Identification of Lanthionine Synthase C-like Protein-1 as a Prominent Glutathione Binding Protein Expressed in the Mammalian Central Nervous System†

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ABSTRACT: Proteomic experiments were performed to identify novel glutathione (GSH) binding proteins expressed in the mammalian central nervous system. Bovine brain lysate was affinity purified using an immobilized glutathione−Sepharose column. Proteins that bound the immobilized glutathione were eluted with free glutathione and identified by one- and two-dimensional electrophoresis coupled with mass spectrometric analysis of tryptic fragments. Major proteins purified by this technique were glutathione *S*-transferase-μ (GST-μ) and GST-π and lanthionine synthase C-like protein-1 (LanCL1). LanCL1 is a mammalian homologue of a prokaryotic enzyme responsible for the synthesis of thioether (lanthionine) cross-links within nascent polypeptide chains, yielding macrocyclic proteins with potent microbicidal activity. An antibody against LanCL1 was generated and applied to immunochemical studies of spinal cord tissue from $SOD1^{G93A}$ transgenic mice, a model for amyotrophic lateral sclerosis (ALS), wherein LanCL1 expression was found to be increased at presymptomatic stages of the disease. These results indicate LanCL1 is a glutathione binding protein possibly significant to neurodegenerative disease.

The tripeptide glutathione (γ-glutamylcysteinylglycine; GSH)[1] is the most abundant cellular sulfhydryl-containing molecule, responsible for maintenance of a reducing intracellular environment and for protection against electrophilic toxins (*1*). As part of the classic phase-II detoxification pathway, GSH reacts with xenobiotics through glutathione *S*-transferase (GST) catalyzed reactions to yield water-soluble conjugates that can be more readily excreted. GSH also acts ubiquitously as a reducing equivalent to remove cellular peroxides through reactions mediated by glutathione peroxidase (GPx) (*1*). In recent years, additional roles have been identified for glutathione as a mediator of redox signal transduction and cytoskeletal dynamics. GSH forms mixed disulfides with protein tyrosine phosphatases, transiently inactivating the enzymes and preventing irreversible oxidation of active site cysteines (*2*−*4*). These disulfide adducts are removed through the action of glutaredoxin and thioredoxin (*2*−*4*). Recently, glutathionylation of actin has been recognized as a means of effecting reversible microfilament dissociation (*3*−*7*). Many other proteins have been identified as targets for glutathionylation (*7*, *8*) though generally the significance of these posttranslational alterations remains to be elucidated. Glutathione may have other functions important to mammalian physiology that have yet to be discovered.

As part of our laboratory's ongoing research into redox signal transduction, we performed a proteomics experiment designed to identify novel GSH binding proteins that might be involved with glutathione conjugation reactions. Bovine brain lysate was eluted over a glutathione−Sepharose column wherein the immobilized glutathione was irreversibly cross-linked to the stationary phase through the central sulfur. Bound protein was eluted from the affinity column using free GSH as a competitive ligand. Eluted proteins were analyzed by one- and two-dimensional SDS−polyacrylamide gel electropheresis (2D PAGE) and mass spectrometry-assisted microsequencing techniques.

The three major identified proteins in order of apparent prevalence were GST-μ and GST-π and a third protein, lanthionine synthase C-like protein 1 (LanCL1). LanCL1 is a mammalian homologue of a prokaryotic enzyme that catalyzes macrocyclization of target peptides via an intramolecular thioether linkage. Subsequent studies found LanCL1 to be elevated 3-fold in the spinal cord tissue of presymp-

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[1] Abbreviations: ALS, amyotrophic lateral sclerosis; CNS, central nervous system; ESI-MS/MS, electrospray ionization−tandem mass spectrometry; $SOD1^{G93A}$, glycine → alanine mutated Cu,Zn superoxide dismutase; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione *S*-transferase; hCys, homocysteine; LK, lanthionine ketimine; LanCL1, lanthionine synthase C-like protein-1; LanA, prokaryotic lantibiotic precursor protein; LanB, prokaryotic lanthionine dehydratase; LanC, prokaryotic lanthionine synthase (cyclase); MALDI-TOF-MS, matrix-assisted laser desorption ionization−time of flight−mass spectromertry.

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tomatic $SOD1^{G93A}$ transgenic mice, a murine model for amyotrophic lateral sclerosis (ALS). This is the first report of an endogenous small-molecule ligand for LanCL1 and suggests that the mammalian homologue may engage in sulfur chemistries analogous to the reactions of the prokaryotic LanC enzyme.

MATERIALS AND METHODS

Protein Extraction. Bovine brain was obtained from the Center for Veterinary Health Sciences, Oklahoma State University Veterinary Diagnostic Laboratory, Stillwater, OK. Animals were evaluated at necropsy for conditions other than central nervous system (CNS) disease. None of the animals had clinical signs of CNS disease, and the brain tissue was grossly normal. Approximately 80 g of brain tissue was homogenized in 200 mL of lysis buffer [20 mM Tris, pH 7.4, plus 0.5% Triton X-100 and 20 mM dithiothreitol (DTT)] using a high-capacity commercial blender. Crude homogenate was centrifuged at 10000*g* for 20 min. Supernatant was adjusted to 100 mM NaCl. This homogenate was eluted over a 20 mL immobilized glutathione−fast-flow Sepharose column (Amersham-Pharmacia, Uppsala, Sweden). The column was washed in 10 volumes of 100 mM NaCl and 20 mM Tris, pH 7.4, containing 20 mM DTT, and eluted in the same buffer containing reduced glutathione (Sigma-Aldrich, St. Louis, MO). In specific experiments an isocratic elution of 50 mM GSH was employed while other experiments designed to optimize resolution employed a gradient of 0−50 mM GSH over 5 column volumes. In other experiments to test for glutathione binding specificity, the elution buffer contained L-cysteine rather than GSH. Chromatography was performed on an ÄKTA Explorer 100 (Amersham-Pharmacia Biotech, Uppsala, Sweden) instrument at a 2 mL/min flow rate and ambient temperature. The most highly purified LanCL1 protein sample was concentrated and buffer exchanged with 20 mM Tris/0.1 N NaCl, pH 7.4, using ultrafiltration membranes (10000 MWCO; Pierce Biotechnology, Rockville, IL). LanCL1 concentration in this essentially pure fraction was estimated using a calculated absorptivity coefficient of 72250 cm^{-1} M^{-1} [1.56 $(mg/mL)^{-1}$ cm^{-1}] at 280 nm.

