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Proteomic Study of Human Glioblastoma Multiforme Tissue Employing Complementary Two-Dimensional Liquid Chromatography- and Mass Spectrometry-Based Approaches

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An extensive data set comprising 2660 unique protein identifications was obtained for the proteome of a human brain tumor (*glioblastoma multiforme*) by combining the results of two complementary analytical strategies based on two-dimensional chromatography and mass spectrometry. A bottom-up method, performing peptide separation in both chromatographic dimensions was employed as well as a semi-top-down method, in which intact proteins were separated in the first and tryptic peptides in the second dimension. The identified proteins were assigned to their molecular functions and compared to previously identified proteins of glioblastoma multiforme (= *astrocytoma* WHO grade IV), lower WHO grade astrocytomas (grade II and III), and nontumor brain tissue. With the use of a subset of 104 identified membrane proteins, the properties of intact protein fractionation in the first dimension of the semi-top-down approach were elucidated in detail. The benefit of the semi-top-down approach was further demonstrated by the identification of a set of endogenous glioblastoma multiforme expressed proteins. These proteins correspond to recombinant antigens which were recently found to be reactive against autoantibodies in glioblastoma multiforme patients. The results indicate the usefulness of the semi-top-down approach for the investigation of immunogenic antigens in human tumor tissue samples.

Keywords: human brain tumor tissue • two-dimensional chromatography • proteome analysis • protein fractionation

Introduction

The advent of high-throughput and high-sensitivity methods for large-scale proteome analysis[1−7] facilitated the study of proteomes derived from a variety of human tissues, for example, the human brain. Proteome analysis involving this highly complex organ is especially intricate and has been pursued independently by a number of research groups.[8−11] The human brain proteome project (HBPP),[12] initiated by the Human Proteome Organization (HUPO) primarily focuses on human neurodegenerative diseases, specifically Alzheimer's disease, Down-syndrome, Parkinson's disease, and aging. The direct comparison between normal and diseased brain at the proteome level is particularly challenging because healthy brain tissue samples are usually not available, especially from the same patient, among other things, due to ethical constraints. Therefore, in the pilot study of HBPP, control brain tissue for proteomic comparison was obtained by surgery from epilepsy patients or autopsy.[13] Collecting protein lists of five independent laboratories including gel-based as well as gel-free approaches led to identification of 1804 human brain proteins.[13,14] Further proteomic information about the nontumor brain proteome was gained by investigation of the fetal brain. Two studies employing two-dimensional gel electrophoresis (2D-GE) resulted in the identification of 437 proteins out of the human fetal cerebellum[8] and 543 out of the whole human fetal brain,[9] respectively.

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Samples of human brain tumors were also studied employing proteomic approaches. Astrocytomas are especially in the focus of research because they account for 60% of all primary brain tumors. This kind of tumor is divided into four grades according to malignancy. The highest malignant astrocytoma is glioblastoma multiforme (WHO grade IV) which is also the most frequent brain tumor in adults.[15] Most glioblastomata multiforme are primary tumors that arise *de novo*, while secondary glioblastomata multiforme develop from lower grade astrocytomas. The average survival time of patients with this type of tumor is less than 16 months with an estimated 5-year survival rate of 5%.[16] As of yet, the etiology of glioblastoma multiforme remains elusive. Specifically, no associations could be verified for glioblastoma multiforme and environmental effects such as smoking, diet, electromagnetic fields, or viral infections.[17−22]

Astrocytomas and especially glioblastomata multiforme were addressed in proteomic studies to investigate the protein composition in different tumor grades and compared to nontumor tissue. Employing a combination of capillary isoelectric focusing (CIEF) and reversed-phase chromatography for multidimensional peptide separation hyphenated to tandem mass spectrometry, 1820 nonredundant protein hits of a human glioblastoma multiforme tissue were identified by at least one unique peptide sequence.[23] Furthermore, glioblastoma multiforme has been analyzed in comparison to low-grade astrocytomas employing two-dimensional gel electrophoresis (2D-GE),[24] differential gel electrophoresis (DIGE),[25] or direct-tissue imaging by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).[26]

Our study targets the proteome analysis of human glioblastoma multiforme tissue by application of two complementary analytical strategies based on two-dimensional chromatographic separation and mass spectrometric identification. The first strategy employs shotgun proteome analysis, including digestion of the isolated proteome into peptides, followed by strong cation-exchange- (SCX) and ion-pair reversed-phase (IP-RP) chromatographic separation of the peptides prior to identification by tandem mass spectrometry (MS/MS). The second strategy involves IP-RP-HPLC separation of the proteome in a first chromatographic dimension at the intact protein level followed by digestion and analysis of the resulting peptides in a second IP-RP-HPLC-MS/MS analysis. Our approach further extends previous setups employing intact protein fractionation in the first separation dimension using either anion-exchange chromatography[27] or chromatofocusing.[28] The innovation in our methodology rests within the use of IP-RP-HPLC to fractionate intact proteins from a tumor tissue, which is known to be capable of efficiently separating proteins having a wide range of chemical properties such as size, charge, or hydrophobicity. A further novelty is the combination of intact protein fractionation with shotgun proteomics to identify the most complex data set of proteins for glioblastoma multiforme known so far.

The identified proteins are further arranged in a biological context and compared with proteins previously identified as promising markers to differentiate glioblastoma multiforme and lower grade astracytomas as well as nontumor tissue. Moreover, the protein elution pattern of the semi-top-down approach is discussed to demonstrate the usability of this approach with respect to targeted protein isolation from complex proteomes. This strategy is evaluated on the example of recently reported, recombinantly expressed antigens that react against serum autoantibodies in patients suffering from glioblastoma multiforme.[29] A panel of these antigens has been combined to provide antigen patterns capable of distinguishing glioblastoma multiforme from other tumors and brain pathologies. We want to provide evidence that these autoantigens are actually expressed in the tumor samples and that their further analysis can be limited to a small number of fractions from the first separation dimension.

