt, Denmark). Mice were killed by intraperitoneal injections of pentobarbital. The thorax was cut open, and the animal was perfused through the left ventricle with 20 ml of 0.15 M NaCl. The spinal cord was dissected by cutting the vertebral column at the base of the skull and just above the hip bone. A syringe was inserted at the lower opening, and the spinal cord was flushed out with 0.15 M NaCl, washed, frozen in liquid nitrogen, and stored at −80 °C. For validation by Western immunoblotting, spinal cords from four G127x mice (age, 184 days), four C57BL/6J BomTac control mice (age, 199 days), and four G85R mice (at their peak body weights; age, 325 days) were collected.

**Homogenization of Tissue**—For 2-D gels, the spinal cords were supplied with 10 volumes of lysis buffer (1 M Tris-HCl, pH 8.5, 2 M thiourea, 7 M urea, 4% CHAPS) and homogenized using an Ultraturrax (IKA, Stufen, Germany) followed by sonication using a Sonifier Cell Disruptor (Branson, Danbury, CT) for 1 min. Tissues for Western blot were supplied with 25 volumes of PBS (10 mM potassium phosphate, pH 7.0, 0.15 M NaCl containing EDTA-free Complete antiproteolytic mixture (Roche Diagnostics)) and homogenized as above.

**Labeling Proteins for DIGE**—Fluorescent DIGE labeling of proteins was performed as described in the manufacturer’s manual (GE Healthcare). For further information, see the supplemental information.

**Analytical Two-dimensional Electrophoresis**—Preparation of 2-D gels for DIGE was carried out as described in the manufacturer’s manual (GE Healthcare). Samples were run on 24-cm pl 3–11 NL and pl 4–7 strips (GE Healthcare). For further information, see the supplemental information.

**Gel Analysis**—CyDye-labeled DIGE gels were scanned without removing the glass plates in a Typhoon 9410 imager (GE Healthcare) using excitation and emission filters specified by GE Healthcare (Cy2, Cy3, and Cy5, excitation 480, 540, and 620 nm and emission 530, 590, and 680 nm, respectively). Individual gels were processed further using DeCyder 6.5 software, and spot detection was done using the differential in-gel analysis module. Data from this application were then loaded into the biological variation analysis software for spot matching between gels and variation analysis. Statistical significance was calculated using the Student’s t test, and spots that were present in all gels, with a difference between the groups of at least 25% and with a p value < 0.05, were considered for further analysis.

**Protein Identification**—Quantitative 24-cm 2-D gels were run using 0.5–1 mg of total protein. For Coomassie staining, gels were fixed in 10% methanol, 7% acetic acid for 1 h. Staining was performed overnight with G-250 staining solution (0.8% phosphoric acid, 8% ammonium sulfamate, 20% methanol, 0.08% Coomassie Brilliant Blue G-250), briefly washed with 25% methanol, and stored in distilled water. Selected spots were picked manually and put in a tube with distilled water. Coomassie-stained spots were dehydrated twice with a solution of 35% (v/v) acetonitrile in 20 mM ammonium hydrogen carbonate and dried in a SpeedVac (Savant; GMI, Inc., Ramsey, MN) and finally incubated with trypsin (V511a; dilution, 10 ng/ml; Promega, Madison, WI) overnight at 37 °C. One microliter of protein digest was applied to a MALDI-TOF target plate (Applied Biosystems) and dried. Then 0.5 μl of a 1:1 mixture of α-cyana-4-hydroxyxicinnamic acid solution (Agilent Technologies, Santa Clara, CA) and 1% trifluoroacetic acid, 50% acetonitrile was added on top. One microliter of calibrant (a 1:1 mixture of 50% acetonitrile, 1% trifluoroacetic acid and α-cyana-4-hydroxyxicinnamic acid solution mixed with Se- quazyme calibration mixtures 1 and 2 (P2-3143-00, Applied Biosys- tems)) was spotted onto the plate next to every protein digest and used for external calibration. When completely dry, the plate was put in a Voyager DE-STR mass spectrometer (Applied Biosystems), and the peptide spectra were recorded. Peptide peak lists were generated using Data Explorer software version 4.1 (Applied Biosystems) with external calibration, advanced base-line correction, noise filter set to 0.7, and deisotoping. A cutoff was generally set to 1–5% of peak base intensity. Advanced peak detection was used for the peptide spectra of some weak protein spots, with few peptides and high background noise, using a higher cutoff in the low molecular weight area for efficient noise reduction. Proteins were identified by searching the Swiss-Prot (version 50.9) and International Protein Index (version mouse_v3.21_20060915) databases using an in-house Mascot server that was licensed to Umeå University by Matrix Science (Boston MA). Following glycine 127, the G127x protein contains five novel amino acids before the carboxyl-terminal truncation. To get a Mowse score for the G127x dimer, this sequence was added to the Swiss-Prot database searched. Search parameters were set to allow mass deviations of ±50 ppm and one or two missed cleavage sites as well as fixed modifications such as carbamidomethylation of cysteine and oxidation of methionine. When human keratin contaminants were present, the “Re-search unmatched” function was used. Identifications that were statistically significant (with a Mowse score in the range of at least 60–66 and a p value < 0.05) and that showed a gel position close to the theoretical mass and isoelectric point were regarded as positive.