Electrophoresis and Mass Spectrometry. Protein fractions were assessed by 1D SDS−PAGE under reducing conditions with 2% β-mercaptoethanol (βME) using 4−20% gradient gels (ISC Biosciences, Kaysville, UT) stained with BioSafe colloidal Coomassie blue (Bio-Rad, Hercules, CA). Two-dimensional electrophoresis was performed as follows. Protein was dialyzed against 10 mM NaCl using 10000 MWCO Slide-A-Lyzer cassettes (Pierce Biotechnology). Samples were mixed 1:1 with 2× sample buffer [7 M urea, 2 M thiourea, 1% DTT, 1% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 1% Biolyte, pH 3−10, 1% Triton X-100]. Samples were isoelectrically focused using ReadyStrip IPG strips, pH 3−10 (Bio-Rad). Rehydration and IEF were programmed as follows: rehydration at 50 V for 14 h and IEF on a linear ramp at 50−250 V for 15 min, 250−6000 V for 3 h, and 6000 V for 6 h. IEF strips were equilibrated with reducing reagent containing 6 M urea, 2% SDS, 375 mM Tris-HCl, pH 8.8, 20% glycerol, and 100 mM DTT for 15 min and then equilibrated with alkylating reagent containing 6 M urea, 2% SDS, 375 mM Tris-HCl, pH 8.8, 20% glycerol, and 100 mM iodoacetamide for 15 min. Strips were then transferred to Bio-Rad Criterion Tris-HCl/12% polyacrylamide gels for second dimensional electrophoresis.

Gel bands were excised and destained in 50% acetonitrile plus 50 mM aqueous NH_4HCO_3 (destaining solution). Tryptic digestion was executed using an in-gel digestion kit (Pierce Biotechnology) according to the manufacturer's instructions, after reductive alkylation. Briefly, destained gel slices were incubated in 50 mM tris(2-carboxyethyl)phosphine (TCEP) in 25 mM NH_4HCO_3 for 60 min at 60 °C. The TCEP solution was discarded, and the sample was incubated with 100 mM iodoacetamide (IAA) in 25 mM NH_4HCO_3 for 60 min at ambient temperature in the dark. Samples were rinsed with 25 mM destaining solution, shrunk in 100% acetonitrile, and dried at ambient temperature. Dried gel samples were rehydrated with trypsin (10 ng/mL) in 25 mM NH_4HCO_3. Samples were digested overnight at 30 °C with agitation. Peptides were extracted in 1% trifluoroacetic acid (TFA).

MALDI-TOF mass spectrometry was performed on a Voyager-DE PRO mass spectrometer (Applied Biosystems, Foster City, CA) operated in the positive ion reflectron mode. The data were converted into monoisotopic form using Data Explorer software (Applied Biosystems) and then submitted to peptide mass fingerprint analysis utilizing the Mascot search engine (www.matrixscience.com <http://www.matrixscience.com/>), searching National Center for Biotechnology protein databases (NCBInr 20051014) with a mass tolerance of ±0.2 Da. For ESI-MS/MS the sample was concentrated using a POROS-50 column (Applied Biosystems) and eluted with 2.0 μL of 0.5% acetic acid in 1:1 methanol/water into a nanospray glass capillary (Proxeon, Odense, Denmark). ESI-MS/MS was performed using a QSTAR ESI-Quad-TOF mass spectrometer operated under Analyst QS software version 1.0 (Applied Biosystems) with an ion spray voltage of 1400 V. Data were collected in the *i*nformation-*d*ependent *ac*quisition (IDA) mode, which consisted of a MS survey scan over a mass range of 300−2000 amu followed by three MS/MS experiments (collisionally induced fragmentation) of the most abundant +2 and +3 ions present in the mass range of m/z = 300−1200. Data were analyzed with the Mascot MS/MS Ion Search routine to search the NCBInr protein databases with a peptide mass tolerance of 0.2 Da and a fragment mass tolerance of 0.1 Da.

Microplate-Based Competition Assays. A competition binding assay was constructed using commercially available glutathione-modified strip plates [ReactiBind plates (Pierce Biotechnology) containing 10 ng per well of GST binding capacity]. All solutions for this experiment were made in 20 mM Tris/0.1 N NaCl, pH 7.4. Plates were blocked with 5% bovine serum albumin for 1 h at room temperature. Approximately 25 μg per well of semipurified LanCL1 (approximately 90% purity) was incubated for 1 h at room temperature. Strips were rinsed three times and then incubated for 0.5 h with varying concentrations of freshly prepared GSH or oxidized glutathione (GSSG). At this point the released protein solutions were carefully collected from each well. Strips were then rinsed, incubated with anti-LanCL1 (described below; 1:1000 dilution) for 1 h at room temperature, rinsed twice, and incubated an additional 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution). After three final rinses, plates were

developed by addition of 20 mM dichlorofluorescein diacetate (H_2DCF-DA) and 2 mM H_2O_2. Peroxidase activity was assayed at 37 °C in a microplate spectrofluorometer at 485 nm excitation and 538 nm emission.