Methods

Chemicals and Materials. Sodium hydrogen phosphate (NaH_2PO_4, min. 99%), was purchased from Merck (Darmstadt, Germany). Trifluoroethanol (TFE, ≥ 99.5%), tributylphosphine (TBP, 97%), acetonitrile (E Chromasolv), α-cyano-4-hydroxycinnamic acid (CHCA), ≥ 98%) and human [Glu^1]-fibrinopeptide B (glu^1-fib) were obtained from Sigma-Aldrich (Steinheim, Germany). Trifluoroacetic acid (TFA, ≥ 99.5%), heptafluorobutyric acid (HFBA, ≥ 99%), ammonium formate (NH_4OAc, ≥ 97%) and ammonium bicarbonate (NH_4HCO_3, ≥ 99.5%) were purchased from Fluka (Buchs, Switzerland). Trypsin (sequencing grade modified) was purchased from Promega (Madison, WI, USA) and 5-bromo-4-chloro-3-indolyl phosphate as well as nitroblue tetrazolium from Applichem (Darmstadt, Germany). For quantification of total protein content prior to digestion or injection of intact proteins into the first dimension the Bradford protein assay was applied (Bio-Rad, Hercules, CA, USA).

Tumor Tissue. The glioblastoma multiforme tissue was obtained with patients' informed consent from the Department of Neurosurgery, Saarland University Hospital. The tumor biopsy sample used in this study was derived from a 54-year-old male patient. Following tumor resection, representative tissue samples containing equivalent cell populations were sectioned and analyzed by immunhistochemistry (Institute of Neuropathology, Saarland University Hospital). The tissue sample for protein extraction was snap-frozen immediately after surgery. Histopathological analysis revealed WHO grade IV glioblastoma multiforme. By cytogenetic analysis, we obtained a 44, XY, −21,−22 karyotype.

Serological Identification of Antigens by Recombinant Expression Screening (SEREX). The SEREX study was conducted as described previously.[29] In brief, a cDNA expression library was constructed using ZAP Express vector arms of λ-phage (Stratagene, La Jolla, CA). Patient serum was diluted and preabsorbed against sonicated *Escherichia coli* XL1blue MRF′ cell lysates. The preabsorbed serum was diluted to a final concentration of 1:100. *E. coli* XL1blue MRF′ cells were transfected with the cDNA expression library and plated to a density of ∼10 000 PFU/plate on NZCYM agar plates. Expressed proteins were bound to nitrocellulose membranes. After blocking steps, the membranes were incubated with preabsorbed patient serum and anti-human IgG antibody conjugated to alkaline phosphatase. Antigen−antibody complexes were detected by 0.0050% (w/v) 5-bromo-4-chloro-3-indolyl phosphate and 0.010% (w/v) nitroblue tetrazolium in 1× color developing solution. The identified antigens were subjected to subsequent serologic spot assays against monoclonal phages. Monoclonal phage and the cDNA library as control at a concentration of ∼5000 PFU/μL were incubated with exponentially growing *E. coli* XL1blue MRF′ and spotted in duplicate on the precoated nitrocellulose membranes. Membranes were incubated with preabsorbed serum samples. Autoantibodies were detected as described above. For each serum, at least two membranes were

screened. An antigen was defined as "positive" if at least two of four replicates showed reactivity.

Preparation of Human Brain Tissue Protein Extracts. Following a published protocol,[30] 100 μL of phosphate buffer (5.0 mM, pH 7.0) was added to two pieces of 10−15 mg of glioblastoma multiforme tissue. After 3 min shaking (Vortex Genie 2, Bender & Hobein AG, Zurich, Switzerland), the samples were incubated for 1 h at room temperature with gentle shaking and then sonicated for 5 min in ice−water. Addition of 100 μL of trifluoroethanol (TFE), incubation for 2 h at 60 °C and sonication for 2 min in ice−water followed. After 5 min of centrifugation (4 °C, 8500 rpm), the protein concentration of the sample supernatant was determined using the Bradford assay. To denature the proteins and reduce the disulfide bonds, the sample was incubated for 30 min at 60 °C after adding 50 mM tributylphosphine (TBP) to a final concentration of 4.5 mM. The proteins were injected immediately after incubation into the first separation dimension for the semi-top-down approach or digested for the bottom-up approach, respectively.

IP-RP-HPLC Prefractionation of Intact Proteins and Digestion in the Semi-Top-Down Approach. Approximately 860 μg of total protein (quantified with the Bradford protein assay) in 500 μL was injected into an analytical HPLC system (Model 1050, Hewlett-Packard, Waldbronn, Germany). The proteins were separated employing two 50 × 4.6 mm i.d. monolithic reversed-phase columns (ProSwift RP-1S, Dionex Corporation, Sunnyvale, CA) connected in-series using a flow rate of 0.80 mL/min and operating at 55 °C, in order to maximize the peak capacity of the first separation dimension.[31−35] The external six-port injection valve (Model 7125, Rheodyne, Rohnert Park, CA) was equipped with a 1-mL sample loop. Proteins were eluted with a 60-min gradient of 15−60% acetonitrile in 0.050% TFA, followed by a washing step of 60−100% acetonitrile in 0.050% aqueous TFA in 3 min and holding for 10 min at 100% acetonitrile containing 0.050% TFA. UV chromatograms were recorded at 280 nm and 2-min fractions were collected.

