

Rapid Discovery of Putative Protein Biomarkers of Traumatic Brain Injury by SDS–PAGE–Capillary Liquid Chromatography–Tandem Mass Spectrometry

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ABSTRACT

We report the rapid discovery of putative protein biomarkers of traumatic brain injury (TBI) by SDS–PAGE–capillary liquid chromatography–tandem mass spectrometry (SDS–PAGE–Capillary LC–MS²). Ipsilateral hippocampus (IH) samples were collected from naive rats and rats subjected to controlled cortical impact (a rodent model of TBI). Protein database searching with 15,558 uninterpreted MS² spectra, collected in 3 days via data-dependent capillary LC–MS² of pooled cyanine dye-labeled samples separated by SDS–PAGE, identified more than 306 unique proteins. Differential proteomic analysis revealed differences in protein sequence coverage for 170 mammalian proteins (57 in naive only, 74 in injured only, and 39 of 64 in both), suggesting these are putative biomarkers of TBI. Confidence in our results was obtained by the presence of several known biomarkers of TBI (including α II-spectrin, brain creatine kinase, and neuron-specific enolase) in our data set. These results show that SDS–PAGE prior to *in vitro* proteolysis and capillary LC–MS² is a promising strategy for the rapid discovery of putative protein biomarkers associated with a specific physiological state (i.e., TBI) without *a priori* knowledge of the molecules involved.

Key words: controlled cortical impact (CCI); differential in-gel electrophoresis (DIGE); sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE); tandem mass spectrometry (MS²); traumatic brain injury (TBI)

INTRODUCTION

TRAUMATIC BRAIN INJURY (TBI), defined as brain damage due to mechanical force applied to the head, has an incidence of approximately 2 million persons annually in the United States with an annual economic cost

of \$25 billion. Thus, accurate diagnosis following TBI is crucial for appropriate clinical management of TBI patients and for reducing costs. Current assessment tools of TBI include computed tomography and magnetic resonance imaging. Despite the accuracy of these techniques, TBI survivors suffer long-term impairment due to late di-

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agnosis and unguided clinical management. Therefore, increased interest in the discovery of biomarkers that are indicative of injury severity and anatomical localization has been realized in recent years.

Several laboratories have examined a number of biological molecules in cerebral spinal fluid (CSF) and blood from TBI patients in an effort to discover TBI-specific molecules (Pike et al., 2002; Varma et al., 2003; Zemlan et al., 2002; Berger et al., 2002; Raabe et al., 2003). For example, our laboratory reported the discovery of non-erythroid α II-spectrin and its protease-specific degradation products as biomarkers of TBI (Pike et al., 2002). However, a major limitation of currently described biomarkers is a lack of TBI specificity and a poor understanding of the biochemical mechanisms of brain trauma. Thus, the discovery of novel protein biomarkers of TBI that serve as reliable indicators of injury severity would be highly beneficial for predicting outcome and managing patients (Denslow et al., 2003). Moreover, novel biomarkers of TBI, particularly neurodegenerative and neuroprotective proteins, provide insights on pathophysiology and may serve as therapeutic targets for various neurological diseases.

Rapid discovery of protein biomarkers in complex samples by state-of-the-art mass spectrometry methods, capable of identifying thousands of proteins in a single sample by protease-specific peptide sequences, is precluded by several limitations. "Shotgun" capillary liquid chromatography (LC)-tandem mass spectrometry (MS²) methods (McDonald and Yates, 2002) require extended analysis times for each sample (days) and information about post-translational modifications (PTMs), particularly protein degradation, is often lost during *in vitro* proteolysis (e.g., trypsination). Liquid-phase protein separation (e.g., 2D gels and LC-LC) prior to *in vitro* proteolysis and capillary LC-MS², preserves more information about PTMs, but can require 10–100-fold more sample and even greater analysis times for complete characterization (weeks). Reproducible replicate analysis, required for preliminary biomarker validation, and limited resources (e.g., mass spectrometer time) further compound these problems.

Recently, the large dynamic range and high quantum yield of cyanine dye-labeled proteins were combined with 2D gels in order to improve gel-to-gel reproducibility and reduce analysis time via sample multiplexing (Gharbi et al., 2002; Leimgruber et al., 2002; Macdonald et al., 2001; Tonge et al., 2001). This technique, differential in-gel electrophoresis (DIGE), provides quantitative information complementary to isotope coded affinity tag (ICAT)-capillary LC-MS² approaches (Gygi et al., 1999), while preserving more information about PTMs. DIGE also provides a reduction in analysis

time because only gel spots with a significant difference in the ratio of their fluorescence signals need to be targeted for protein identification by mass spectrometry (Gharbi et al., 2002; Kernec et al., 2001; Shaw et al., 2003; Tonge et al., 2001; Yan et al., 2002). However, poorly resolved proteins elude identification, while well-resolved, multiply labeled, proteins produce redundant identifications. Given our emphasis on rapid analysis, rather than more comprehensive characterization, we selected the limited resolving power of SDS-PAGE as an effective means to reduce redundant identifications and accelerate the discovery of putative protein biomarkers.

In this report, we describe the application of a novel differential analysis strategy, SDS-PAGE-capillary liquid chromatography-tandem mass spectrometry (SDS-PAGE-Capillary LC-MS²), to the discovery of putative protein biomarkers of TBI in hippocampus tissue. Herein, protein database searching of uninterpreted MS² spectra, collected via data-dependent capillary LC-MS² of pooled cyanine dye-labeled samples separated by SDS-PAGE, was combined with differential proteomic analysis. We hypothesized that a subset of putative protein biomarkers of TBI, including some with PTMs, would be rapidly revealed by comparing the protein sequence coverage of naive and injured samples.

MATERIALS AND METHODS

Chemicals and Reagents

The chemicals and reagents used are described elsewhere (Haskins et al., 2001). Tryptic digests were purchased from Michrom Bioresources (Auburn, CA) for use as quality control standards. Cyanine dye labeling reagents were purchased from Amersham Biosciences (Piscataway, NJ).

Controlled Cortical Impact

The controlled cortical impact (CCI) device used to model TBI in male Sprague-Dawley rats was described in detail elsewhere (Pike et al., 2002). The magnitude of injury used in these studies produces significant cortical contusions and less overt injury that often extends into the region of the hippocampus (Posmantur et al., 1997; Dixon et al., 1991). Although overt hippocampal damage is not usually associated with this model, there is evidence of increased pathological calpain-mediated proteolysis in the hippocampus following cortical impact injury (Newcomb et al., 1997). Cortical impact injury is usually associated with intraparenchymal hemorrhage and dural disruption, but extensive subdural hemorrhage is not a primary feature of this model. The

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adult rats were anesthetized with 4% isoflurane in a carrier gas of 1:1 O₂/N₂O (4 min) followed by maintenance anesthesia of 2.5% isoflurane in the same carrier gas. Core body temperature was monitored continuously by a rectal thermistor probe and maintained at 37 ± 1°C by placing an adjustable temperature controlled heating pad beneath the rats. Animals were mounted in a stereotactic frame in a prone position and secured by ear and incisor bars. A midline cranial incision was made, the soft tissues were reflected, and a unilateral (ipsilateral to site of impact) craniotomy (7 mm diameter) was performed adjacent to the central suture, midway between bregma and lambda. The dura mater was kept intact over the cortex. Brain trauma was produced by impacting the right cortex (ipsilateral cortex) with a 5-mm-diameter aluminum impactor tip (housed in a pneumatic cylinder) at a velocity of 3.5 m/sec with a 1.6-mm compression and 150-msec dwell time (compression duration). Velocity was controlled by adjusting the pressure (compressed N₂) supplied to the pneumatic cylinder. Velocity and dwell time were measured by a linear velocity displacement transducer (Lucas Shaevitz™ model 500 HR, Detroit, MI) that produced an analog signal that was recorded by a storage-trace oscilloscope (BK Precision, model 2522B, Placentia, CA). At 48 h post-injury, the animals were anesthetized with 4% isoflurane in a carrier gas of 1:1 O₂/N₂O (4 min) and subsequently sacrificed by decapitation. Hippocampus samples were rapidly dissected, washed with saline solution, snap-frozen in liquid nitrogen, and stored at -80°C until further processing. Naive animals underwent identical surgical procedures but did not receive an impact injury. Appropriate pre- and post-injury management was maintained to insure compliance with guidelines set forth by the University of Florida Institutional Animal Care and Use Committee and the National Institutes of Health guidelines detailed in the *Guide for the Care and Use of Laboratory Animals*.