**Solubilization and Reduction of the 33-kDa SOD1 Species**—Formic acid efficiently solubilizes aggregated proteins (11). Homogenates were prepared as for Western blots, and an aliquot was dried in a SpeedVac and then incubated in 100% formic acid for 15 min. The acid was evaporated under a stream of argon, and the dry material was dissolved in sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate, reductant) for immunoblotting. Because 2-mercaptoethanol has been shown to occasionally induce aggregation (12), both 10% 2-mercaptoethanol and 100 mM dithiothreitol were tested as reductants prior to immunoblotting (13).

**Immunoblotting**—One-dimensional immunoblotting and quantitation of proteins were performed as described previously (10). All immunoblots were run at least in triplicate. Two-dimensional immunoblotting was performed with 11-cm pl 3–11 NL strips (GE Healthcare) run on regular SDS-PAGE (Criterion IPG + 1 gels (Bio-Rad)). For further information, see the supplemental information.

**Immunohistochemistry**—Brain and spinal cords from 200-day-old G127x and G85R transgenic mice and C57BL/6J BomTac control mice were fixed with 4% paraformaldehyde in phosphate-buffered solution (10 mM potassium phosphate, pH 7.4, 0.15 M NaCl) and embedded in paraffin. Four-micrometer-thick transverse paraffin sections were prepared for immunohistochemistry, which was carried out using the same set of antibodies as for immunoblotting. For enhancement, microwave heating for 20 min in citrate buffer (pH 6.0) was performed, and the sections were blocked for 30 min in goat serum prior to staining procedures. Single immunohistochemistry was performed on the sections using the Ventana 3-amino-9-
Protein spots, based on \( p(\text{corr})p \), and recalculating the model until the difference was eliminated. This was done by successively removing the 20 most significant protein spots that contributed most to the calculated OPLS-DA model (VIP analysis).

Network Analysis—Network analysis was performed using the Ingenuity Pathways Knowledge Base. Protein identifiers of the altered proteins were uploaded into the Ingenuity Pathways Knowledge Base to reveal networks of interacting proteins. Function and canonical pathways analysis revealed the possible impact of the proteins identified on cellular functions and pathways. See Ref. 14 and the supplemental information for further information.

Statistical Analysis—Orthogonal projections to latent structures-discriminant analysis (OPLS-DA) (15) was used for multivariate statistical evaluation in the SIMCA-P+ software version 11.5 (Umetrics AB, Umeå, Sweden). OPLS-DA is a supervised multivariate classification method used to extract the systematic differences between known classes of samples in predictive variation (systematic variation correlated to the class separation) and orthogonal variation (systematic variation unrelated to the class separation). Cy3 and Cy5 values were corrected by dividing by the corresponding Cy2 values. The data were mean-centered and Pareto scaled before being subjected to OPLS-DA. The number of significant OPLS-DA components was decided by full cross-validation (16). Protein spots that differed significantly between wild-type and G127X SOD1 mice were detected through a simultaneous inspection of the OPLS-DA covariance and correlation loadings (\( \omega_p \) and \( p(\text{corr})p \), respectively). In addition, an iterative threshold approach, a modified version of the approach presented by Karp et al. (17), was applied to identify the protein spots that contributed most to the calculated OPLS-DA model (VIP analysis). This was done by successively removing the 20 most significant protein spots, based on \( p(\text{corr})p \), and recalculating the model until the cross-validated score vector \( t_{cv} \) revealed the first overlap between the two classes. For this separation, the cross-validated predictive ability (VIP-Q2) and the \( p \) value were calculated.