Each fraction obtained from the first dimension was evaporated to dryness in a vacuum concentrator (Model Concentrator 5301, Eppendorf AG, Hamburg, Germany), and redissolved in 100 μL of 50 mM ammonium bicarbonate to give a clear solution without visibly insoluble material. The data from Bradford assay and the signal intensity in the UV chromatogram of the first separation dimension were used to adjust the enzyme/protein ratio to approximately 1:20 (40 μg trypsin for 860 μg protein). The enzyme was activated at 37 °C and 550 rpm for 30 min in the thermo mixer (Model Comfort, Eppendorf) and then added to the fractions of the first dimension. After incubation overnight, the reaction was quenched by addition of 1% (v/v) TFA and the fractions were frozen at −30 °C. Prior to injection into the nanoflow HPLC-MALDI-TOF/TOF system, 100 μL of 0.10% aqueous HFBA solution was added to each fraction.

Tryptic Digestion of the Extracted Proteins in the Bottom-Up Approach. The reduced protein solution was diluted 1:4 with ammonium bicarbonate (50 mM, pH 7.9) to reduce the TFE concentration. The enzyme-to-protein ratio was adjusted to approximately 1:50 with a solution of 1.0 μg/μL trypsin in 50 mM acetic acid. After activation of trypsin, 8.5 μL was added to the protein extract and incubated for 15 h in the thermo mixer at 37 °C and 550 rpm. The digest was quenched by addition of TFA to 1.0% (v/v) final concentration. The solvent was evaporated to dryness in the vacuum concentrator and stored at −30 °C. Prior to injection into the first separation dimension, peptides were redissolved in 200 μL of ammonium formate (10 mM, pH 3.0 which is the starting eluent in SCX-HPLC).

SCX-HPLC Prefractionation of Peptides for the Bottom-Up Approach. About 426 μg/200 μL of peptides was injected into an analytical HPLC system (Model 1050, Hewlett-Packard). Peptides were separated on an SCX-column (Polysulfethyl A, 200 × 2.1 mm i.d., 5 μm, 200 Å, PolyLC, Columbia, MD) using a 10 × 2.1 mm i.d. guard column and a flow rate of 0.20 mL/min operating at 25 °C. The external six-port injection valve (Model 7125, Rheodyne) was equipped with a 200-μL sample loop (Rheodyne). Eluent A consisted of 10 mM ammonium formate, pH 3.0, and eluent B of 500 mM ammonium formate, pH 6.8. Both mobile phases contained 25% (v/v) acetonitrile. After injection, the system was held for 10 min at 10 mM ammonium formate, pH 3.0. Then, a gradient of 10−250 mM ammonium formate within 40 min was started, followed by ramping to 500 mM ammonium formate, pH 6.8, within 10 min. For the last 10 min, the system was held at 500 mM ammonium formate, pH 6.8. The UV chromatogram was recorded at 214 nm. Two-minute fractions were collected, evaporated to 10 μL and frozen at −30 °C. Prior to injection into the second dimension, 100 μL of 0.1% aqueous HFBA solution was added to the fractions.

Nanoflow IP-RP-HPLC-MALDI-MS/MS of Peptides. The instrumental setup was the same as described earlier.[36] It consisted of a nanoflow HPLC unit (Model Ultimate, LC Packings, Amsterdam, The Netherlands), an automatic injection unit (Model Famos, LC-Packings), and a loading pump (Model K-1001, Knauer, Berlin, Germany) with a 10-port switching valve (Model C2−1000D (stator) and 06A-8029C (rotor), VICI, Schenkon, Switzerland). The detector was equipped with a 3 nL Z-shaped flow cell (Model Ultimate, LC Packings) and the UV chromatogram was recorded at 214 nm. The separation column (60 × 0.10 mm i.d.) as well as the preconcentration column (10 × 0.20 mm i.d.) contained a monolithic PS-DVB-based stationary phase material synthesized according to a published protocol[37] (available from Dionex Benelux, Amsterdam, The Netherlands).

In the semi-top-down approach, the analysis of each fraction was performed in triplicate, for the bottom-up approach in duplicate, respectively. For both, 10 μL of the peptide sample was injected into the second-dimension nanoflow HPLC system, preconcentrated and desalted in the monolithic trap column for 3 min with a 10 μL/min flow of 0.10% aqueous HFBA. Subsequently, the peptides were eluted in back-flush mode onto the separation column and separated with a shallow 50-min gradient of 0−30% acetonitrile in 0.050% aqueous TFA at a flow rate of 0.70 μL/min, followed by ramping to 100% acetonitrile in 10 min.

The eluate was spotted with a frequency of 1 spot per 5 s (258 nL per spot) onto a stainless steel target (Opti-TOF 123 × 81 mm, Applied Biosystems, Framingham, MA) using an automated spotting unit (Model Probot, Dionex, Germering, Germany). Matrix solution containing 3.0 mg/mL α-cyano-4-hydroxy-cinnamic acid and 15 fmol/μL glu[1]-fib as internal calibrant in 70% acetonitrile and 0.10% aqueous TFA was added through a T-piece at a flow rate of 3.1 μL/min. A matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer (ABI 4800 TOF/TOF Analyzer, Applied Biosystems) was utilized for protein identification. The

spectra were acquired in positive ion reflector mode in a mass range of m/z 800−3000. In MS mode, 25 laser shots were accumulated to one subspectrum and 40 subspectra were summed to the final spectrum (1000 shots) with a frequency of 200 Hz and a laser wavelength of 355 nm. Fragmentation of the six highest abundant peptide ions per spot was conducted in MS/MS mode exploiting metastable decay with reacceleration.[38] The fragment ion data acquisition stopped after 3000 laser shots or after a signal-to-noise ratio (S/N) of 35 for at least 10 peaks, was obtained.

The mass spectrometer was calibrated using a six-peptide calibration mix (Applied Biosystems), on eight external calibration spots for each MALDI plate (Plate Model Calibration) resulting in a mass accuracy better than 50 ppm (= default calibration). For optimized mass accuracy (5 ppm), internal calibration on the m/z of glu[1]-fib was performed during data acquisition. When this additional calibration failed due to signal suppression especially in spectra with high sample peptide signal intensity, default calibration was automatically used (50 ppm) by the software. Calibration of the instrument in MS/MS mode was performed using four fragments of glu[1]-fib.