Sample preparation. Hippocampus samples were homogenized in a glass tube with a Teflon dounce pestle in 15 volumes of ice-cold detergent-free buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM EGTA, 0.33 M sucrose, 1 mM DTT) containing a broad-range protease inhibitor cocktail (Roche Molecular Biochemicals, no. 1-836-145) and sonicated. Samples were then centrifuged at 9000g for 5 min at 4°C. The supernatant was stored at -80°C. The protein concentration of each sample was determined by DC protein assay (Biorad, Hercules, CA) with albumin standards. Proteins were diluted to 5 µg/µL in DIGE lysis buffer containing a 1% protease inhibitor cocktail (P8340, Sigma, St. Louis, MO) to prevent proteolysis during labeling.

SDS-PAGE. The Cyanine dye labeling reaction was performed with minimal labeling conditions (50 µg of protein at 5 µg/µL) per the manufacturer's instructions unless stated otherwise (Amersham, Piscataway, NJ). Labeled proteins from pooled and individual (naive or injured) samples were reduced with 5 mM DTT, alkylated with 55 mM iodoacetamide, and heated to 95°C for 2 min prior to separation with Tris-tricine SDS-PAGE gels (10–20% polyacrylamide, Invitrogen, Carlsbad, CA) at 4°C. Fluorescence imaging was performed with 1-sec exposure times (ProExpress, PerkinElmer, Boston, MA). Alternatively, unlabeled proteins were separated with the same gel system and stained with Coomassie blue. In both cases, image analysis (ImageJ, NIH) was performed to target specific regions of the gel; however, 1.5 mm × 4 mm gel slices spanning the entire gel lane were excised and stored at -80°C for trypsinization.

In vitro proteolysis. Excised gel bands were destained, reduced with 5 mM DTT, and alkylated with 55 mM iodoacetamide prior to overnight digestion with 400 ng of trypsin (Trypsin gold, Promega, Madison, WI) in 100 mM NH₄HCO₃.

Preparation of capillary LC columns with integrated electrospray emitters. The preparation of capillary LC columns with integrated electrospray emitters is described elsewhere (Haskins et al., 2001); however, 5 cm of 3-µm C18 particles (Alltima C18, Alltech, Deerfield, IL) and 50-µm-i.d. capillary LC columns were used in this work.

Automated two-pressure capillary LC-MS² system. The capillary LC-MS² system is described elsewhere (Haskins et al., 2001). The system utilizes 2 six-port valves to select the pump and flow path for preconcentration, desalting, and separation/electrospray steps. During the preconcentration and desalting steps the high-flow-rate pump was selected without splitting of the sample in order to minimize the sample loading time. During the separation/electrospray step, the low-flow-rate pump was selected with splitting of the gradient in order to maximize the separation and electrospray efficiency and to minimize the delay time of the gradient, respectively.

In this work, 4.5 µL from a 12-µL sample of tryptic peptides was transferred into a 2-µL sample loop with an autosampler and analyzed every 38 min by preconcentrating/desalting at 600 nL/min and separating/electrospraying at 60 nL/min. All measurements were made with the following capillary LC-MS² parameters, unless specified otherwise: preconcentration time = 3.3 min (2.0 µL), desalting time = 3.3 min (2.0 L), separa-

tion/electrospray time = 30 min (10 min pump gradient from 5% to 45% mobile phase B; mobile phase A = 2% acetonitrile: 1% acetic acid; mobile phase B = 98% acetonitrile: 1% acetic acid), re-equilibration time = 1.4 min. The mass spectrometer was a QIT (LCQ-Deca XP+, ThermoFinnigan, San Jose, CA) with the following parameters, unless specified otherwise: automatic gain control (AGC) on, max AGC time = 300 msec, $q = 0.25$, isolation width = 3 m/z , normalized collision energy = 35%, activation time = 0.25 msec and the default number of microscans and target count values. Data-dependent MS/MS spectra (MS, 4 \times MS/MS) were collected using a precursor ion window of m/z 400–1800 and a product ion window calculated for $z = +2$.

Differential proteomic analysis. Protein database searching (RefSeq 785,143 sequences (Pruitt and Maglott, 2001) with uninterpreted MS² spectra and differential proteomic analysis of unmodified proteins were performed with Sequest (Yates et al., 1998) and DTA-Select (Tabb et al., 2002), respectively. The default precursor and product ion tolerances of 1.5 and 0.0 were selected for Sequest, while only singly, doubly, and triply charged tryptic peptide sequences with Xcorr > 1.8, 2.5, and 3.5 were considered significant for DTASelect. No molecular mass constraints were placed on protein identification by protein database searching. A TBI database containing unmodified peptide and protein sequences that were observed in naive only, injured only, or both conditions was constructed in-house (from the DTASelect files via Microsoft Access 2002) as a function of the 1D-DIGE gel position (gel slices were numbered 1–50 from high to low molecular mass). PTMs were investigated with Mascot (Perkins et al., 1999) using the same protein database as Sequest but with the recommended precursor and product ion tolerances of 2.0 and 0.8, respectively. PTMs were considered significant if the Mascot score indicated homology with greater than 95% probability.

RESULTS

SDS-PAGE-Capillary LC-MS²

Naive and injured hippocampal protein samples were processed and labeled with Cy-3 and Cy-5 dye separately. Labeled proteins from pooled and individual samples were separated side-by-side, and naive and injured samples were run on separate lanes (Fig. 1). Our results show the consistency in protein loading, cyanine dye labeling, and separation efficiency. Alternatively, unlabeled proteins were separated with the same gel system and stained with Coomassie blue (data not shown). In general, we did

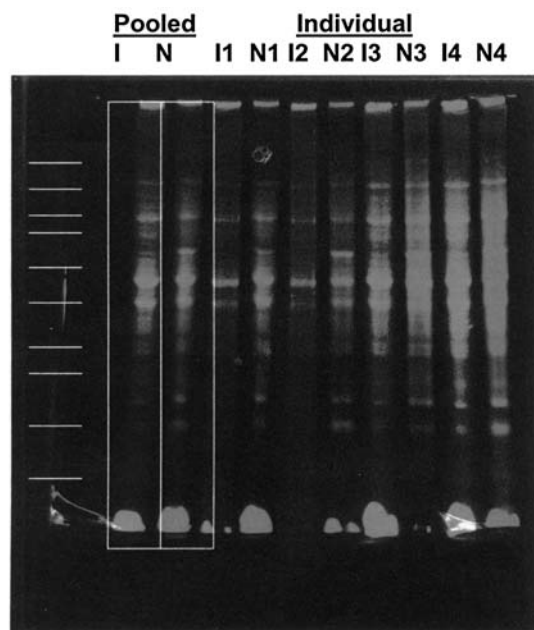


FIG. 1. Cyanine-dye labeled proteins separated by SDS-PAGE. Fluorescence image of 25 μ g of Cy3- and Cy5-labeled proteins from naive (N) and injured (I) ipsilateral hippocampus (IH) samples separated on a Tris-tricine SDS-PAGE gel (10–20% polyacrylamide).

not find a significant advantage of cyanine-dye labeling for our purposes. Fifty 1.5 mm \times 4 mm gel slices spanning each (naive or injured) gel lane were excised, trypsinized and subjected to automated capillary LC-MS². We collected 15,558 uninterpreted MS² spectra in 3 days for pooled cyanine dye-labeled samples separated by SDS-PAGE. Protein database searching identified more than 306 unique proteins. Overall, we obtained 156 ± 60 MS² spectra per gel slice and 1–3 tryptic peptide sequences per protein. Figure 2 shows the correlation between the database-derived molecular mass (M_r calc), and SDS-PAGE-predicted molecular mass (M_r obs). The migration of proteins in the SDS-PAGE gel inversely correlates with M_r calc for unmodified proteins identified by capillary LC-MS² and database searching (solid line), as expected. Accordingly, M_r obs directly correlates with M_r calc. In addition, protein sequence coverage shows an inverse correlation with M_r calc (dashed line). That is, the higher the molecular mass of the protein, the less sequence coverage is obtained. However, it is important to note that we have successfully identified (by peptide sequences rather than by peptide masses) more than 20 proteins of high molecular mass (150–300 kDa). In contrast, proteins in this molecular mass range are almost impossible to visualize and identify by 2D gels (Fountoulakis et al., 1999b).