Antibodies and Immunoblots. A rabbit polyclonal antibody against an internal sequence of LanCL1 (LQQMERGLKSADPRDGTG) was produced and affinity purified by BioSynthesis, Inc. (Lewis, TX). Tissues were lysed in 10 mM sodium acetate, pH 6.5, containing 0.1% Triton X-100, 100 μM sodium orthovanadate, and 1:1000 diluted mammalian protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). After centrifugation, supernatants and resuspended pellet fractions were assayed for total protein by Lowry assay (*9*), adjusted to 2 mg/mL concentration, mixed 1:1 with loading dye (50% glycerol, 10% Tris, 0.01% bromophenol blue, 2% βME), and electrophoresed across 4−20% gradient polyacrylamide gels. Samples were electroblotted onto polyvinylidene difluoride (PVDF) membranes, blocked overnight in 4% BSA, and then probed with anti-LanCL1 at 1:1000 dilution. Blots were developed using chemiluminescence with horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody.

For immunohistology mice were perfused with 4% paraformaldehyde in physiological saline. Spinal cords were removed, postfixed with perfusant for 2 h, cryoprotected in a graded series of 20% glycerol/2% DMSO, and frozen sectioned into 5 μm slices. Sections were probed with anti-LanCL1 and phycoerythrin-conjugated anti-rabbit secondary IgG. For immunocytology, cell cultures were grown on chambered coverslips (Fisher, St. Louis, MO) and fixed in 4% paraformaldehyde in phenol red-free Dulbecco's modified essential medium (DMEM). Fixed cells were rinsed with phosphate-buffered saline, labeled with anti-LanCL1 (1:1000) and fluorescein isothiocyanate (FITC) conjugated secondary anti-rabbit IgG (10 g/mL). Controls were performed routinely wherein the primary antibody was omitted.

Transgenic Mice. Mice expressing high copy numbers of human mutant $SOD1^{G93A}$ were obtained from Jackson Laboratories [Bar Harbor, ME; strain designation B6SJL-TgN-(SOD1 G93A)-1-Gur or $SOD1^{G93A}$]. Transgenic mice were maintained in the hemizygous state by mating $SOD1^{G93A}$ males with B6SJL-TGN females. Animals were housed in the Oklahoma Medical Research Foundation Laboratory Animal Resource Center.

Cell Cultures. Human LN229 and rat C6 glioma cells and NSC-34 motor neuron-like cells were grown in Dulbeco's modified essential medium (DMEM) plus 5% fetal bovine serum (FBS) and 1% penicillin plus 1% streptomycin (Pen/Strep). Mouse EOC-20 microglia were grown in DMEM containing 20% L929 fibroblast-supplemented medium. L929 fibroblasts were grown in RPMI plus 10% FBS and 1% Pen/Strep. Dopaminergic SHSY5Y neuroblastoma cells were grown in 50% DMEM/50% F12 medium plus 1% Pen/Strep. All cell lines were obtained from the American Type Culture Collection (Gaithersville, MD) except for NSC34 cells, which were a gift from Dr. Neil Cashman of the University of Toronto, Canada.

RESULTS

A number of brain proteins bound to the immobilized glutathione column in a fashion amenable to elution with free GSH (Figures 1 and 2). Chromatographic fractions were assessed for protein distribution by 1D and 2D PAGE (Figures 1 and 2). Excised bands from 1D and 2D gels were individually subjected to tryptic digestion. MALDI-TOF mass fingerprint analysis of the tryptic digests produced significant MOWSE score identifications for three major protein bands as GST-μ (MOWSE = 164), GST-π (MOWSE = 80), and LanCL1 (MOWSE = 128) (Figure 3). The putative LanCL1 migrated very close to its predicted molecular mass of 45 kDa and isoelectric point of 7.9 (Figures 1, 2, and 4).

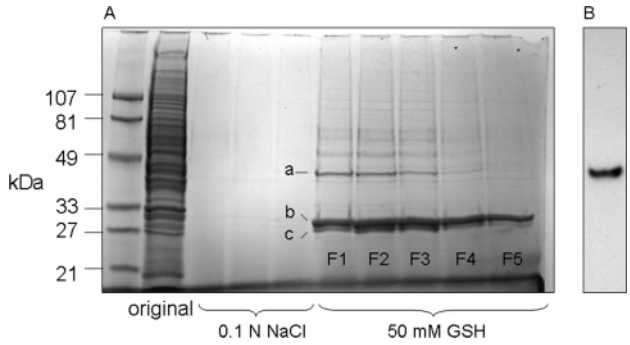


FIGURE 1: Fractionation of bovine brain proteins across an immobilized glutathione column. (A) Coomassie blue-stained, 1D SDS−PAGE gel illustrating the distribution of bovine brain proteins before passage over a glutathione−Sepharose column (original) and within fractions eluted from the column with saline buffer (100 mM NaCl, 20 mM Tris, pH 7.4, plus 20 mM DTT) or 50 mM GSH in the same buffer. Protein bands labeled a, b, and c were identified by tryptic digestion and MALDI-TOF-MS as LanCL1, GST-μ, and GST-π, respectively. The first lane left of the original protein lysate contained molecular mass markers. (B) Western blot of pooled fractions F1−F3 using a polyclonal antibody raised against a polypeptide sequence within LanCL1.