Protein Identification and Validation. The MS/MS spectra were smoothed with the Savitsky-Golay algorithm, which averaged three neighboring raw data points into one employing least-squares regression with a polynomial order of four. For generating peak lists and exporting data to create Mascot generic files (.mgf), the following settings were used in the MS/MS peak filter of the 4000 Series Explorer Software (Applied Biosystems, version 3.5.3): Mass range, m/z 60 to precursor mass minus 35 Da; peak density, maximum 20 peaks per 200 Da; minimum S/N, 10; minimum area, 200 and maximum 65 peaks/precursor. To identify proteins, the Mascot generic files (.mgf-files) were sent to the Mascot software (version 2.2.03, Matrix Science, London, U.K.) which uses the MOWSE (Molecular weight search) algorithm.[39] The applied settings were database, Swiss-Prot (version 54.7, January 15th, 2008); taxonomy, *Homo sapiens* (18 117 sequences); enzyme, trypsin; variable modification, methionine oxidation; peptide tolerance, 50 ppm; MS/MS tolerance, 0.2 Da; maximal missed cleavages, 1 and ion-score cutoff for peptides, 0.05 ($P \geq 95\%$). The probability of an identification as a random event for proteins was set to 0.01 ($P \geq 99\%$). The false positive discovery rate (FDR) was determined by Mascot for the protein identifications using an automatically generated randomized decoy database. Moreover, for all identified proteins, Mascot directly delivers the theoretical isoelectric points and intact molecular masses calculated on the basis of the primary amino acid sequence.

Scaffold Proteome Software (version 2 Scaffold Proteome Software_2_02_02, Proteome Software, Inc., Portland, OR) was utilized to validate MS/MS based peptide and protein identifications obtained by Mascot. Scaffold, in contrast to Mascot, groups proteins similar in their primary structure and observed with the same subset of peptides to satisfy the principle of parsimony, counts them as only one hit, and reports the result as a protein group. Here, all MS/MS spectra were analyzed using both Mascot and X! Tandem (www.thegpm.org; version 2007.01.01.1) for protein identification. Mascot and X! Tandem searches in the human subset of the Swiss-Prot database were conducted with the same parameters as described above for Mascot only. Peptide identifications were assigned by the Peptide Prophet algorithm[40] and proteins by the Protein Prophet algorithm[41] of the Scaffold package with at least two unique peptides.

Classification of the Proteins by Gene Ontology. To assign the identified proteins of the glioblastoma multiforme tissue to their location in the cell, Gene Ontology pathway analysis using the Gene Trail software (http://genetrail.bioinf.uni-sb.de/links.php)[42] was performed. The whole human genome served as reference set for the statistical analysis. The subset of integral membrane proteins was selected for characterization of retention behavior for intact proteins in the first dimension of the semi-top-down approach. The following parameters were set: The organism selected was human; identifier type, Swiss-Prot; significance threshold, 0.01% and the minimal number of genes of a category shown, 100. The GeneTrail software is based on Gene Ontology (GO) terms.[43] PANTHER software[44] (www.pantherdb.org) was utilized to subdivide the identified proteins into functionally related subfamilies based on their gene function.

Results and Discussion

Complementary Strategies for Proteome Analysis of Glioblastoma Multiforme Tissue. A recently published protocol for the preparation of brain tissue samples using trifluoroethanol instead of a detergent was applied for cell disruption and proteome isolation.[30] Two different two-dimensional chromatography- and mass spectrometry-based methods were employed for separation and protein identification, as illustrated in Figure 1. In the first approach, a commonly used bottom-up method (Figure 1a), the extracted proteome was digested using trypsin, and the resulting peptide mixture was separated by SCX-HPLC in the first dimension. After collection of 2-min fractions followed by partial evaporation of the solvent, peptides were preconcentrated and desalted on a short trap column, separated by IP-RP-HPLC in a second dimension, spotted onto a MALDI target, and identified with MALDI-MS/MS. For the semi-top-down approach, the extracted protein mixture was separated by IP-RP-HPLC at the intact protein level in the first dimension (Figure 1b). After collection of 2-min fractions, proteins were digested and the resulting tryptic peptides were subsequently separated and analyzed in the same second dimension setup as employed for the bottom-up approach. Because of intact protein separation in the first dimension and peptide separation in the second dimension, we designate this strategy "semi top-down approach" in the following text. In both approaches, the peak lists generated from the MALDI-MS/MS spectra were submitted to Mascot for protein identifications with the parameters given in the methods section.

Protein Identifications in Glioblastoma Multiforme Tissue. After pooling the peptide identification results of both approaches, we identified 2660 proteins (for complete list see Supporting Information) out of a human glioblastoma multiforme brain tumor tissue. In subsequent data evaluation, proteins identified only by a single peptide sequence were excluded from the protein list according to commonly accepted rules in proteome analysis.[45] This resulted in 1670 protein hits without "one-hit-wonders" (lists provided in the Supporting Information). Scaffold Proteome Software was applied for further validation of the proteins identified by Mascot, which rendered 1401 proteins or protein groups identified with more than one peptide sequence (complete list provided in the Supporting Information). To the best of our knowledge, this represents the most extensive data set of proteins confidently identified so far in glioblastoma multiforme tissue.