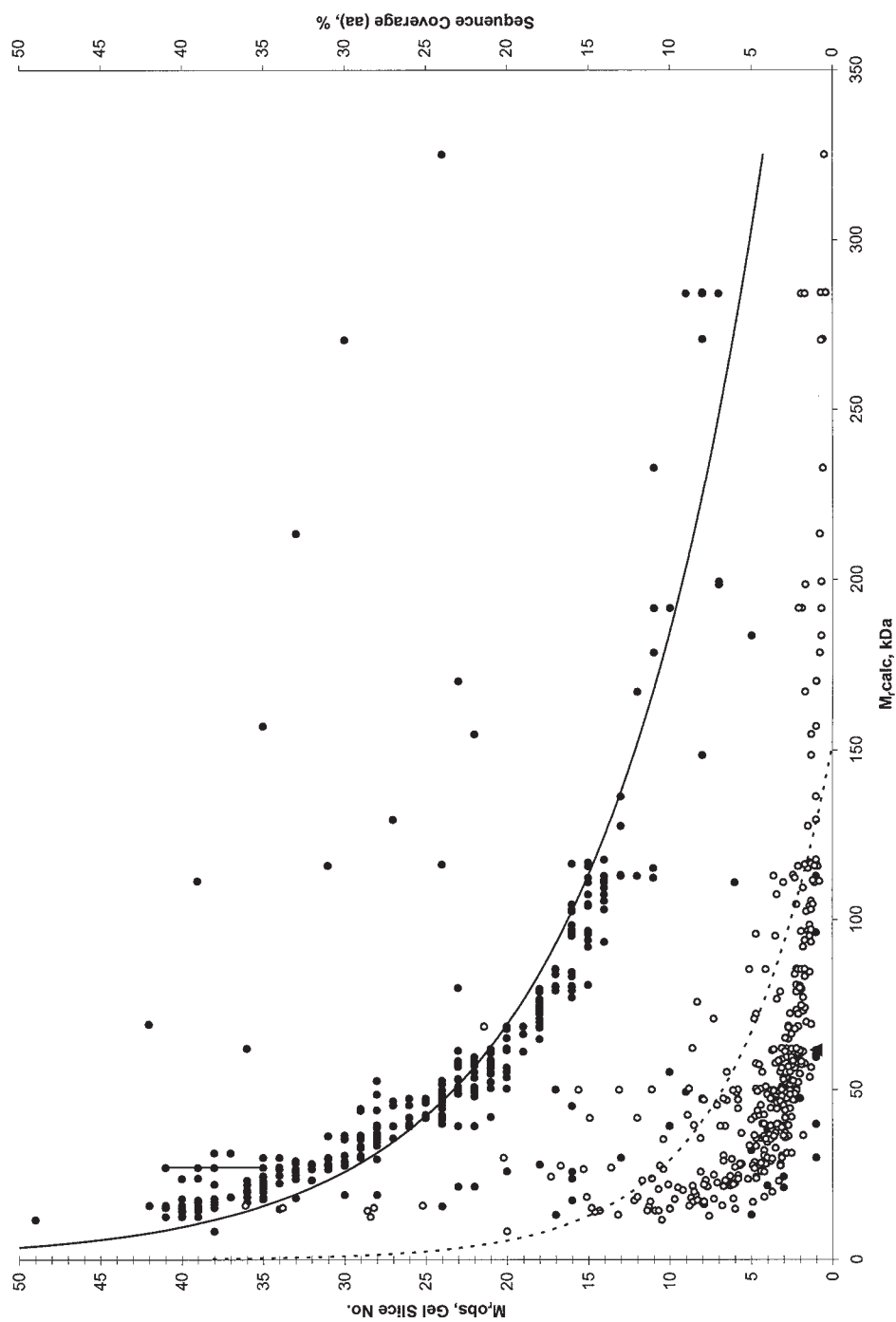


FIG. 2. Correlation between the database-derived molecular mass ($M_{r,calc}$), and SDS-PAGE-predicted molecular mass ($M_{r,obs}$). The migration of proteins in the SDS-PAGE gel inversely correlates with $M_{r,calc}$ for unmodified proteins identified by capillary LC-MS² and database searching (solid line), as expected. Accordingly, $M_{r,obs}$ directly correlates with $M_{r,calc}$. In addition, protein sequence coverage shows an inverse correlation with $M_{r,calc}$ (dashed line).

TABLE 1. DIFFERENTIAL PROTEOMIC ANALYSIS OF MAMMALIAN PROTEIN

<i>RefSeq accession number</i>	<i>Protein description</i>	<i>M_{r,calc}</i> (kDa)	<i>M_{r,obs}</i> (kDa)
Protein appears in naïve animals only			
NM_022007	FXYD domain-containing ion transport regulator 7 [<i>Mus musculus</i>]	8	13–17
NM_181029	casein alpha-S1 [<i>Bos taurus</i>]	25	>250
NM_012966	heat shock 10 kDa protein 1 (chaperonin 10) [<i>Rattus norvegicus</i>]	11	10–13
NM_017236	phosphatidylethanolamine binding protein [<i>Rattus norvegicus</i>]	21	15–25
NM_018947	cytochrome c [<i>Homo sapiens</i>]	12	<10
NM_057114	peroxiredoxin 1 [<i>Rattus norvegicus</i>]	22	15–25
NM_022511	profilin [<i>Rattus norvegicus</i>]	15	10–15
NM_174294	casein kappa [<i>Bos taurus</i>]	21	>250
NM_028207	dual specificity phosphatase 3 [<i>Mus musculus</i>]	20	15–25
NM_016956	hemoglobin, beta adult minor chain; beta min; beta minor globin [<i>Mus musculus</i>]	16	10–15
NM_017169	thioredoxin peroxidase 1 [<i>Rattus norvegicus</i>]	22	15–25
NM_017055	transferrin [<i>Rattus norvegicus</i>]	76	50–75
NM_182839	RIKEN cDNA 2900041A09 [<i>Mus musculus</i>]	23	17–27
NM_010471	hippocalcin [<i>Mus musculus</i>]	22	15–25
NM_023716	tubulin, beta [<i>Mus musculus</i>]	50	35–50
NM_021316	BM88 antigen [<i>Mus musculus</i>]	15	15–25
NM_053511	neural F box protein NFB42 [<i>Rattus norvegicus</i>]	34	30–35
NM_009610	Actin, gamma 2 (smooth muscle) [<i>Mus musculus</i>]	42	40–60
NM_022922	triosephosphate isomerase 1 [<i>Rattus norvegicus</i>]	27	17–27
NM_019131	tropomyosin 1, alpha [<i>Rattus norvegicus</i>]	29	27–33
NM_012498	aldehyde reductase 1 [<i>Rattus norvegicus</i>]	36	13–17
NM_000410	hemochromatosis protein 1 [<i>Homo sapiens</i>]	10	35–50
NM_017025	lactate dehydrogenase A [<i>Rattus norvegicus</i>]	36	27–33
NM_008617	malate dehydrogenase, mitochondrial [<i>Mus musculus</i>]	35	27–33
NM_023716	RIKEN cDNA 2410129E14 gene [<i>Mus musculus</i>]	50	35–50
NM_002634	prohibitin [<i>Homo sapiens</i>]	30	17–27
NM_011553	t-complex protein 10b [<i>Mus musculus</i>]	49	>250
NM_001069	tubulin, beta polypeptide [<i>Homo sapiens</i>]	50	35–50
NM_002301	lactate dehydrogenase C [<i>Homo sapiens</i>]	36	27–33
NM_012949	enolase 3, beta; [<i>Rattus norvegicus</i>]	47	35–50
NM_014364	glyceraldehyde-3-phosphate dehydrogenase, testis-specific [<i>Homo sapiens</i>]	44	27–33
NM_013506	eukaryotic translation initiation factor 4A2 [<i>Mus musculus</i>]	46	35–50
NM_133977	transferrin; hypotransferrinemia with hemochromatosis [<i>Mus musculus</i>]	77	50–75
NM_003026	SH3-domain GRB2-like 2 [<i>Homo sapiens</i>]	40	33–40
NM_139254	tubulin, beta 3 [<i>Rattus norvegicus</i>]	50	35–50
NM_022399	calreticulin [<i>Rattus norvegicus</i>]	48	35–50
NM_031140	vimentin [<i>Rattus norvegicus</i>]	54	35–50
NM_012497	aldolase C, fructose-biphosphate [<i>Rattus norvegicus</i>]	39	33–40
NM_031034	guanine nucleotide binding protein (G protein) alpha 12 [<i>Rattus norvegicus</i>]	44	30–35
XM_236277	protein phosphatase PP2A [<i>Rattus norvegicus</i>]	65	50–75
NM_019225	solute carrier family 1, member 3 [<i>Rattus norvegicus</i>]	60	>250
NM_017009	glial fibrillary acidic protein [<i>Rattus norvegicus</i>]	50	35–50
NM_025407	ubiquinol-cytochrome c reductase core protein 1 [<i>Mus musculus</i>]	53	35–50
NM_145614	dihydroipoamide S-acetyltransferase [<i>Mus musculus</i>]	68	50–75