RESULTS

The DIGE analysis of the gels covering pI 3–11 found about 1,800 potential protein spots that could be matched between the different gels and that could be subjected to statistical analysis. When analyzed by OPLS-DA, it was found by VIP-Q2 scoring that 420 of these spots significantly contributed to the separation between the groups (Fig. 1). The criteria we used regarding choice for identification (differences greater than 25% with \( p < 0.05 \) by Student’s \( t \) test) yielded 178 apparent protein spots. Because of streaking, multiple protein isoforms (trains of spots), differences in staining between DIGE and Coomassie, and other uncertainties, 111 spots could be reliably picked. The omissions occurred mainly at molecular masses above 80 kDa. Finally the MALDI-TOF analysis resulted in 65 identified proteins, most located in the 15–80-kDa range. Most failures in identification occurred in low abundance proteins below 15 kDa because of the presence of few significant peptides and difficulties in achieving high coverage.

A problem in analysis of DIGE data sets is that the number of variables/protein spots is much greater than the number of observations and that the variables often are correlated and contain noise. Multivariate methods are well suited for extraction of systematic information from such materials, and they can robustly and reliably detect differences between groups. Combined with full cross-validation, the number of false positives can be minimized. In our final analysis of alterations caused by expression of the G127X mutant SOD1, we only considered proteins that showed good correlations (\( p(\text{corr})p > 0.70 \)) by the multivariate OPLS-DA and that differed from controls with significances better than \( p < 0.01 \) using the parametric \( t \) test. This gave us a final list of 53 proteins (Fig. 2 and Table I). Eleven proteins with borderline significance levels of between 0.01 and 0.05 and one spot that was identified as a mixture of two proteins are listed in supplemental Table 1.

As a partial technical replicate and to achieve a higher resolution, the transgene/control set was also subjected to separation in pI 4–7 gels and DIGE analysis. In the optimally resolved area, 25 of the proteins in the pI 3–11 gels that fulfilled all criteria for inclusion were also found to be differentially regulated \( (p < 0.01) \) in the pI 4–7 gels. One such protein was not replicated in the pI 4–7 gels and was omitted from Table I. Another protein, complexin, which was poorly resolved in the pI 3–11 gels, fulfilled the significance criterion \( (p < 0.01) \) and was added to Table I.

Compartment, Networks, and Functions—The proteins were divided into groups defined by their main tissue compartments (Fig. 3) based on information from European Molecular Biology Laboratory-European Bioinformatics Institute or Swiss-Prot. Altered proteins were mainly from the cytoplasm or mitochondria. In a recent comprehensive study, Pollak et al. (18) tried to identify as many proteins as possible in another part of the murine central nervous system (the
The hippocampus of the brain) by the same techniques as those used here, i.e. 2-D gels and MALDI-TOF. The compartment distributions of the 469 different proteins they identified were compared with the present findings (Fig. 3). The most distinct difference is the overrepresentation of altered mitochondrial proteins in the ALS model mice.

The proteins identified were further evaluated by Ingenuity Pathways Knowledge Base analysis (14). This tool builds protein networks based on known direct or indirect interactions described in the literature and defines common functional and canonical pathways. Of the 53 proteins, 44 could be used for network analysis, and 42 could be used for functional/canonical pathway analysis. The discrepancy was due to protein triplicates or duplicates (e.g. actin-γ) and to a few proteins having very limited information in the literature. Four protein networks were identified with high significance and contained 41 of the 44 proteins included (Fig. 4). The networks generated were composed of the proteins identified and their interaction partners known from the literature. Network A contained 13 identified proteins and their interaction partners and involved functions such as cellular assembly/organization (filament dynamics) and the cell cycle. Networks B and C contained 12 and 11 identified proteins, respectively, and involved functions in nervous system development/function and cellular assembly/organization. Network D contained five identified proteins, and the main functions involved the cell cycle.

The material was then subjected to functional analysis (Table II). In this analysis, the $p$ value is calculated by comparing the number of identified proteins that participate in a given function or pathway relative to the total number of occurrences of these proteins in all functional pathway annotations stored in the Ingenuity Pathways Knowledge Base. The four top functional pathways involved fundamental mechanisms of cell survival but also pointed at proteins known earlier to take part in neurological disease (nucleic acid metabolism, neurological disease, cellular assembly/organization, and cellular function/maintenance). Ten canonical pathways reached significance levels of $<0.01$ (Table III). Pyruvate metabolism was the top pathway ($p < 0.001$) represented by four identified proteins followed by the citrate cycle (three proteins, all up-regulated), and the NRF-2-mediated oxidative stress response (five proteins, all up-regulated).