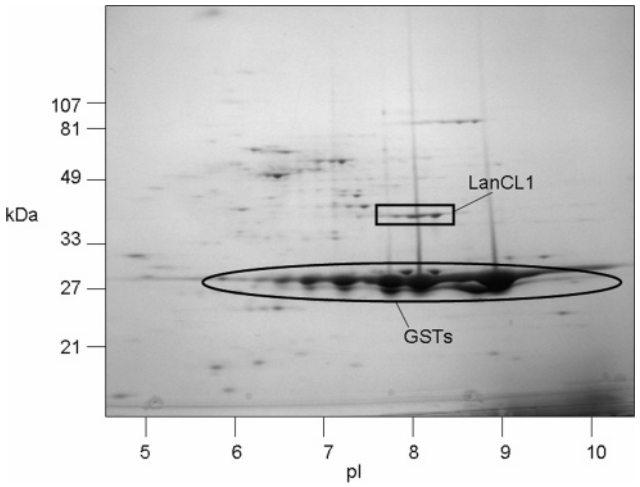
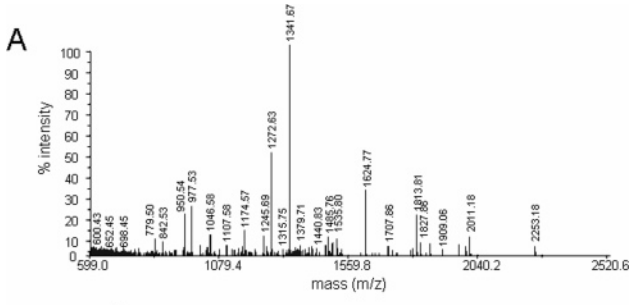


FIGURE 2: Fractionation of bovine brain proteins across an immobilized glutathione column. Samples corresponding to fractions F1−F3 in Figure 1 were separated by 2D PAGE. Bands identified by MALDI-TOF-MS as GST and LanCL1 are indicated.

Fractions containing LanCL1 were pooled and subjected to a second fractionation over an immobilized glutathione column, this time eluted with a 0−50 mM GSH ramp over 5 column volumes (Figure 4). The protein identified by MALDI-TOF as LanCL1 eluted at approximately 20−30 mM GSH whereas GSTs eluted at 30−50 mM GSH (Figure 4), suggesting a relatively high affinity of LanCL1 for immobilized glutathione. A fraction of almost homogeneous protein was thus obtained at a yield of 1.2 mg from



C

peptide	m/z observed	m/z predicted	sequence
1-15	1827.8615	1827.8522	-.MAQRAFPNPYADYNK.S
5-15	1341.6730	1341.6159	R.AFPNPYADYNK.S
28-35	977.5347	977.5274	R.LTPEFSQR.L
28-39	1475.8040	1475.7892	R.LTPEFSQRLTNK.I
42-49	1046.5785	1046.5226	R.ELLQQMER.G
50-57	842.5322	842.4843	R.GLKSADPR.D
137-142	779.5004	779.4886	R.LIHLNK.I
240-255	1970.1424	1970.0567	K.LHSLVKPSVDYVCQLK.F
351-364	1813.8145	1813.7348	K.FAEWCLEYGEHGCR.T
392-399	950.5364	950.5021	K.ARFPAFEL.-

FIGURE 3: MALDI-TOF-MS fingerprint profile for the trypsin-digested 43 kDa protein band identified in this study as LanCL1. (A) Representative spectrum from one typical in-gel digest of fractions illustrated in Figures 1 and 2. (B) Probability distribution plot for matched proteins from a Mascot search of the NCBInr database. (C) Matched peptide sequences corresponding to major peaks labeled in (A) that were not observed in sample blanks.

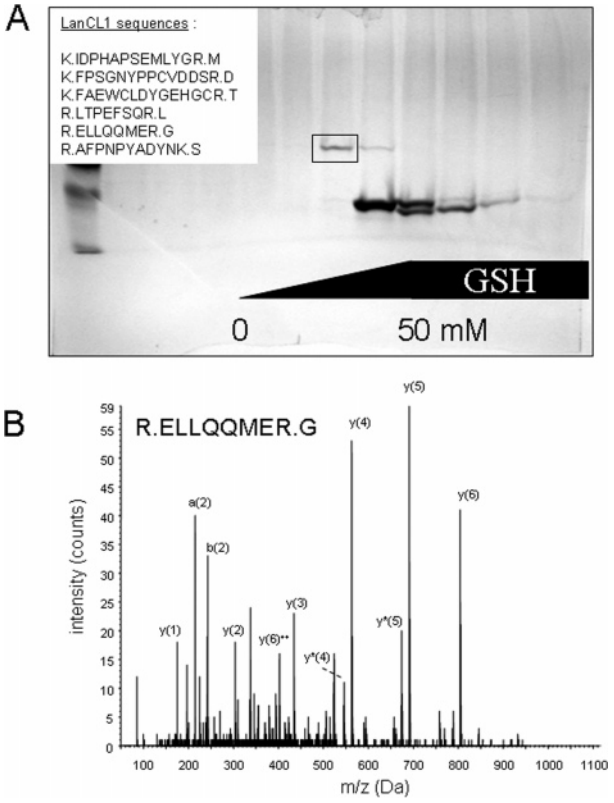


FIGURE 4: Refined fractionation of LanCL1 from GST and ESI-MS/MS sequence analysis. (A) Pooled fractions corresponding to F1−F3 from Figure 1 were repurified by glutathione affinity chromatography utilizing a GSH concentration ramp. Protein distribution in the resulting fractions was visualized by 1D SDS−PAGE. Each lane represents a separate column fraction; the leftmost lane contained molecular mass markers. The Coomassie blue-stained band (box) corresponding to suspected LanCL1 was excised, trypsin digested, and sequenced by ESI-MS/MS. The listed sequences of LanCL1 tryptic peptides were derived from ESI-MS/MS data, and the highly purified LanCL1 fraction was used for further experimentation as described in the text. (B) Representative ESI-MS/MS spectrum of one identified tryptic peptide, ELLQQMER, with labeled peaks corresponding to major anticipated fragment ions. The average difference ± SD between observed and anticipated *m*/*z* values for the indicated peaks was 0.041 ± 0.001 Da.

approximately 1 g of total brain protein in the original lysate (Figure 4). This protein band was excised, trypsin-digested in the gel slice, and subjected to electrospray ionization−mass spectrometric (ESI-MS/MS) microsequence analysis. From this sample, several peptides were identified corresponding to specific LanCL1 tryptic fragments including sequences not previously identified in the MALDI-TOF-MS experiment (Figure 4). The identified tryptic fragments from both MS experiments totaled approximately 27% coverage of the LanCL1 polypeptide.