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TABLE 1. DIFFERENTIAL PROTEOMIC ANALYSIS OF MAMMALIAN PROTEIN (CONTINUED)

<i>RefSeq accession number</i>	<i>Protein description</i>	<i>M_{r,calc} (kDa)</i>	<i>M_{r,obs} (kDa)</i>
NM_022229	heat shock protein 60 (chaperonin) [<i>Rattus norvegicus</i>]	61	40–60
NM_145518	NADH dehydrogenase (ubiquinone) Fe-S protein 1 [<i>Mus musculus</i>]	80	50–75
NM_175199	heat shock protein 12A [<i>Mus musculus</i>]	75	50–75
NM_008449	kinesin heavy chain 5C, neuron-specific [<i>Mus musculus</i>]	109	80–130
NM_031715	phosphofructokinase, muscle [<i>Rattus norvegicus</i>]	86	60–90
NM_145779	pregnancy-zone protein [<i>Rattus norvegicus</i>]	167	115–205
NM_013559	HSP105 [<i>Mus musculus</i>]	97	80–130
NM_031604	H ⁺ transporting ATPase, lysosomal (vacuolar proton pump) [<i>Rattus norvegicus</i>]	96	>250
NM_021343	spermatogenesis associated factor [<i>Mus musculus</i>]	97	75–105
NM_007804	cut-like 2 [<i>Mus musculus</i>]	155	35–50
NM_152296	ATPase, Na ⁺ /K ⁺ transporting, alpha 3 polypeptide [<i>Homo sapiens</i>]	112	80–130
NM_054004	TBP-interacting protein 120A [<i>Rattus norvegicus</i>]	136	105–160
NM_019167	beta-spectrin 3 [<i>Rattus norvegicus</i>]	271	>250
Protein appears in injured animals only			
NM_003509	H2A histone family, member C [<i>Homo sapiens</i>]	14	10–15
NM_080777	synuclein, beta [<i>Rattus norvegicus</i>]	15	10–15
NM_000976	ribosomal protein L12 [<i>Homo sapiens</i>]	18	15–25
NM_025562	RIKEN cDNA 2010003O14 [<i>Mus musculus</i>]	17	13–17
NM_016068	CGI-135 protein [<i>Homo sapiens</i>]	17	13–17
NM_025313	RIKEN cDNA 0610008F14 [<i>Mus musculus</i>]	18	10–15
NM_026369	actin-related protein 2/3 complex, subunit 5 [<i>Mus musculus</i>]	16	13–17
NM_012038	visinin-like 1 [<i>Mus musculus</i>]	22	15–25
NM_009923	cyclic nucleotide phosphodiesterase 1 [<i>Mus musculus</i>]	47	35–50
NM_133796	Rho GDP dissociation inhibitor (GDI) alpha [<i>Mus musculus</i>]	23	17–27
NM_014231	VAMP-1A; synaptobrevin [<i>Homo sapiens</i>]	13	10–15
NM_017101	peptidylprolyl isomerase A (cyclophilin A) [<i>Rattus norvegicus</i>]	18	10–15
NM_009001	RAB3A, member RAS oncogene family [<i>Mus musculus</i>]	25	15–25
NM_000518	beta globin [<i>Homo sapiens</i>]	16	10–15
NM_024349	adenylate kinase [<i>Rattus norvegicus</i>]	21	15–25
NM_031603	14-3-3 epsilon [<i>Rattus norvegicus</i>]	29	15–25
NM_017051	superoxide dismutase 2, mitochondrial [<i>Rattus norvegicus</i>]	25	15–25
NM_026267	RIKEN cDNA 1200016B17 [<i>Mus musculus</i>]	30	30–35
NM_008907	peptidylprolyl isomerase A; cyclophilin A [<i>Mus musculus</i>]	18	13–17
NM_053610	peroxiredoxin 5 precursor [<i>Rattus norvegicus</i>]	22	13–17
NM_016131	ras-related GTP-binding protein RAB10 [<i>Homo sapiens</i>]	23	15–25
NM_011670	ubiquitin carboxy-terminal hydrolase L1; gracile axonal dystrophy; protein gene product 9.5 [<i>Mus musculus</i>]	25	15–25
NM_019376	14-3-3 protein gamma [<i>Rattus norvegicus</i>]	28	17–27
NM_011739	14-3-3 theta [<i>Mus musculus</i>]	28	17–27
NM_010312	guanine nucleotide-binding protein, beta-2 subunit [<i>Mus musculus</i>]	37	30–35
NM_023200	protein phosphatase-1 regulatory subunit 7 [<i>Mus musculus</i>]	41	35–50
NM_019632	N-ethylmaleimide sensitive fusion protein attachment protein beta; brain protein I47 [<i>Mus musculus</i>]	34	27–33
NM_017327	GTP-binding protein alpha o; RATBPGTPC [<i>Rattus norvegicus</i>]	40	30–35
NM_026646	RIKEN cDNA 1300006L01 [<i>Mus musculus</i>]	35	30–35
NM_005165	aldolase C, fructose-bisphosphate; Aldolase C, fructose- bisphosphatase [<i>Homo sapiens</i>]	39	33–40

(continued)

TABLE 1. DIFFERENTIAL PROTEOMIC ANALYSIS OF MAMMALIAN PROTEIN (CONTINUED)