**G127X SOD1 Dimer**—A spot with a molecular mass of 17 kDa, representing G127X SOD1 monomer, was found by the DIGE analysis and was not considered further (Fig. 2). In the pI 4–7 2-D gels, another spot was found at pI 32 kDa, pI 5.5 that was only present in the transgenic samples (Fig. 5 A). When analyzed by MALDI-TOF, all the major peaks were found to represent peptides from G127X hSOD1 with 90% sequence coverage (Fig. 5B). If these were removed from the peptide spectrum, the remaining peaks yielded only an insignificant hit for human keratin. With one-dimensional and 2-D Western blotting for G127X hSOD1, in addition to the mono-
### Table I

**Proteins significantly down- or up-regulated**

Gel IDs are numbers from Fig. 1, and accession numbers are from Swiss-Prot. Abbreviated gene names are as indicated from the Ingenuity Pathways Knowledge Base (14). -Fold change refers to ratios as identified by DeCyder software. Further data on identifications and statistics are presented in supplemental Table 2.

| Gel ID | Abbreviation/gene | Protein accession no. | Name of protein | -Fold change |
|--------|-------------------|-----------------------|-----------------|-------------|
| 1      | MBP               | P04370                | Myelin basic protein | −3.10       |
| 2      | ETFA              | Q0THD7                | Electron transfer flavoprotein subunit α | −2.12       |
| 3      | MTAP              | Q9COQ5                | S-Methyl-5-thioadenosine phosphorlyase | −2.03       |
| 4      | DSTDN             | Q9R0P5                | Destrin (actin-depolymerizing factor) | −1.97       |
| 5      | INSL3             | Q5RL10                | Insulin-like 3 | −1.92       |
| 6      | ABAT              | P61922                | 4-Aminobutyrate aminotransferase | −1.76       |
| 7      | FABP5             | Q05816                | Fatty acid-binding protein, epidermal | −1.65       |
| 8      | ALDH5A1           | Q05193                | Succinate-semialdehyde dehydrogenase | −1.56       |
| 9      | VDAC1             | Q05816                | Voltage-dependent anion-selective channel protein 1 | −1.50       |
| 10     | FABP5             | Q05816                | Fatty acid-binding protein | −1.46       |
| 11     | ESF1              | P59999                | Actin-related protein 2/3 complex subunit 4 | −1.48       |
| 12     | HINT3             | Q8CPS6                | Histidine triad protein 4 | −1.47       |
| 13     | FABP5             | Q05816                | Fatty acid-binding protein | −1.46       |
| 14     | DPYS12            | Q9DBJ1                | Dihydropyrimidinidase-related protein 2 | −1.44       |
| 15     | HAGH              | Q99KB8                | Hydroxyacylglutathione hydrolase | −1.42       |
| 16     | DBI               | P31786                | Acyl-CoA-binding protein | −1.39       |
| 17     | CNP               | P16360                | 2’,3’-Cyclic-nucleotide 3’-phosphodiesterase | −1.39       |
| 18     | CPLX1             | P63040                | Complexin | −1.38       |
| 19     | DNM1              | Q9OJHR7               | Insulin-degrading enzyme | 1.31       |
| 20     | SUCLA2            | Q22219                | Succinyl-CoA ligase | 1.34       |
| 21     | STIP1             | Q60864                | STI (stress-induced) phosphoprotein 1 | 1.35       |
| 22     | CYC1              | Q8D9M3                | Cytochrome c, heme protein, mitochondrial precursor | 1.35       |
| 23     | SOD2              | P09671                | Mn-SOD | 1.36       |
| 24     | C3ORF10           | Q91VR8                | Brick 1 | 1.37       |
| 25     | APOA1BP           | Q8K4Z3                | ApoA-I-binding protein | 1.29       |
| 26     | IDE               | Q9JJH7                | Insulin-degrading enzyme | 1.31       |
| 27     | DMH1              | P61922                | Destrin | 1.46       |
| 28     | PGAM1             | Q9DBJ1                | Phosphoglycerate mutase | 1.32       |
| 29     | ACTG1             | Q3TSB7                | Actin-γ | 1.34       |
| 30     | SUCLA2            | Q9OJHR7               | Insulin-degrading enzyme | 1.31       |
| 31     | STIP1             | Q60864                | STI (stress-induced) phosphoprotein 1 | 1.35       |
| 32     | CYC1              | Q8D9M3                | Cytochrome c, heme protein, mitochondrial precursor | 1.35       |
| 33     | SOD2              | P09671                | Mn-SOD | 1.36       |
| 34     | C3ORF10           | Q91VR8                | Brick 1 | 1.37       |
| 35     | CAPNS1            | Q9SS56                | Calpain smaller subunit | 1.40       |
| 36     | MLP2              | Q9KX1                 | Myeloid leukemia factor 2 | 1.40       |
| 37     | AKR1A1            | Q9JI6                 | Alcohol dehydrogenase (NADP⁺) | 1.42       |
| 38     | MDH1              | P14152                | Malate dehydrogenase | 1.43       |
| 39     | COPS4             | Q8544                 | COP9 (signalosome complex) subunit 4 | 1.43       |
| 40     | CKB               | Q04447                | Creatine kinase B-type | 1.44       |
| 41     | YWHAH             | P61922                | 14-3-3 protein γ | 1.44       |
| 42     | BLVRA             | Q9CY64                | Biliverdin reductase A precursor | 1.47       |
| 43     | COPS8             | Q9BV7                 | COP9 (signalosome complex subunit 8 | 1.47       |
| 44     | TPRG1L            | Q9EBS2                | Tumor protein p63-regulated gene 1-like protein | 1.49       |
| 45     | MDH1              | P14152                | Malate dehydrogenase | 1.52       |
| 46     | IDH1              | Q9BO44                | Isocitrate dehydrogenase (NADP) cytoplasmic | 1.52       |
| 47     | ERP29             | P57759                | Endoplasmic reticulum protein 29 | 1.53       |
| 48     | ACTG1             | Q3TSB7                | Actin-γ | 1.53       |
| 49     | ACTG1             | Q3TSB7                | Actin-γ | 1.56       |
| 50     | CKB               | Q04447                | Creatine kinase B-type | 1.62       |
| 51     | COPS8             | Q9BV7                 | COP9 (signalosome complex) subunit 8 | 1.71       |
| 52     | UCHL1             | Q9R0P9                | Ubiquitin carboxy-terminal hydrolase isozyme L1 | 1.75       |
| 53     | UBE2L3            | P68037                | Ubiquitin-conjugating enzyme E2 L3 | 1.81       |
mers, a major band/spot was found at about 32 kDa, i.e. close to the expected molecular mass of a dimer (Fig. 5C). A similar band is commonly seen in spinal cord extracts from humans (7) and transgenic mice expressing various mutant hSOD1s (9, 19), and it has recently been observed also in sporadic ALS patients (20). The present analysis shows conclusively that the 32-kDa band is an hSOD1 dimer at least in the case of G127X mice. To further study the nature of this dimerization, the protein samples were subjected to solubilization with SDS or formic acid to disrupt any non-covalent binding (Fig. 5D). This treatment had no effect on the ratio of monomer to dimer. To determine whether the dimer is attached by way of inefficiently reduced disulfide bonds, homogenates were treated with two different reducing agents (Fig. 5E), both of which failed to change the quantity of dimer, showing that the SOD1 dimer is linked covalently but by means other than disulfide bonding.