To date, we have been unable to ascribe specific enzymatic activities to LanCL1. The most highly purified protein fraction had no discernible GST activity using chlorodinitrobenzene (CDNB) as a substrate (data not shown). The protein likewise possessed no discernible peroxidase activity using dichlorofluorescein and H_2O_2 as substrates (data not shown). The protein did not catalyze GSH or GSSG attachment to actin in vitro, nor could the protein catalyze removal of disulfide-bound glutathione from chemically glutathionylated actin in vitro even when excess free GSH, NAD^+, NADH, or NADPH was included in the reaction mixture (data not shown). Addition of 1 mM Zn^{2+} did not encourage any of the aforementioned activities (data not shown).

A rabbit polyclonal antibody was raised against a predicted immunogenic and specific target sequence within LanCL1 and was affinity-purified using immobilized polypeptide antigen. When this antibody was used to immunoblot chromatographic fractions containing LanCL1, GST, and other proteins eluted from the glutathione column, only the band corresponding to LanCL1 was recognized by the antibody (Figure 1). No immunoreactivity was inherent to preimmune rabbit serum (not shown). The polyclonal antibody also recognized nondenatured LanCL1 bound to glutathione-modified microplates (Figure 5). Taking advantage of this feature, we next performed competition binding experiments to assess the relative affinity of LanCL1 for GSH versus GSSG. Semipurified LanCL1 was allowed to bind GS-modified microplates and then was competitively eluted with either free GSH or GSSG. Residual LanCL1 was detected by incubation of the plates with anti-LanCL1 and peroxidase-conjugated secondary antibodies. LanCL1 was liberated from the microplate surface by submicromolar concentrations of either free GSH or GSSG with GSSG being approximately twice as effective (Figure 5). These results indicate that LanCL1 binds GSH and GSSG with very similar affinities. The apparent K_d in both cases was below 1 μM. This value is approximately 1 log lower than the K_d of GST for glutathione and close to the K_d of GST for some of its GS-conjugated products (*10*).

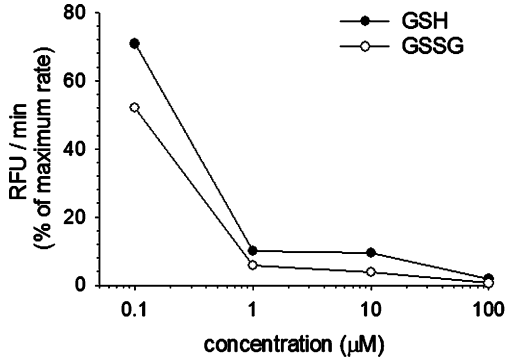


FIGURE 5: LanCL1 binds GSH and GSSG with similar affinity. LanCL1 from semipurified fractions (approximately 90% LanCL1) was allowed to bind glutathione-modified microplate surfaces and then was competitively eluted with free GSH or GSSG at the indicated concentrations. Residual LanCL1 remaining bound to the plate was detected by indirect immunoperoxidase labeling with spectrofluorometric detection of the peroxidase reaction. The maximum peroxidase rate (100% scale) was observed at zero GSH or GSSG, and all other data were normalized to this value.

Unfortunately, experiments designed to extract a precise K_d using BiaCore surface plasmon resonance were unsuccessful for technical reasons. The estimation by BiaCore requires at least five dilutions of enzyme when the ligand is bound to the BiaCore chip surface. We found that concentrating LanCL1 above approximately 0.5 mg/mL resulted in formation of protein precipitates and SDS-insoluble aggregates (data not shown). LanCL1 bound specifically to GSH or GSSG-modified BiaCore surfaces at low concentrations of protein in the flow stream but lost ligand-specific binding above this concentration (data not shown), probably due to protein aggregation. More accurate binding constants must await further experiments such as equilibrium dialysis, which we are not able to perform at this time due to time constraints and limited availability of highly purified LanCL1.

In Western blots of bovine, mouse, and human brain and mouse spinal cord, the anti-LanCL1 recognized a prominent band that comigrated with authentic purified LanCL1 (Figure 6). In better resolved Western blots LanCL1 migrated as a major band corresponding to authentic purified bovine LanCL1, plus several minor bands of ±3 kDa molecular mass that might indicate significant degrees of posttranslational regulation in vivo (Figure 6). A minor band of approximately 8 kDa higher molecular mass, in the anticipated mass range of LanCL2, also was observed (Figure 6). The intensity of this band generally correlated with that of the primary LanCL1 band. Because LanCL2 contains a sequence (LQQMEEGLKTADPHDCSA) similar to that of the antigenic peptide, some cross-reactivity of the polyclonal antibody is possible. Alternatively, this higher molecular mass band might indicate ubiquitinylation, which would add approximately 8 kDa to LanCL1. All bands recognized by anti-LanCL1 could be effectively outcompeted with antigenic peptide (data not shown).