<i>RefSeq accession number</i>	<i>Protein description</i>	<i>M_rcalc (kDa)</i>	<i>M_robs (kDa)</i>
NM_025942	RIKEN cDNA 2810409H07 [<i>Mus musculus</i>]	45	35–50
NM_019291	carbonic anhydrase 2 [<i>Rattus norvegicus</i>]	37	27–33
NM_017215	solute carrier family 1, member 2 [<i>Rattus norvegicus</i>]	62	>250
NM_005917	cytosolic malate dehydrogenase [<i>Homo sapiens</i>]	36	17–27
NM_006032	copine 6; neuronal copine; N-copine [<i>Homo sapiens</i>]	62	50–75
NM_006136	F-actin capping protein alpha-2 [<i>Homo sapiens</i>]	33	27–33
NM_002074	G protein, beta-1 subunit; transducin beta chain 1I [<i>Homo sapiens</i>]	37	30–35
NM_014203	adaptin, alpha A; [<i>Homo sapiens</i>]	108	80–130
NM_002635	phosphate carrier precursor isoform 1b; mitochondrial [<i>Homo sapiens</i>]	40	>250
NM_018754	stratifin; 14-3-3 sigm [<i>Mus musculus</i>]	28	17–27
NM_024221	pyruvate dehydrogenase (lipoamide) beta [<i>Mus musculus</i>]	39	30–35
NM_025899	ubiquinol cytochrome c reductase core protein 2 [<i>Mus musculus</i>]	48	35–50
NM_002300	lactate dehydrogenase B [<i>Homo sapiens</i>]	37	27–33
NM_012570	glutamate dehydrogenase 1; memory related gene 2 [<i>Rattus norvegicus</i>]	61	40–60
NM_138828	apolipoprotein E [<i>Rattus norvegicus</i>]	36	27–33
NM_013681	synapsin II [<i>Mus musculus</i>]	52	40–60
NM_033235	malate dehydrogenase 1[<i>Rattus norvegicus</i>]	36	27–33
NM_011861	protein kinase C and casein kinase substrate in neurons 1 [<i>Mus musculus</i>]	51	40–60
NM_004077	citrate synthase precursor; citrate synthase, mitochondrial [<i>Homo sapiens</i>]	52	35–50
NM_007505	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, alpha subunit, isoform 1 [<i>Mus musculus</i>]	60	35–50
NM_057118	contactin 1 [<i>Rattus norvegicus</i>]	113	105–160
NM_010481	heat shock protein, A [<i>Mus musculus</i>]	74	50–75
NM_019703	phosphofructokinase [<i>Mus musculus</i>]	86	60–90
NM_012491	adducin 2, beta [<i>Rattus norvegicus</i>]	81	80–130
NM_003178	synapsin IIb [<i>Homo sapiens</i>]	52	40–60
NM_009947	copine VI; copine 6; neuronal copine [<i>Mus musculus</i>]	62	50–75
NM_011393	solute carrier family 1, member 2; glial high affinity glutamate transporter [<i>Mus musculus</i>]	61	>250
NM_153781	brain glycogen phosphorylase [<i>Mus musculus</i>]	97	75–105
NM_006644	heat shock 105 kD [<i>Homo sapiens</i>]	92	80–130
NM_031783	neurofilament, light polypeptide [<i>Rattus norvegicus</i>]	61	50–75
NM_006950	synapsin Ia [<i>Homo sapiens</i>]	74	50–75
NM_013066	microtubule-associated protein 2 [<i>Rattus norvegicus</i>]	199	>250
NM_021979	heat shock 70 kDa protein 2 [<i>Homo sapiens</i>]	70	50–75
NM_012607	neurofilament, heavy polypeptide [<i>Rattus norvegicus</i>]	115	115–205
NM_181092	synaptic Ras GTPase activating protein 1 [<i>Rattus norvegicus</i>]	128	105–160
NM_005348	heat shock 90 kDa protein 1, alpha [<i>Homo sapiens</i>]	85	75–105
NM_010438	hexokinase 1; downeast anemia [<i>Mus musculus</i>]	106	80–130
NM_000477	albumin precursor [<i>Homo sapiens</i>]	69	10–15
NM_001385	dihydropyrimidinase [<i>Homo sapiens</i>]	57	50–75
NM_001127	Seta-adaptin [<i>Homo sapiens</i>]	105	80–130
NM_003334	ubiquitin-activating enzyme E1 [<i>Homo sapiens</i>]	118	80–130
NM_001835	Clathrin, heavy chain [<i>Homo sapiens</i>]	179	115–205
NM_005657	tumor protein p53 binding protein, 1 [<i>Homo sapiens</i>]	214	15–25
NM_002374	microtubule-associated protein 2a [<i>Homo sapiens</i>]	199	>250

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TABLE 1. DIFFERENTIAL PROTEOMIC ANALYSIS OF MAMMALIAN PROTEIN (CONTINUED)

<i>RefSeq accession number</i>	<i>Protein description</i>	<i>M_{r,calc}</i> (kDa)	<i>M_{r,obs}</i> (kDa)
Higher sequence coverage in injured than naïve (protein appears in both)			
NM_005530	mitochondrial; isocitrate dehydrogenase (NAD ⁺) alpha [<i>Homo sapiens</i>]	40	30–35
NM_024398	mitochondrial aconitase [<i>Rattus norvegicus</i>]	85	60–90
NM_005566	lactate dehydrogenase A [<i>Homo sapiens</i>]	37	27–33
NM_057143	fertility protein SP22 [<i>Rattus norvegicus</i>]	200	15–25
NM_013083	heat shock 70 kD protein 5 [<i>Rattus norvegicus</i>]	72	50–75
NM_019169	synuclein, alpha [<i>Rattus norvegicus</i>]	15	10–15
NM_024398	mitochondrial aconitase [<i>Rattus norvegicus</i>]	85	60–90
XM_237718	tubulin alpha 6 [<i>Rattus norvegicus</i>]	50	35–50
NM_171983	alpha-spectrin 2 [<i>Rattus norvegicus</i>]	285	>250
NM_139325	enolase 2, gamma; neuronal [<i>Rattus norvegicus</i>]	50	35–50
NM_006597	heat shock 70 kDa protein 8 isoform 1 [<i>Homo sapiens</i>]	71	50–75
NM_015981	CaM kinase II alpha subunit; isoform 1 [<i>Homo sapiens</i>]	55	40–60
NM_011738	14-3-3 eta [<i>Mus musculus</i>]	28	17–27
NM_017042	protein phosphatase 3 (calcineurin) subunit A beta [<i>Rattus norvegicus</i>]	59	40–60
NM_005507	cofilin 1 (non-muscle) [<i>Homo sapiens</i>]	19	15–25
NM_146100	hypothetical protein MGC25352 [<i>Mus musculus</i>]	55	50–75
XM_217040	tubulin alpha-1 [<i>Rattus norvegicus</i>]	50	35–50
Same sequence coverage in naïve and injured			
NM_006870	destrin [<i>Homo sapiens</i>]	19	15–25
NM_173102	tubulin, beta 5 [<i>Rattus norvegicus</i>]	50	35–50
NM_006000	tubulin, alpha 1; testis-specific [<i>Homo sapiens</i>]	50	35–50
NM_001102	actinin, alpha 1 [<i>Homo sapiens</i>]	103	80–130
NM_008634	microtubule-associated protein 1b [<i>Mus musculus</i>]	270	27–33
NM_008084	glyceraldehyde-3-phosphate dehydrogenase [<i>Mus musculus</i>]	36	30–35
NM_023964	glyceraldehyde-3-phosphate dehydrogenase type 2 [<i>Rattus norvegicus</i>]	47	30–35
NM_153629	(NM_153629) heat shock 70 kDa protein 4 [<i>Rattus norvegicus</i>]	94	80–130
NM_012529	(NM_012529) creatine kinase, brain [<i>Rattus norvegicus</i>]	43	33–40
NM_012734	hexokinase 1 [<i>Rattus norvegicus</i>]	103	75–105
NM_009497	vesicle-associated membrane protein 2; synaptobrevin II [<i>Mus musculus</i>]	13	10–15
NM_016774	ATP synthase, H ⁺ transporting mitochondrial F1 complex, beta subunit [<i>Mus musculus</i>]	58	35–50
NM_010777	(NM_010777) myelin basic protein; myelin deficient [<i>Mus musculus</i>]	27	10–27
NM_031728	synaptosomal-associated protein (AP180) [<i>Rattus norvegicus</i>]	94	80–130
NM_000517	alpha 2 globin [<i>Homo sapiens</i>]	15	10–15
NM_002965	S100 A9; calgranulin B [<i>Homo sapiens</i>]	13	I: >250, N: 60–90
NM_030873	profilin II [<i>Rattus norvegicus</i>]	15	10–15
NM_012673	thymus cell surface antigen [<i>Rattus norvegicus</i>]	18	15–25
NM_012635	(NM_012635) pancreatic trypsin 1 [<i>Rattus norvegicus</i>]	26	75–105
NM_013177	Glutamate oxaloacetate transaminase 2 mitochondrial [<i>Rattus norvegicus</i>]	27	33–40
NM_012504	ATPase, Na ⁺ K ⁺ transporting, alpha 1 [<i>Rattus norvegicus</i>]	113	105–160
NM_010324	glutamate oxaloacetate transaminase 1, cytosolic [<i>Mus musculus</i>]	26	33–40
NM_080583	adaptor-related protein complex 2, beta 1 subunit; beta adaptin [<i>Rattus norvegicus</i>]	105	75–105