Transglutaminases cross-link proteins via isopeptide linkages, and such cross-linking has been implicated in the pathogenesis of several neurodegenerative diseases (21). 2-D immunoblots from G127X and control mice were carried out using an anti-isopeptide antibody. More spots were indeed found in the blots from the ALS model mice, but none of them with different isoelectric points. The species detected of them with different isoelectric points. The species detected.

When quantified from Western immunoblots, the dimer appears to represent about 40–50% of the total G127X hSOD both in transgenic mice (Fig. 5C) and in human cases (7). However, on CyDye- and Coomassie-stained 2-D gels the dimer appears to represent about 5–10% of the total amount of G127X hSOD1. This suggests that the dimer has a very high immunoreactivity and that it becomes overestimated relative to the monomer in immunoblots. Difficulties in finding the dimer in protein-stained 2-D gels may explain why its composition has not been determined previously.

**Validations**—To examine the validity of the differences found by the DIGE procedure and the biological variation analysis software, the G127X and control mice were randomized into new groups (e.g. 2 + 3 from each group) that were still mixed regarding Cy3 and Cy5 (which is a confounder if not mixed). Generally fewer than 10 proteins were found to be significantly different between the groups using the initial criteria for selection (p < 0.05 and ±25% regulation). If p was set to <0.01 in practice no significant spots remained in the randomized setup.

Performing a response permutation test in the SIMCA-P+ software (VALIDAT) showed that the Q2 values for OPLS-DA models calculated against permuted response vectors (class identity) were significantly decreasing with declining correlation to the original response vector.Twenty permutations gave an intercept for the extrapolation of Q2 of −0.296, implying that a permuted response with zero correlation to the original response would not yield a significant model. Of the 20 permutations none gave a Q2 higher or even close to the Q2 for the original response.