Our laboratory has a particular interest in the biology of neurodegenerative diseases especially amyotrophic lateral sclerosis (ALS). Our preferred model for studying protein expression changes in ALS is the SOD1^{G93A} mouse, which ubiquitously expresses a mutant human Cu,Zn superoxide dismutase enzyme responsible for a heritable form of ALS. In these animals, protein oxidation and neuroinflammatory

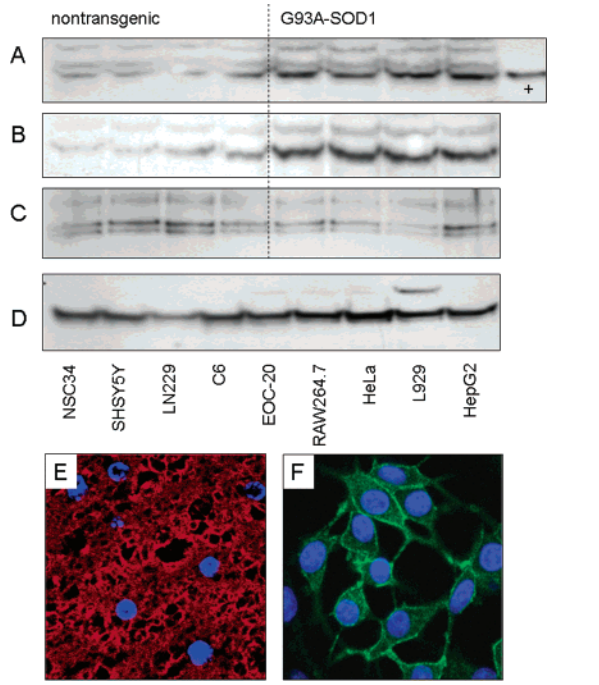


FIGURE 6: LanCL1 was expressed in CNS cells as well as other cell types and elevated in presymptomatic spinal cord lysates obtained from SOD1^{G93A} transgenic mice. (A−C) Immunoblots of spinal cord lysates from 75-day-old (A, B) and 120-day-old (C) SOD1^{G93A} or matched nontransgenic mice, probed with anti-LanCL1. Each lane represents 10 μg of protein from individual mice of the indicated age. The positive control (+) is semipurified LanCL1 from bovine brain. (D) Western blots were performed using cell lysates from several cell lines representing neuron-like cells (NSC-34 and SHSY5Y cells), glia (LN229 and C6 glioma), microglia or macrophage (EOC-20 and RAW264.7), fibroblasts (L929), hepatocytes (HepG2), and epithelial cells (HeLa). Each lane represents 10 μg of total protein. (E) Histochemical stain of a nontransgenic mouse spinal cord section stained for LanCL1 (red) and DAPI-stained for nuclei (blue). (F) NSC-34 motor neuron-like cells stained for LanCL1 (green) and DAPI-stained to highlight nuclei (blue).

gene induction occur in an accelerating fashion from approximately 80 days until death at 120−130 days (*11*). Significant motor neuron loss and frank paralysis occurs between 100 and 120 days. Accordingly, we undertook to determine whether LanCL1 levels might be affected in the SOD1^{G93A} mouse at disease stages prior to and following frank paralytic disease. The anti-LanCL1 polyclonal antibody was blotted against spinal cord lysates from 75- or 120-day-old SOD1^{G93A} mice. LanCL1-immunoreactive protein was detected in cytosolic fractions but not nuclear fractions or membrane fractions (data not shown). LanCL1 was elevated 3-fold at 75 days (late presymptomatic stage, prior to loss of spinal cord motor neurons; $p < 0.01$ by two-tailed *t*-test; Figure 6A,B) and decreased by approximately 50% at 120 days, a time point where significant motor neuron loss is known to occur; however, this end-stage decrease was not statistically significant (Figure 6C). The minor "satellite bands" recognized by anti-LanCL1 correlated very well with the major LanCL1 protein band as a function of disease state (Figure 6A−C). Immunoblot analysis of spinal cord lysates from mice overexpressing wild-type human SOD1 (*10*) indicated no alteration in LanCL1 expression (data not shown).

Initial studies were undertaken to explore LanCL1 protein expression among different cell types relevant to neurobiology, in order to identify cell lines that might prove useful in future studies of LanCL1 function and in the hope of uncovering clues regarding any cell type-specific roles for this novel protein. Immunohistology of mouse spinal cord sections indicated expression of LanCL1 in neurons and surrounding neuropil (Figure 6F). In cell culture experiments prominent LanCL1 expression was observed in NSC-34 motor neuron-like cells [derived from the fusion of embryonic mouse motor neurons with neuroblastoma cells (*12*)] and SHSY5Y dopaminergic neuroblastoma cells (Figure 6D). Several glioma cell lines including C6 glioma and LN229 glioblastoma cells likewise expressed LanCL1 as did macrophage, microglia, epithelial, and fibroblast cell lines (Figure 6D). The LanCL1 + 8 kDa band was particularly prominent in L292 fibroblasts. Additionally, strongly immunoreactive bands <25 kDa were observed in lysates from EOC-20 and L292 fibroblasts (data not shown) probably indicative of significant LanCL1 turnover in these cells. Confocal microscopy of NSC-34 cells suggested a predominant localization peripheral to the surface membrane (Figure 6F), consistent with a previous identification of LanCL1 as a peripheral membrane protein in erythrocytes (*13*).

DISCUSSION

We have identified a glutathione binding ability inherent to LanCL1, a protein of unknown function previously found to be expressed at highest levels in mammalian central nervous system tissue. We have been unable to demonstrate a specific glutathione-dependent reaction catalyzed by semipurified LanCL1 preparations. These preparations appear to lack notable glutathione *S*-transferase-like activity, peroxidase activity, or glutaredoxin-like activity. These activity data must be interpreted cautiously and considered provisional due to the possibility that any enzyme activity inherent to LanCL1 might have been lost due to protein denaturation during purification or might require other enzymes, cofactors, and substrates that were not considered during the course of our experiments. The presence of conserved cysteine and histidine residues in LanCL1 analogous to active site residues of prokaryotic LanC (discussed below), combined with the observed affinity of LanCL1 for glutathione, suggests that LanCL1 might catalyze alkylation reactions between GSH and as yet unidentified electrophiles.