(continued)

TABLE 1. DIFFERENTIAL PROTEOMIC ANALYSIS OF MAMMALIAN PROTEIN (CONTINUED)

RefSeq accession number	Protein description	M_r ,calc (kDa)	M_r ,obs (kDa)
NM_026508	(NM_026508) RIKEN cDNA 2410002K23 [<i>Mus musculus</i>]	80	75–105
NM_006310	puromycin-sensitive aminopeptidase; metalloproteinase MP100 [<i>Homo sapiens</i>]	99	75–105
Higher sequence coverage in naïve than injured (protein appears in both)			
NM_030773	beta tubulin 1, class VI [<i>Homo sapiens</i>]	50	35–50
NM_019299	clathrin, heavy polypeptide (Hc) [<i>Rattus norvegicus</i>]	192	160–250
NM_003127	alpha-spectrin 2 (alpha-fodrin) [<i>Homo sapiens</i>]	284	>250
NM_013096	hemoglobin, alpha 1 [<i>Rattus norvegicus</i>]	15	10–15
NM_018753	14-3-3 protein beta [<i>Mus musculus</i>]	28	17–27
NM_000944	protein phosphatase 3 (calcineurin A alpha) [<i>Homo sapiens</i>]	59	40–60
NM_057213	ATPase, H ⁺ transporting, lysosomal beta 2 [<i>Rattus norvegicus</i>]	57	40–60
NM_138548	nucleoside diphosphate kinase (NM23A) [<i>Rattus norvegicus</i>]	17	10–15
NM_033234	Hemoglobin, beta [<i>Rattus norvegicus</i>]	16	10–15
NM_053543	neurochondrin [<i>Rattus norvegicus</i>]	79	50–75
NM_003406	14-3-3 zeta [<i>Homo sapiens</i>]	28	17–27
NM_031353	voltage-dependent anion channel 1 [<i>Rattus norvegicus</i>]	31	27–33
NM_138597	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit [<i>Mus musculus</i>]	23	15–25
NM_053291	phosphoglycerate kinase 1 [<i>Rattus norvegicus</i>]	45	35–50
NM_000034	aldolase A; fructose-biphosphate aldolase [<i>Homo sapiens</i>]	39	33–40
NM_017245	translation elongation factor 2 [<i>Rattus norvegicus</i>]	95	75–105
NM_023119	enolase 1, alpha non-neuron [<i>Mus musculus</i>]	47	35–50
NM_005918	mitochondrial malate dehydrogenase [<i>Homo sapiens</i>]	36	27–33
NM_053297	/pyruvate kinase, muscle [<i>Rattus norvegicus</i>]	58	40–60
NM_080689	dynamin 1 [<i>Rattus norvegicus</i>]	96	75–105
NM_011123	myelin proteolipid protein [<i>Mus musculus</i>]	30	I, N: 15–30, N: 105–160
NM_134326	albumin [<i>Rattus norvegicus</i>]	69	50–75

The RefSeq (Pruitt and Maglott, 2001) accession number, protein description, database-derived molecular mass (M_r ,calc), and SDS-PAGE-predicted molecular mass (M_r ,obs) are shown for putative protein biomarkers of TBI.

Differential proteomic analysis of the gel slices (high to low M_r ,obs) revealed differences in protein sequence coverage for 170 mammalian proteins (57 in naïve only, 74 in injured only, and 39 of 64 in both) as listed in Table 1. Inspection of the proteins falling into each of the three categories of protein markers shows that several well-studied proteins involved in TBI were observed in both naïve and injured samples, including brain creatine kinase (CKB), α II-spectrin, neuron-specific enolase (NSE), α -synuclein (α -Syn), microtubule associated protein 2a and 2b (MAP2), neurofilament (NF), proteolipid protein (PLP), and myelin basic protein (MBP). The injured-to-naïve ratio of protein sequence coverage suggests putative biomarkers that may exhibit significant differences in protein concentration between naïve and injured samples. However, protein sequence coverage is only a semi-quantitative measure of protein concentration. This is particularly true for protein identifications based on single

tryptic peptide sequences, and it is even more pronounced for degraded proteins. However, proteins observed only in naïve samples, or proteins observed with greater sequence coverage in naïve samples than in injured samples, suggest a subset of putative biomarkers that are down-regulated, released, or degraded during TBI, for example, (α II-spectrin (Pike et al., 2002), MAP2 (Huh et al., 2003), NF (Posmantur et al., 1996, 1998) and PLP (Banik et al., 1985; Domanska-Janik et al., 1992). Likewise, proteins observed only in injured samples, or proteins observed with greater sequence coverage in injured samples than in naïve samples, suggest a subset of putative biomarkers that are up-regulated, accumulated, or aggregated during TBI, for example, NSE (Varma et al., 2003), amyloid precursor protein, amyloid β 1-42, tau (Franz et al., 2003), and α -Syn (Uryu et al., 2003; Bramlett and Dietrich 2003; Newell et al., 1999; Smith et al., 2003). Since the fragments of degraded proteins, for ex-

ample, breakdown products of α II-spectrin (Pike et al., 2002) may also be observed, it is important to relate $M_{r,calc}$ to $M_{r,obs}$ for putative protein biomarkers of TBI.

In order to evaluate whether any of our biomarkers were fragments of degraded proteins rather than intact proteins, we performed differential proteomic analysis as a function of $M_{r,obs}$. Degraded protein biomarkers may not be revealed by differences in protein sequence coverage using current differential proteomic analysis tools, even when proteins are separated prior to *in vitro* proteolysis and capillary LC-MS², because $M_{r,obs}$, which is encoded in SDS-PAGE–Capillary LC–MS² data, may not be preserved during data reduction. For example, MBP was identified by database searching ($M_{r,calc} = 27$ kDa) in both naive and injured samples with a sequence coverage of 13.6%, incorrectly suggesting that it is not a putative biomarker of TBI. However, the vertical line in Figure 2 illustrates that MBP was observed in gel slices 35–41 ($M_{r,obs} \sim 27$ kDa to 10 kDa, respectively) in injured (and not naive) samples, suggesting possible degradation, as confirmed by Western blot (Liu et al., unpublished observations).