After Mascot analysis of the identification data from the two individual methods, 1245 proteins were assigned in the bottom-

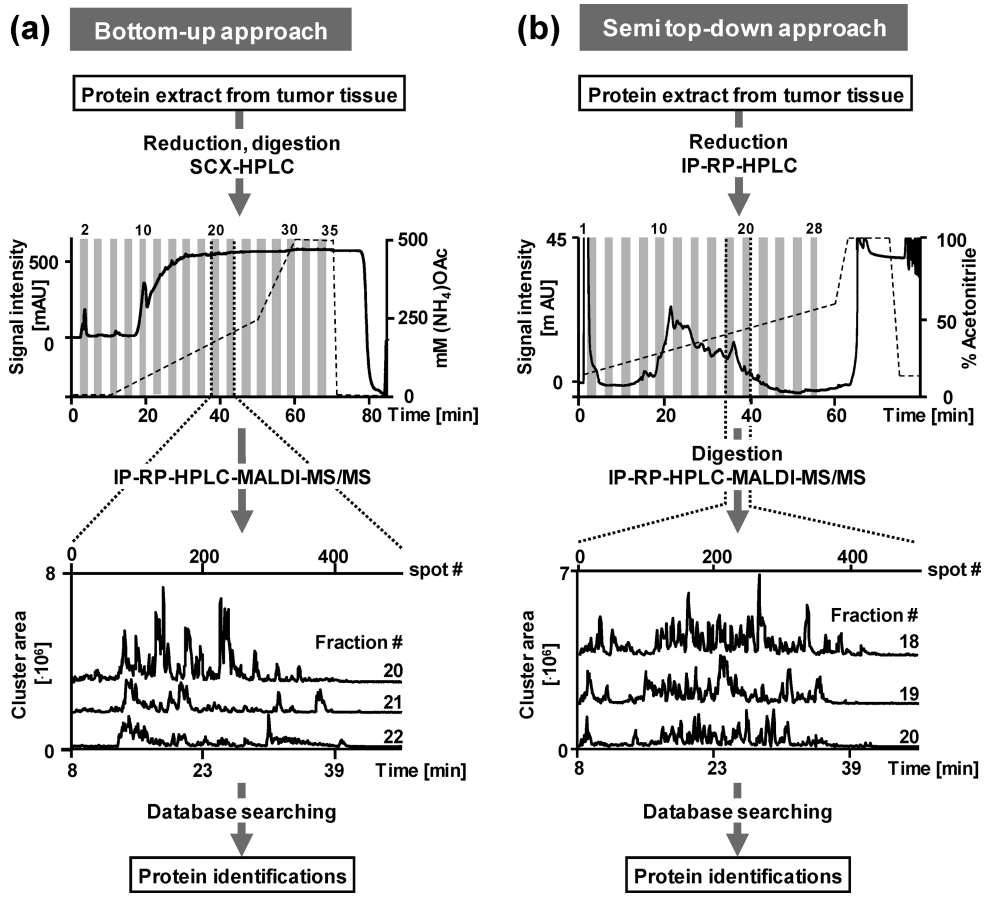

Figure 1. Analytical strategies for gel-free proteome analysis of human glioblastoma multiforme tissue: (a) bottom-up and (b) semi-top-down approach. UV chromatograms of the first separation dimension and reconstructed total ion current chromatograms for three analyses of the second dimension are shown. For details see Methods section.

up and 1005 for the semi-top-down approach, which represents an overlap of 580 proteins or 34.7% between the two methods (Figure 2a). A search against a decoy-database generated by Mascot was performed resulting in a 1.3% false discovery rate (FDR) for the bottom-up and a 1.8% FDR for the semi-top-down approach, respectively. The Scaffold results were quite similar to the Mascot results with an overlap of 33.2% (Figure 2b), suggesting that the proportions of identification were not changed upon further validation. Because of the considerable undersampling of the proteome, we cannot distinguish at this point whether additional identifications applying the alternative analytical technique are simply because of performing another replicate of the analysis or because of their complementarity. Nevertheless, compared to repetitive analyses using the same analytical method with overlaps usually above 50%, the relatively low protein identification overlap between the two methods of 33% strongly indicates a complementary character of both approaches.

Characterization of the Identified Proteins in Terms of Covered Mass and p*I* Range. An advantage of chromatographic methods in proteome analysis compared to gel-based approaches is the increased mass and pI range coverage of the identified proteins.[46,47] To characterize these two parameters for the pool of both chromatographic approaches, the computed logarithmic molecular protein masses were plotted as a function of the calculated isoelectric points for each protein identified by at least two peptides, as shown in Figure 3. Of the 1401 identified proteins, 380 proteins (26.4%) were detected with theoretical p*I*-values higher than 8. Even 49 (3.5%) proteins with p*I*-values above 10 were observed. Moreover, a number of large proteins with a molecular mass higher than 100 kDa were identified (211 proteins, 15.1%). Small proteins were also detected to a significant extent (162 proteins <20 kDa, 11.6%). The population of proteins with p*I* values around physiological pH was low due to the poor solubility of proteins at a pH equal to their p*I* value. This low presentation of proteins around pH 7 was also observed in a computational study.[48] The shown data confirms that a wide p*I* and mass range could be covered in our approaches utilizing the pooled protein data set.

Elution Behavior of Membrane Proteins in the First Dimension of Separation. Because of their numerous functions as receptors, enzymes, or transporters, membrane proteins are promising targets for biomarker identification studies. However, due to their poor solubility in aqueous solvent, membrane proteins are generally underrepresented in proteome analyses, especially in top-down approaches.[49] The analysis of membrane proteins therefore usually demands detergent-based methods including a strong denaturant for bringing them into solution. The main drawback of these methods is the necessity to remove the denaturant and detergent prior to mass spectrometric analysis, which usually comes along with considerable sample losses. To investigate membrane proteins selectively, sedimentation or partition techniques are required to separate membrane components from the cytosolic bulk.[50]

Although cytosolic proteins were preferentially recovered in our study, the sample preparation method employed was