LanCL1 has been identified previously by Prohaska and colleagues as a mammalian homologue of bacterial lanthionine synthase-C (LanC) enzymes whose mRNA is expressed most prominently in brain (*13*−*17*). Prior to our studies no biochemical activities, substrates, or small molecule ligands had been reported for the mammalian protein although two protein binding partners were known. LanCL1 was first isolated as a peripheral membrane protein and possible G-protein-coupled receptor that bound to lipid rafts and associated with the erythrocyte protein stomatin (band 7.2), which is implicated in the hemolytic disease hereditary overhydrated stamatocytosis (*13*, *15*−*16*). More recent studies by Blisnick and colleagues have identified LanCL1 as a binding partner for the protein PfSBP1, an integral membrane protein expressed by the parasite *Plasmodium falciparum* (*18*). PfSBP1 is a component of Maurer's clefts, which are parasite-derived structures that facilitate delivery of parasite proteins to host cell surface cytoadherence complexes. Our immunochemical investigations suggest a similar localization of LanCL1 with surface membranes of motor neuron-like and glial cells as has previously been found for erythrocytic cell membranes. We do however note a rather ubiquitous expression of LanCL1 protein in multiple common cell lines commonly used to investigate cell biology of both the CNS and peripheral organ systems.

Although LanCL1 was initially proposed to be a G-protein-coupled receptor, this was later found not to be the case, and a second LanCL1 homologue, LanCL2, was reported (*17*). Like LanCL1, LanCL2 appears to be widely expressed with highest levels found in the central nervous system and testes (*14*−*17*). LanCL2 is a bystander gene that is coamplified and overexpressed with epidermal growth factor receptor in approximately 20% of all glioblastomas (*19*). LanCL2 expression increases cellular sensitivity to adriamycin, apparently by encouraging transcriptional suppression of multidrug-resistance protein-1 (MDR1)/P-glycoprotein (*19*). In our proteomics investigation we found no evidence for LanCL2 binding to glutathione; however, this lack of recovery for LanCL2 must be interpreted cautiously for several reasons. First, LanCL2 may not be as highly expressed in the CNS as is LanCL1, thus resulting in low efficiency of capture by the glutathione column. Second, recent findings indicate that LanCL2 is myristoylated upon its unique N-terminus and also is associated with nuclear fractions where it may coregulate MDR1/P-glycoprotein expression (*20*). Our extraction technique may not have been appropriate to recover such posttranslationally modified, membrane-anchored or perhaps even chromatin-associated LanCL2 from post-mortem bovine brain tissue. Finally, posttranslational modifications may affect LanCL1/2 affinity for glutathione. Similar reasoning may explain why the antibody against LanCL1 detected multiple protein bands of similar molecular mass in CNS tissue lysates whereas the immobilized glutathione column only captured a single protein.

The LanCL1 polypeptide is approximately 30% homologous to prokaryotic LanC expressed by many Gram-positive bacteria (*16*). The degree of homology between LanCL1 and particular LanC polypeptides is similar in magnitude to the homology among different LanC polypeptides from different bacterial species, which can vary substantially in terms of primary sequence (*21*). Specific motifs within LanCL1 are highly conserved relative to LanC proteins, including a WC-X-G-X-PGV-X_{38}-I/L-CHG motif (where X = any amino acid) containing two cysteine residues located 46 amino acids apart near the C-terminal region of both LanCL1 and all known bacterial LanC enzymes (*21*). LanCL1 shares no significant homology to other known GSH-utilizing mammalian enzymes, including GSTs and glutaredoxin. Thus LanCL1 likely represents a new class of glutathione binding protein.

Suggestions of possible LanCL1 functions may be found in the known biochemistry of prokaryotic LanC enzymes. These bacterial zinc-containing enzymes act in concert with specific dehydratases to facilitate intramolecular conjugation of cysteine to serine or threonine residues, producing macrocyclic thioether (monosulfide) containing peptides called "lantibiotics" that possess potent antibiotic activity (*21*) (Figure 7). The bacterial dehydratases, or LanB enzymes,

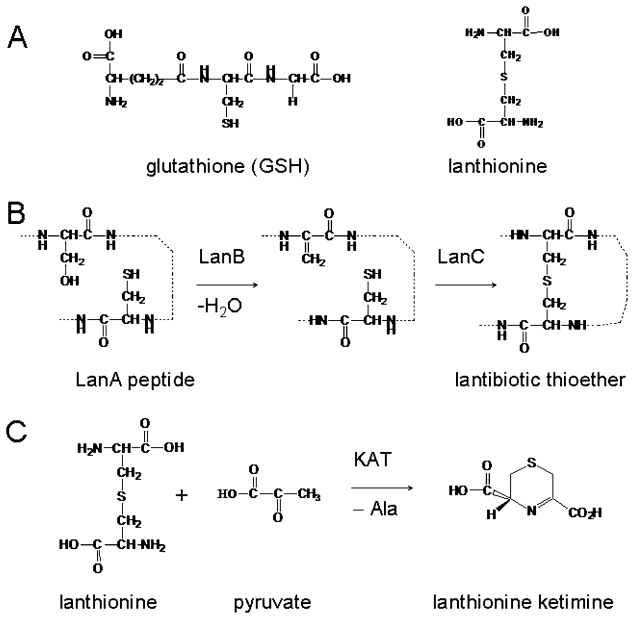


FIGURE 7: Structures and reactions discussed in the text. (A) Structures of reduced glutathione (GSH) and lanthionine. (B) Lantibiotic synthesis scheme sequentially catalyzed by prokaryotic LanB dehydratase and LanC cyclase enzymes. (C) Biosynthesis of the neurochemical lanthionine ketimine from the kynurenine amino transferase (KAT) catalyzed reaction of pyruvate with lanthionine.