Classification of Putative Protein Biomarkers of TBI

Stratification of the putative protein biomarkers discovered in this work, based on function and distribution, suggests several classes of proteins are of interest (Figs. 3 and 4). Careful examination of the fraction of proteins from each class that were observed in naive only, injured only, and both naive and injured samples highlights the most promising classes for biomarkers of TBI. For example, Figure 3 shows that 10% of the putative biomarkers observed only in injured samples were neuronal proteins including: PLP, Syn (α and β), NSE, NF (light and heavy), synapsin (I and II), vesicle associated membrane protein 1, and apolipoprotein E. Other promising classes of biomarkers observed only in injured samples include heat shock proteins (e.g., chaperonin 10) and kinases (e.g., calcium/calmodulin protein kinase II). These observations are reflected by peaks in the line plot shown in Figure 4. Thus, neuronal proteins, heat shock proteins, and kinases are a promising class of biomarkers that are up-regulated, accumulated, or aggregated during TBI. In contrast, the valley for dehydrogenases (e.g., lactate dehydrogenase) only in naive samples indicates a promising class of biomarkers that are down-regulated, released, or degraded. A complete discussion of the putative protein biomarkers discovered in this work is beyond the scope of this paper. While some ambiguity is expected, for example, glutamate dehydrogenase was observed only in injured samples while the neuronal protein glial

fibrillary acidic protein (GFAP) was observed only in naive samples, the classification of putative protein biomarkers of TBI, combined with differential analysis methods such as this one, provides direction for biomarker research.

Preliminary Validation

The relative concentration of several putative protein biomarkers of TBI was investigated by targeted capillary LC-MS² (Haskins et al., 2001) of selected tryptic peptides (Fig. 4). Two- to ten-fold changes in tryptic peptide concentration for injured versus naive samples reflect the semi-quantitative differences in protein sequence coverage observed. For example, glutamate dehydrogenase (memory related gene 2), shown in Figure 5C, was ~ 10 -fold higher in injured samples than in naive samples: corresponding to 2.9% protein sequence coverage in injured samples and 0.0% protein sequence coverage in naive samples (i.e., no tryptic peptides were observed in naive samples). A high yield of sequence-specific b- and y-type product ions was observed following isolation and fragmentation of selected tryptic precursor ions by collision-induced dissociation. Absolute quantification (AQUA) (Gerber et al., 2003) of these proteins can be readily achieved by incorporating an isotopically labeled tryptic peptide as an internal standard during trypsin digestion (publication in preparation). Assuming that the analytical variability exceeds the biological variability in pooled samples such as these, a false-positive rate as high as 30% is expected for data-dependent capillary LC-MS² of complex mixtures (unpublished work). While only a 29% overlap of proteins conserved between naive and injured samples underscores the need for higher-resolution protein separation methods, this must be balanced with the need for faster results. Indeed, preliminary validation of biomarkers is a significant bottleneck for proteomics as the speed of discovery continues to outpace the speed of validation (Bodovitz and Joos, 2004).

Comparison with Previous Work

This is the first report of SDS-PAGE–Capillary LC–MS² for biomarker discovery. Several of the putative protein biomarkers described herein at 48 h post-injury were suggested previously by a microarray- and RNA-based gene expression experiment (Matzilevich et al., 2002) In 10 oligonucleotide array pairs, 261 of 8800 genes were significantly affected at 24 h post-injury, including NF (light), MAP2, GFAP, and beta-tubulin.

More recently, a proteomics approach using 2D gels and database searching of 2D gel images (Fountoulakis et al., 1999a) at 24 h post-injury was presented (Jenkins et al., 2002). In that work, 50 (<95 kDa proteins) of

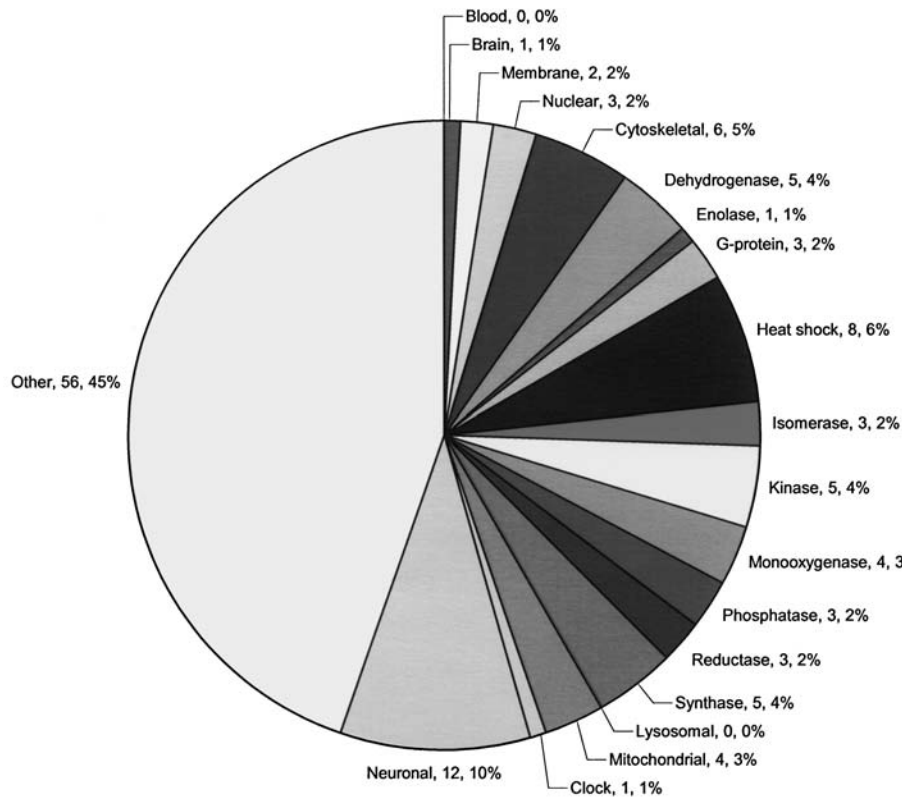


FIG. 3. Stratification of the putative protein biomarkers discovered in injured hippocampus only. Protein class, number of proteins, fraction of protein biomarkers. Proteins were sorted into classes based on function and localization with increasingly stringent specificity: blood, brain < membrane < nuclear < cytoskeletal < dehydrogenase, enolase, clock, G-protein, heat shock, isomerase, kinase, monoxygenase, phosphatase, reductase < synthase < lysosomal, mitochondrial < neuronal.