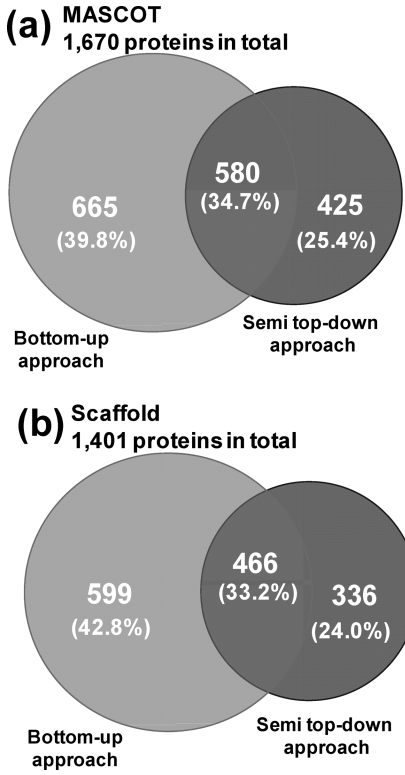

Figure 2. Venn diagrams of the proteins identified with both analytical strategies showing the unique proteins for each method and the overlap of both approaches. Exclusively proteins of human glioblastoma multiforme tissue identified with more than one peptide were considered both for (a) Mascot and (b) Scaffold data evaluation.

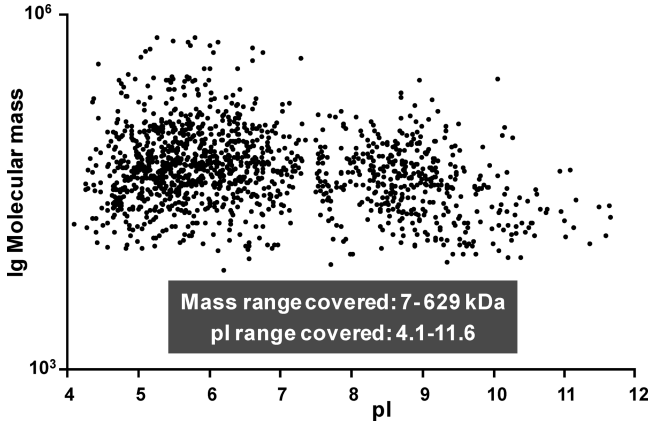

Figure 3. LogM_r/p*I* plots of the 1401 identified proteins with at least two peptides identified for human brain tumor tissue (glioblastoma multiforme). A mass range of 7−629 kDa and a p*I* range of 4.1−11.6 were covered.

previously shown to facilitate the identification of a substantial number of membrane proteins.[30] Indeed, gene ontology analysis of the proteins in our data set revealed 236 membrane proteins (14.1%) in the pool of results from both analytical methods, of which 191 were assigned in the bottom-up approach, and 104 in the semi-top-down approach. This improved representation of membrane proteins is due to the addition of trifluoroethanol (TFE) which is known to enhance protein solubility, to stabilize peptide and protein conformation, and to mimic, at least partially, membrane environments,[51] thus, preventing loss of proteins by precipitation. Furthermore, a high concentration of TFE (40−50% v/v) decreases the dielectric constant of the solubilization medium, resulting in superior protein extraction performance.[52] Finally, it easily evaporates during the sample concentration process, and hence, no additional cleanup step is required prior to HPLC-MS/MS analysis. The good representation of membrane proteins is also a consequence of the high recovery of membrane proteins from the poly-(styrene-divinylbenzene)-based monolithic columns utilized in the first separation dimension, having the very low unspecific adsorption tendency even for very hydrophobic membrane proteins.[53]

With the subset of proteins identified from this class, three typical elution patterns during IP-RP-HPLC separation in the first dimension of the semi-top-down approach are discussed in more detail. Table 1 shows the retention behavior of six selected membrane proteins: calnexin and neurofascin eluted over more than two fractions, transmembrane protein 65 and gamma-glutamyltransferase 5 precursor over two fractions, and mitochondrial import receptor subunit TOM22 homologue as well as adipocyte plasma membrane-associated protein in one fraction, respectively. Calnexin, a protein of medium size (592 amino acids, 67.6 kDa), was distributed over seven consecutive fractions (fractions 22−28). Neurofascin, a large protein composed of 1347 amino acids resulting in a molecular mass of 150 kDa, eluted in two distinct peaks and the later eluting peak was distributed over at total of seven fractions. One possible explanation for the elution of proteins over a large number of fractions is their high abundance, resulting in peak broadening and smearing due to overloading of the separation column. The relative protein abundance was estimated employing Scaffold Proteome Software, which gave spectral counts higher than 80 only for 9.9% of the 802 proteins identified with the semi-top-down approach. Spectral counts of 118 and 89 for calnexin and neurofascin, respectively, are indeed indicative of their high abundance, which is a very likely source of their elution over several fractions.

The occurrence of a protein in more than one chromatographic peak can be caused by degradation due to residual protease activity in the sample. Moreover, the high sequence homology of many human proteins as well as post-translational modifications might result in the identification of the same protein represented by different peptides in different chromatographic fractions. Another reason for elution of proteins in nonconsecutive fractions might be the disassembly of the quaternary protein structure under denaturing conditions. For example, gamma-glutamyltransferase 5, composed of 586 amino acids (62.3 kDa) was identified in two isolated fractions, which were separated by eight fractions. In fact, the peptide "LWLGFDLR" identified the light chain of this heterodimer in fraction 14, whereas three peptides of the heavy chain were found in fraction 23.

Transmembrane protein 65, consisting of 240 amino acids (25.5 kDa) was distributed over two consecutive fractions. This is reasonable as the peak elution window might not fully coincide with the fractionation window. Twenty-three of the 105 membrane proteins, for example, mitochondrial import receptor subunit (42 amino acids, 15.5 kDa) or adipocyte plasma associated protein (416 amino acids, 46.5 kDa), eluted in a single fraction, which suggests that elution in more than one fraction is not necessarily linked to protein size. Moreover, 42% of the 104 proteins eluted in one or two fractions. This represents a major advantage compared to the bottom-up approach where the peptides belonging to the same proteins

Table 1. Retention Behavior of Six Selected Membrane Proteins Representing Different Elution Patterns

	Protein	Fraction 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28	Sequence coverage [%]	Unique peptides
1	Calnexin precursor		19.0	9
2	Neurofascin precursor		7.6	8
3	Transmembrane protein 65		20.0	4
4	Gamma-glutamyltransferase 5 precursor		7.3	4
5	Mitochondrial import receptor subunit TOM22 homolog		62.0	5
6	Adipocyte plasma membrane-associated protein		19.0	4

are generally distributed over a broad range of fractions obtained from first dimension separation. Especially for the isolation and further investigation of proteins, this focused elution is very useful as demonstrated in a later section for tumor antigens related to glioblastoma multiforme.