As to the differences in amounts of protein, seven of the proteins found to be altered were analyzed by Western immunoblots (Fig. 6A and supplemental Fig. 2). Spinal cords from independent sets of four G127X and C57BL/6J BomTac controls were used as were tissues from transgenic mice representing another ALS model, G85R hSOD1, at peak weight. Of the four up-regulated proteins, isocitrate dehydrogenase and HSC70 (with borderline significance in DIGE analysis; see supplemental Table 1) were found to be increased in both ALS models. From this additional analysis, it can be concluded that HSC70 is significantly up-regulated in transgenic ALS models. The increases found on Western blot for Mn-SOD and COP9s8 were not statistically significant. Of the three down-regulated proteins, FABP was significantly reduced in the G127X mice but not in those expressing G85R hSOD1. FIS1 was significantly reduced in the G127X mice but could not be compared in the G85R ALS model because of a different immunoblot pattern with double bands (supplemental Fig. 2). No changes could be found for CNP1 in the validation sets (Fig. 6B and supplemental Fig. 2). We investigated this result further by immunoblotting 2-D gels for CNP1 (Fig. 6B). CNP1 was found to exist in multiple isoforms, most of them with different isoelectric points. The species detected by the DIGE analysis in controls appears to be in much lower amounts in the G127X sample, which in turn contains a species with a lower isoelectric point (arrow). The latter was not detected by the DIGE analysis possibly because of shielding by other proteins stained by the CyDyes. The other differences between DIGE analysis and the one-dimensional Western immunoblots may have similar explanations, i.e. differences in isoforms with distinct isoelectric points that are only resolved in the 2-D analysis.

To investigate the cellular specificity of these proteins, sections from brain and cervical, thoracic, and lumbar spinal cord were examined in G127X, G85R, and control mice using the above mentioned antibodies. Two animals were studied for
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Network Shapes
- Chemical or Drug
- Cytokine
- Enzyme
- G-protein Coupled Receptor
- Group or Complex
- Growth Factor
- Ion Channel
- Kinase
- Ligand-dependent Nuclear Receptor
- Peptidase
- Phosphatase
- Transcription Regulator
- Translation Regulator
- Transmembrane Receptor
- Transporter
- Other

Relationships
- Binding only
- Acts on
- Inhibits AHR acts on

Note: "Acts on" and "inhibits" edges may also include a binding event.
each group. The FABP and CNP1 antibodies failed to give a reliable staining. Staining with the Mn-SOD antibody showed small dotlike specific staining in the cytoplasm of spinal motor neurons in all mice investigated (Fig. 6C). Mn-SOD was also seen in neurons of the dorsal horn, a few reactive astrocytes, and the neuropil. Staining with the Cop9s8, FIS1, and HSC70 antibodies showed diffuse immunoreactivity in the cytosol of ventral horn motor neurons as well as in dorsal horn neurons and in a few reactive astrocytes (Fig. 6C). No staining was seen in the majority of astrocytes or the neuropil, indicating that the staining principally was neuron-specific. If this pattern can be extrapolated to the rest of the proteins, it may indicate that the majority of protein alterations in ALS take place in neurons.

**DISCUSSION**

In this study we analyzed ALS model mice at their peak body weight, a time point at which there is significant pathology in the spinal cord but still limited deterioration of the global status of the animals. In the murine transgenic ALS models, the motor neurons in the ventral horns of the spinal cords are primarily affected. Because murine spinal cords are small (weighing about 70 mg), whole spinal cords had to be used. By stereology, we found that the ventral horns account for 20% of the volume of the spinal cords. Motor neuron somas and neurites have been estimated to occupy 20% of the volume of the ventral horns (22), and they thus account for about 4% of the whole spinal cord. Changes in a specific cell type (e.g. motor neurons) in proteins that are expressed in multiple cell types therefore run the risk of being diluted in the present study, whereas changes in more motor neuron-specific proteins will be less affected. There is evidence, however, that the demise of motor neurons is caused by toxic effects of mutant SOD1s in multiple cell types (for a review, see Boilleé et al. (23)), which is why many protein changes can be expected to be widespread among the cells in the spinal cord.