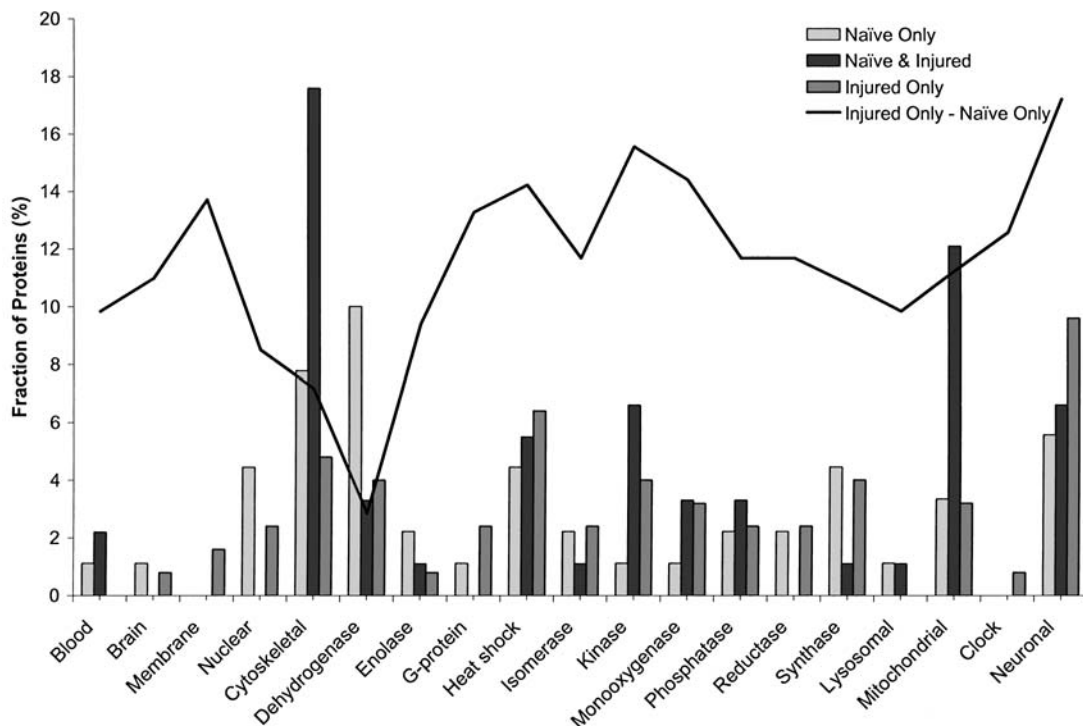


FIG. 4. Stratification of putative protein biomarkers discovered in naive only, injured only, and both naive and injured hippocampal samples. The fractions of biomarkers in “Naive Only” (light grey columns), “Injured Only” (dark grey), and both “Naive and Injured” samples (black), were plotted against each function and localization class. In addition, the difference in the fraction between the “Injured Only” group and the “Naive Only” group was plotted as a line graph on the same scale. Peaks in the line plot suggest classes of proteins that are elevated, upregulated, or aggregated (e.g., neuronal, kinase) in injured hippocampus, while valleys in the line plot are those that are down-regulated, released, or degraded (e.g., dehydrogenase) in injured hippocampus.

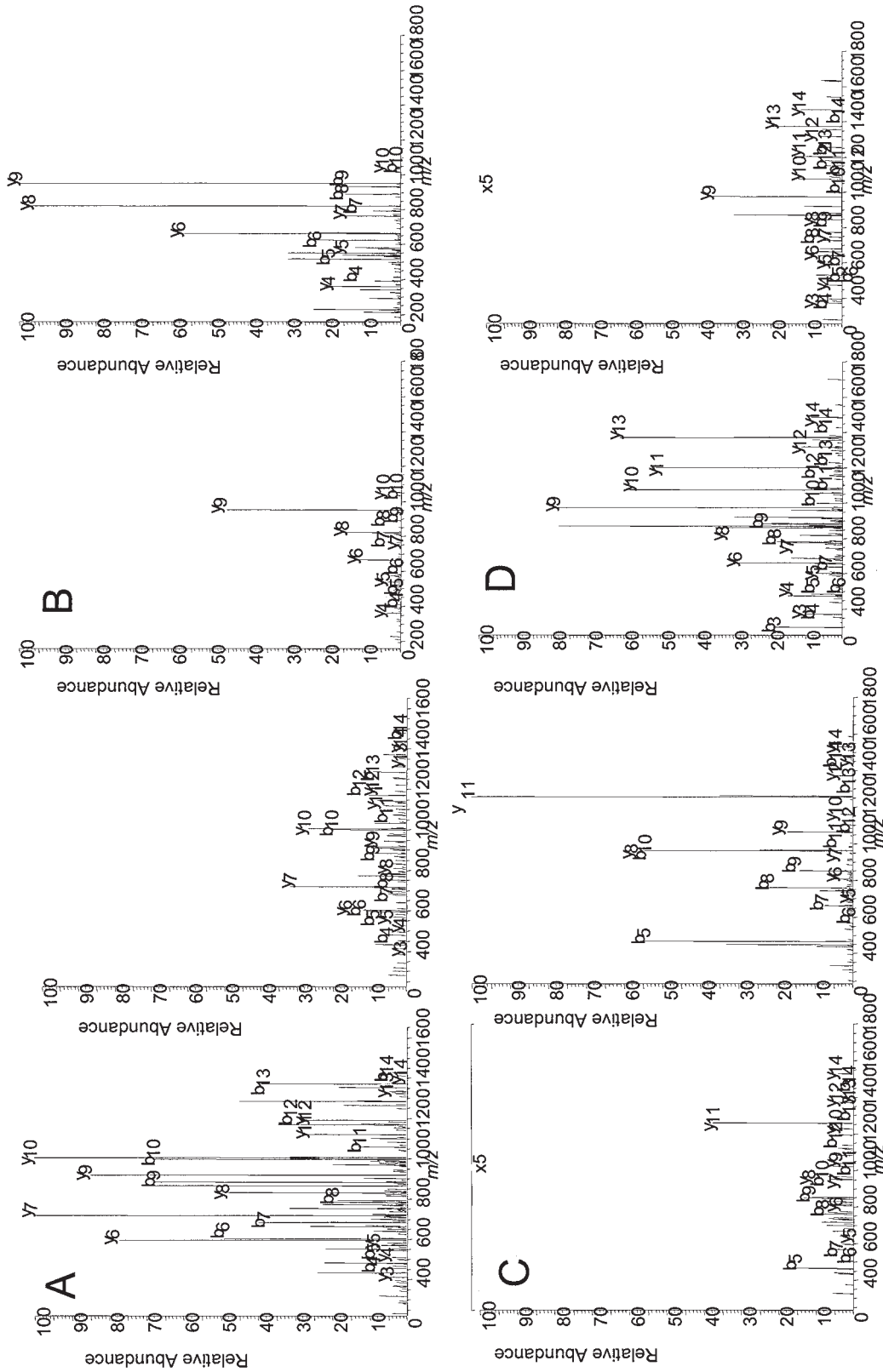


FIG. 5. Representative targeted MS² spectra collected in naive (left) and injured (right) hippocampus samples. (A) CKB-rat, (B) α -Syn, (C) Memory-related gene 2, (D) α II-Spectrin. The tryptic peptide sequences corresponding to these spectra are LAVEALSSLDGDLGR (A), KEGVLYVGSK (B), HGGTIPVYPTAEFQDR (C), and DLAAALGDKVNSLGETAQR (D), respectively.

~1500 protein spots were tentatively identified by matching the 2D gel-derived molecular masses and isoelectric points of the protein spots with a rat brain database of 210 proteins. However, only six putative protein biomarkers were revealed by significant changes across six of six gel pairs (individual rather than pooled samples). Interestingly, an increase in the mitochondrial protein Cu/Zn superoxide dismutase, and a decrease in the cytoskeletal proteins α - and β -tubulin, were also observed in this work.

Confidence in previously reported putative biomarkers is significantly strengthened by sequence-specific discovery of these proteins by SDS-PAGE–Capillary LC–MS². Protease-specific peptide sequences provide a means to unambiguously identify putative protein biomarkers and various PTMs (e.g., degradation) from large protein databases (e.g., RefSeq 785,143 sequences) (Pruitt and Maglott, 2001). In contrast, microarray experiments suffer from our incomplete understanding of the interaction between transcription and translation; that is, RNA levels do not accurately reflect protein levels, and database searching of 2D gel images suffers from a low success rate for protein identification. Despite the limitations of these techniques, the unambiguous identification of several previously reported putative biomarkers by SDS-PAGE–Capillary LC–MS² provides evidence for the validity of this approach to biomarker discovery.

DISCUSSION

Using differential proteomic analysis, we revealed differences in protein sequence coverage for 170 mammalian proteins (57 in naive only, 74 in injured only, and 39 of 64 in both). Our data suggest that these are putative biomarkers of TBI in hippocampus tissue, as these are expected to either accumulate in the CSF and blood, or form aggregate in the extracellular compartment of the brain. However, we must further establish if these markers can distinguish TBI from various other brain diseases, and the kinetics for their degradation and clearance from tissue to CSF and blood must be favorable in order to obtain reliable indicators of injury severity. A subset of the putative protein biomarkers of TBI described herein, particularly the neuronal proteins, are expected to meet these criteria for biomarker validation. In the meantime, these biomarkers may also find use in the laboratory setting. For example, β 3-tubulin and GFAP are used to distinguish neuronal differentiation in stem cell research (Kornblum and Geschwind, 2001). Lastly, this work provides proof-of-principle for more rapid and comprehensive sequence-specific biomarker discovery strategies incorporating protein separation prior to capillary LC–MS².

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