remove water from specific serine or threonine residues within LanA precursor polypeptides. This yields alkene side chains dehydroalanine or dehydrobutyrine, respectively (*21*) (Figure 7). LanC then catalyzes the electrophilic attack of a distal Cys upon these modified side chains, yielding a Cys-S-Cys monosulfide (i.e., lanthionine) cross-link (Figure 7). The two aforementioned cysteine residues that reside near the C-terminus of both LanC and LanCL1 are thought to coordinate Zn^{2+} in LanC enzymes and are obligatory for LanC catalysis of thiol alkylations (*21*−*22*). Identification of an inherent affinity of LanCL1 for GSH suggests that the mammalian homologue may catalyze similar sulfur-dependent reactions using GSH as one substrate. This suggestion is not completely unprecedented, as Li et al. previously noted that the crystal structure of nisin (a bacterial LanC) contained folding similarities to mammalian farnesyl transferases, suggesting that mammalian homologues might act upon a protein cysteine substrate (*23*). Our data presented in this report are the first to identify one such specific mammalian cysteine-containing ligand.

The presence of macrocyclic lanthionine-containing polypeptides in mammals, similar to the lantibiotic structures, has not been documented. Such structures are thought unlikely because mammals do not possess homologues to prokaryotic dehydratases that are necessary for initial dehydration of Ser and Thr. Nonetheless, Ser and Thr are prone to base-catalyzed dehydration especially when phosphorylated, and the predicted dehydroalanine and dehydrobutyrine products have been detected in cataractogenous human lens tissue (*24*). In support for the presence of protein-bound lanthionine, Lenitsky and Legrande recently presented evidence for a nonreducible glutathione cross-link inherent to proteins within aged mammalian lens (*25*). These researchers inferred the presence of this modification as an epitope recognized by an anti-glutathione antibody that could not be removed by thiol reducing agents (*26*). The implication of these data is that some fraction of protein glutathionylation likely occurs through a thioether or lanthionine linkage rather than the classical, reducible disulfide configuration. Further research is required to determine whether LanCL1 may be implicated in the formation or removal of such lanthionine−cross-linked glutathione−protein adducts.

The unusual amino acid lanthionine has been detected in bovine brain as a free molecule where generally it is thought to form as a side reaction of the transulfuration pathway (*26*). Normally the transulfuration enzyme cystathionine β-synthase conjugates Ser with homocysteine (hCys, a byproduct of methyl transfer reactions) to yield cystathionine. Cystathionine then is recycled to free cysteine by means of cystathionine γ-lyase (CγL). In this way the brain and other tissues salvage reduced sulfur while supporting biological methylations. If Cys were to substitute for hCys in the CβS-catalyzed reaction, the resulting product would be lanthionine (*26*). While such an origin for brain lanthionine is highly plausible, direct proof for this pathway is lacking. It is conceivable that LanCL1 might function within the transulfuration pathway or adjacent to this conduit to initiate lanthionine synthesis from GSH. The purpose of such hypothetical transformation is not readily apparent because there is no known biological function for free lanthionine.

A role for free lanthionine has been postulated by Cavallini and colleagues (*27*−*29*), who find that lanthionine reacts with pyruvate through a reaction catalyzed by kynurenine aminotransferase (KAT/cysteine conjugate β-lyase/glutamine transpeptidase K) (*26*, *28*) (Figure 7). The resulting product is a cyclic lanthionine ketimine (LK, Figure 7) that Cavallini was able to detect in mammalian brain (*27*−*29*) but for which no biological activity has yet been ascribed. Nonetheless, Cavallini reported that lanthionine ketimine binds cortical synaptosomes with nanomolar affinity, suggesting specific receptor−ligand interactions (*30*). It is conceivable that lanthionine exists not as a metabolic waste product but as a neurochemical metabolite with specific but currently unappreciated functions. The presence of glutathione binding activity inherent to mammalian lanthionine synthase-C-like protein suggests that further investigation into the source and dispensation of brain lanthionine might prove to be a fruitful strategy for future neurochemical research.

Our finding that LanCL1 is upregulated in presymptomatic phases of disease in a murine model for amyotrophic lateral sclerosis suggests that this mouse and this disease might provide an experimental setting in which to conduct future research regarding LanCL1, lanthionine, and derived products. Protein upregulation might suggest a protective or compensatory function in the time frame prior to protein oxidation, neuroinflammatory acceleration, and motor neuron death (*11*). The late-stage decrease of protein in the $SOD1^{G93A}$ mouse spinal cord likely is a consequence of neuron loss because Prohaska's group found a predominately neuronal pattern of LanCL1 mRNA expression in rodents detected by in situ hybridization (*16*), and we find abundant protein expression in cultured neuron-like cells (this work). If LanCL1 provides a support function for mammalian neurons, elucidation of this role could suggest new strategies to mitigate neurodegeneration in ALS and other diseases of the central nervous system. It may be noteworthy that Pericak-Vance and colleagues report a genetic locus for early Alzheimer's disease susceptibility on a region of chromo-

some 2q34 within several centimorgans of the *LanCL1* gene (*31*). Considering the well-established involvement of oxidative stress and protein modification with Alzheimer's disease, LanCL1 cannot be discounted as a candidate gene in this region. The possible involvement of LanCL1 with neurodegenerative etiologies is currently being explored in our laboratory.

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