**TABLE II**

Functional analysis

Proteins identified were uploaded to the Ingenuity Pathways Knowledge Base and subjected to functional analysis. This identifies functions and diseases in which the proteins have previously been found to be altered. Abbreviations are as in Table I. Up-regulated proteins are shown in bold.

| Global function                              | p value | Proteins                                      |
|----------------------------------------------|---------|-----------------------------------------------|
| Nuclei acid metabolism                       | 0.0003  | DPYSL2, MTAP, NUDT2, VDAC1, IDH1              |
| Neurological disease                         | 0.0005  | MBP, CNP, DPYSL2, VDAC1, SOD2, IDE, UCHL1, MDH1 |
| Cellular assembly and organization           | 0.0006  | CNP, DPYSL2, DSTN, MBP, ANXA5, TST, CPLX1, FIS1, GRB2, CAPNS1, SOD2, UCHL1, DNM1 |
| Cellular function and maintenance            | 0.001   | VDAC1, SOD2                                   |

**TABLE III**

Canonical pathways

Proteins identified were uploaded to the Ingenuity Pathways Knowledge Base and subjected to canonical pathway analysis. This identifies canonical pathways that are overrepresented among the identified proteins in a way that is not likely to be by chance. Abbreviations are as in Table I. Up-regulated proteins are shown in bold. GABA, γ-aminobutyric acid.

| Canonical pathways                   | p value | Proteins                                      |
|--------------------------------------|---------|-----------------------------------------------|
| Pyruvate metabolism                 | 0.0001  | HAGH, ALDH5A1, MDH1, AKR1A1                   |
| Citrate cycle                       | 0.0002  | MDH1, SUCLA2, IDH1                           |
| NRF-2-mediated oxidative stress response | 0.0004 | AKR1A1, SOD2, ERP29, STIP1, ACTG1             |
| GABA receptor signaling             | 0.0006  | ABAT, ALDH5A1, DNM1                          |
| β-Alanine metabolism                | 0.0009  | ABAT, DPYSL2, ALDH5A1                        |
| Propanoate metabolism               | 0.001   | ABAT, ALDH5A1, SUCLA2                        |
| Glycolysis/glucoseogenesis          | 0.005   | ALDH5A1, AKR1A1, PGAM1                       |
| Integrin signaling                  | 0.005   | GRB2, ARPC4, CAPNS1, ACTG1                    |
| Actin cytoskeleton signaling        | 0.006   | GRB2, ARPC4, CSORF10, ACTG1                   |
| Glutamate metabolism                | 0.008   | ABAT, ALDH5A1                                |

Fig. 4. Networks of interacting proteins. Networks of interacting proteins were generated through Ingenuity Pathways Analysis. Proteins identified (displayed as abbreviated gene names) were connected with interaction partners known from the literature and information stored in the Ingenuity Pathways Knowledge Base. Red means up-regulated, and green means down-regulated. Solid lines represent direct interaction, and dashed lines represent indirect interaction. A, network A contained 13 identified proteins and involved top functions in cellular assembly/organization, the cell cycle, and cell death. B, network B contained 12 identified proteins and top functions in nervous system development/function and inflammatory disease. C, network C contained 11 identified proteins and involved top functions in cellular assembly/organization, cellular compromise, and cell morphology. D, network D contained five proteins and top functions in the cell cycle, cancer, and cellular development.
Multiple comparisons of the present magnitude are likely to give many “false positives.” This led us to use a combination of parametric ($p < 0.01$) and multivariate analysis (OPLS-DA $p(\text{corr})p > 0.7$) criteria to reduce this risk. A subset of 53 (Table I) of all the proteins contributing to the differences between ALS/model mice and controls (Fig. 2) was finally selected for evaluation. Most proteins accepted for network analysis (44 of 53) could be divided into four networks of interacting proteins (Fig. 4). This indicates that the proteins identified were not altered in a random way but rather showed a high degree of interaction, which points to functional relationships. Functional analysis revealed changes in pathways for basal cellular functions such as nucleic acid metabolism and cellular organization. One can hypothesize that the mechanisms behind most of these reflect compensatory actions taken from cells that are affected by the disease. The alterations in eight proteins were classified as common with other neurological diseases.

The NRF-2-mediated oxidant stress response was found to be one of the top canonical pathways differentially regulated. All identified proteins were up-regulated. The presence of the covalently linked SOD1 dimers also supports the occurrence of oxidant stress because protein-protein cross-linkages are often generated by oxidants (24). The findings are in accordance with evidence of increased oxidative stress reported previously in the ALS context (25, 26). Furthermore in mice expressing mutant SOD1 and a reporter for NRF-2 (27), activation was found in several cell types in the spinal cord during the course of the disease. However, it should be noted that in cultured cells as well as in motor neurons isolated from rats expressing the stable high level G93A mutant SOD1 (28, 29) NRF-2 and its downstream targets have been reported to be down-regulated.