Biological Context of the Identified Proteins. To put the identified proteins in a biological context, molecular functions of the 1401 protein hits obtained by the Scaffold Proteome Software were assigned using the PANTHER classification system.[54] A subset of 1429 proteins, which was obtained by ungrouping of the Scaffold groups, was uploaded to PANTHER. To these proteins, 1761 molecular functions were allotted. For 186 hits, the molecular function was unknown (10.6%). These were excluded as well as the 85 hits with miscellaneous function (4.8%). The distribution of the remaining 1490 molecular function hits is depicted in Figure 4. More than one-third (35.8%) of the identified proteins were assigned as enzymes. Altogether 10 different classes of enzymes could be identified of which the oxidoreductases with 24.2% were most highly represented.

Comparison of Protein Identifications in Human Glioblastoma Multiforme Tissue with Published Data. To discuss the compliance of our identified set of proteins with previously observed human brain and brain tumor proteins, we considered four different published proteomic studies of astrocytomas including different WHO grades (grade II−IV) and one study of nontumor human brain tissue (see Table 2 for details of the studies). In the study performed by Schwartz et

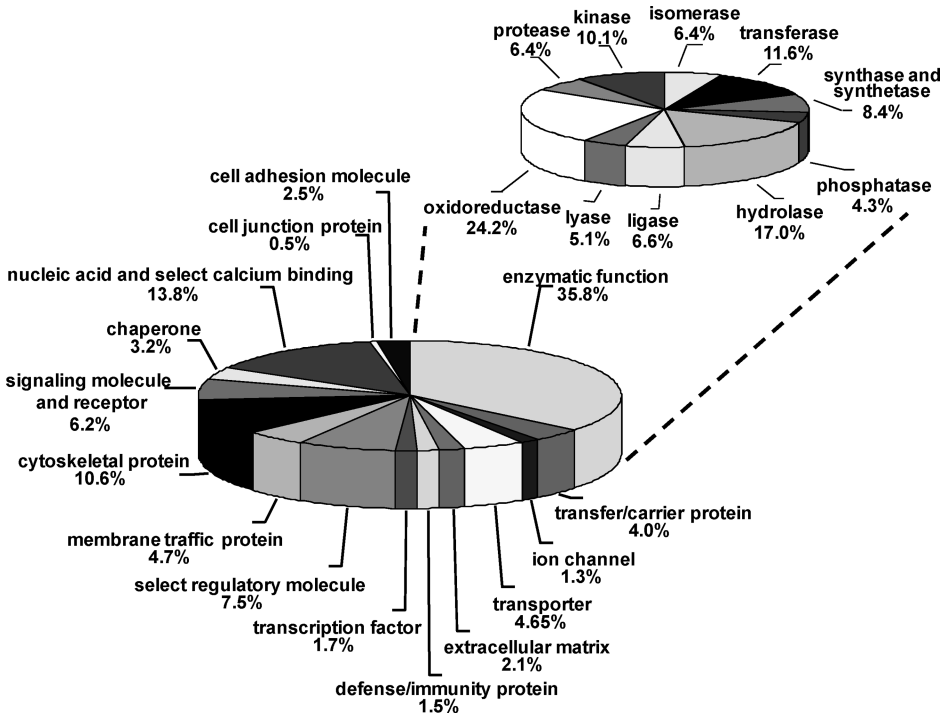

Figure 4. Molecular function of proteins identified in the pool of the semi-top-down and the bottom-up approach for human glioblastoma multiforme tissue. Unknown and miscellaneous function hits were excluded.

Table 2. Proteins Identified in Three Proteomic Studies of Astrocytoma Tissues and One Nontumor Tissue Study[a]

study reference	tissue samples controls	tumor	proteins identified
Mueller et al. (ref 13)	non tumor tissue from autopsy or biopsy samples obtained during epilepsy surgery	-	1302
Schwartz et al. (ref 26)	19 nonneoplastic	29 grade II 22 grade III 57 grade IV	6 glioma biomarkers
Odreman et al. (ref 24)	-	10 grade II 10 grade IV	70 conserved 9 higher represented in GBM 6 higher represented in low-grade astrocytoma
Khalil (ref 25)	7 (obtained from epilepsy surgery)	5 grade II 4 grade III 28 grade IV	90 differentially expressed

[a] GBM = glioblastoma multiforme.