Proper degradation of protein is essential for cells to function, particularly neurons as they lack the ability to regenerate. The ubiquitination system is one of the major degradation pathways, and here it was found to be differentially regulated. Ubiquitin is reduced, and ubiquitin-conjugating enzyme E2 L3 and ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) are up-regulated in G127X mice (Table I, up-regulated proteins). Oxidation and altered expression of UCH-L1 have been found previously in brain tissue in Alzheimer and Parkinson disease (30). Furthermore intense immunostaining for UCH-L1 was seen in motor neuron somata from both a patient and transgenic mice carrying the G127X SOD1 mutation (7). The COP9 signalosome complex, which is preferentially seen in neurons in the CNS (31), is multifunctional and involved in protein degradation and apoptosis for example (for a review, see Wei et al. (32)). An important function is to inhibit ubiquitination through deneddylation of the large cullin-RING family of ubiquitin E3 ligases. The up-regulation of subunits 4 and 8 suggests that inhibition of ubiquitination of many substrates and hence interference with proper protein degradation might occur.

![Image](https://example.com/image.png)
Validation. A, to validate quantitative data from the DIGE experiment, Western immunoblotting was performed in four new spinal cords each from control, G127X, and G85R mice. A series of proteins was tested, and gels were run at least in triplicate. Down-regulation of FABP and FIS1 was statistically significant in G127X, and up-regulation of isocitrate dehydrogenase (ICD) was statistically significant in G85R mice. HSC70 was significantly up-regulated in both strains. Western blots to detect COP9s8 and Mn-SOD did not reveal any statistically significant changes, but the trend was in the same direction as in the DIGE experiment. DIGE ratios are indicated by a dashed line (in the case of FABP and COP9s8 as means of the two spots) (see Table I). The error bars indicate standard deviations.

B, Western blots for detection of CNP1 did not reveal any sign of down-regulation of protein levels. We therefore performed two-dimensional immunoblotting on 11-cm 3–11 NL strips for isoform resolution. As compared with material from control mice, we could see a change in isoform pattern with one spot shifted toward a lower isoelectric point (arrows).

C, immunohistochemical staining of mouse ventral horn sections from control and G127X SOD1 transgenic mice with antibodies to HSC70, FIS1, COP9s8, and Mn-SOD.
Recently knock-out of components involved in the autophagic pathway for protein degradation has been shown to cause neurodegeneration in mice (33). This points to the neuronal dependence on proper protein degradation and the potentially deleterious consequences of such disturbances.

A few proteomics studies of spinal cord from the highly expressing G93A ALS transgenic mice have been published (34, 35). Lukas et al. (34) performed a study including non-transgenic mice and transgenic mice expressing wild-type human SOD1 (wthSOD1) mice as controls. Using fractionation of spinal cord homogenates and LC-MS/MS, they compared the proteome from different fractions on the basis of functional categories. Many of these categories overlap with ours (e.g. mitochondria and oxidative stress) despite the fact that very different techniques and mouse models were used. In a study comparing G93A mice with wthSOD1-expressing mice using Coomassie-stained 2-D gels and MALDI-TOF, Massig-nan et al. (35) reported a mutant SOD1-specific change in 15 proteins in presymptomatic mice. As in our study, alterations in mitochondrial proteins were found, but none of the specific protein changes are common. This may be due in part to the use of wthSOD1 mice as controls as they have been shown to show pathology, although delayed, similar to that of the ALS-inducing mutants (8, 36).

Several previous studies of ALS have implicated oxidative stress and mitochondrial impairment in mutant SOD1-induced injury. Such changes have also been found in ALS cases lacking SOD1 mutations. In these studies, mostly wild-type-like mutants such as G93A have been used. Such SOD1s have been found to carry out increased catalysis of strong oxidant formation (37, 38) and to artificially overload into mitochondria (10). The truncation mutant G127X is present in minute quantities, lacks SOD activity, and is unlikely to bind potentially oxidant-producing copper ions in vivo (7, 9). Furthermore there is no evidence of association between G127X protein and mitochondria in mice (10). The fact that we found signs of both oxidant stress and mitochondrial disturbances in this study suggests that such effects are not necessarily directly caused by mutant SOD1s but rather could be secondary to attacks on other primary targets. Recent studies in cells lacking Apaf1 are compatible with the occurrence of mutant SOD1-induced feedback loops, which originate in the cytosol and attack mitochondria (39). In conclusion, our findings suggest that interventions with antioxidants, agents that support mitochondria, and agents that preserve or enhance degradation of misfolded proteins might be interesting to test in transgenic ALS models even if such interventions do not affect the primary cytotoxic mechanisms of mutant SOD1s.

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