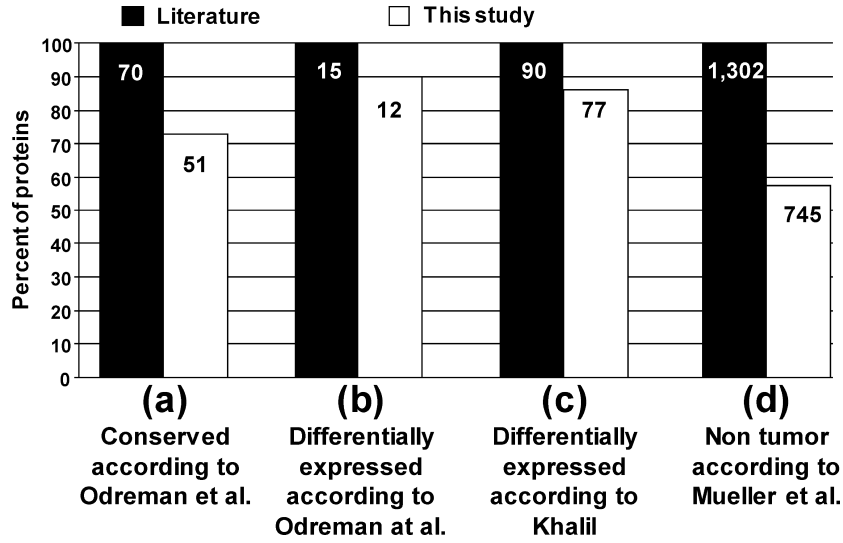

Figure 5. Comparison of proteins identified in our study with proteins (a) conserved[24] and (b and c) differentially expressed between low- and high grade astrocytoma[25] as well as (d) nontumor[13] human brain proteins. Numbers in the bars refer to absolute number of protein identifications.

al.,[26] MALDI-MS imaging was utilized to obtain tumor-specific mass patterns suitable to distinguish between nontumor and tumor tissue, to obtain grade-to-grade expression patterns, and additionally to predict short- and long-time patient survival. Furthermore, two-dimensional chromatography−mass spectrometry facilitated the identification of six potential tumor biomarkers, which showed expression patterns that enable the distinction of different tumor grades. In our sample, we were able to confirm two of the six proteins found by Schwartz et al., namely, tubulin-specific chaperone A and astrocystic phosphoprotein PEA-15, which corroborate the important role of these two proteins in grade IV tumors.

The proteomes of astrocytoma samples of different grades were compared by Odreman et al.[24] using two-dimensional gel electrophoresis (2D-GE). The study aimed at the identification of differentially expressed proteins in astrocytomata compared to nontumor or lower grade astrocytoma tissue. Of 85 identified proteins, 70 were found to be conserved in expression level in low- and high-grade astrocytomas, while the remaining 15 proteins were found to be differentially expressed: six of them were more represented in low-grade and nine in high-grade astrocytomas. A comparison with our identification results revealed that 51 of the 70 conserved proteins (72.9%) and 12 of 15 differentially expressed proteins (80%) were also identified in our study (Figure 5a,b, identities of the differentially expressed proteins are given in Table 5 of the Supporting Information). In a study performed by Khalil, differential quantitative information was obtained by utilizing differential gel electrophoresis (DIGE) and nontumor samples obtained from epilepsy surgery as control samples. Khalil identified 90 differentially expressed proteins, of which we were able to confirm 77 (85.6%) in the investigated glioblastoma multiforme tissue (Figure 5c).

Finally, we compare our protein identifications in brain tumor tissue with the brain proteome investigated in nontumor brain samples.[13] Of 1302 proteins identified with the Swiss-Prot database in the nontumor tissue study, 745 were also observed in our study (57.2%, Figure 5d). As expected, these results confirm that many proteins expressed in tumor tissue are present in nontumor tissue as well. Nevertheless, additional quantitative studies on a larger number of tumor samples, as well as nontumor and preferentially nonpathological brain control samples, are necessary to obtain more information about the tumor relevance of the identified proteins.

Confirmation of Potential Tumor Antigens for Glioblastoma Multiforme Identified by Immunological Analysis. Antigens that cause a humoral immune response in glioblastoma multiforme patients are considered as potential biomarkers for this type of tumors. Serum autoantibodies

Table 3. Tumor Antigens of Glioblastoma Multiforme Identified by SEREX and Confirmed by Two-Dimensional Chromatography-Mass Spectrometry in an Individual Patient's Sample

antigen identified by SEREX	Swiss-Prot accession number	Expasy entry[a]	antigen confirmed in this study
Actinin, alpha 4	ACTN4	O43707	x
Inhibitor of growth family, member 4	ING4	Q9UNL4	
Reticulon 4	RTN4	Q9NQC3	x
Cytoplasmic linker 2	CLIP2	Q9UDT6	x
Hematopoietic cell-specific Lyn substrate 1	HCLS1	P14317	x
U2(RNU2) small nuclear RNA auxiliary factor 1-like 1	U2AFL	Q15695	
Zinc finger protein 232	ZN232	Q9UNY5	
Heat-shock protein 105 kDa	HS105	Q92598	x
PHD finger protein 3	PHF3	Q92576	
PHD finger protein 20	PHF20	Q9BVI0	
Translocated promoter region (to activated MET oncogene)	TPR	P12270	
Cytospin-A (NY-REN-22 antigen)	CYTSA	Q69YQ0	
Golgi autoantigen, golgin subfamily a	GOGA1	Q92805	

[a] www.expasy.org.

Table 4. Distribution of Peptides Observed for SEREX Proteins[a]

Fraction	1	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	25	27	28	31	Unique peptides	Sequence coverage
Protein	Number of peptides																							
RTN4		1	1	1				1	1	1	1	1			1	1	1	1		1			5	5.7
	4					2	2																6	9.7
HS105															1								1	1.7
																2	1	1					3	4.5
ACTN4													4	4	3	2			1		2	1	14	23.1
CLIP2							1	1															2	3.3
HCLS1	1		1	1	4		1																6	22.2

[a] White numbers belong to bottom-up and black numbers to semi-top-down results.

against these antigens can be detected by analyzing the patients' blood employing the method of serological identification of antigens by recombinant expression screening (SEREX), in which molecular cloning techniques are implemented into serological analysis.[55] More than 2000 tumor-associated antigens of 15 different tumor types were identified conducting SEREX.[56] By applying SEREX to pooled sera of glioblastoma multiforme patients, we were able to identify 13 tumor antigens listed in Table 3.[29] ACTN4, ING4, U2AFL, ZN232 and HS105 were identified from a glioblastoma multiforme cell line expression library by heterologous expression screening, PHF20, PHF3, TPR, GOGA1 and CYTSA from a glioblastoma multiforme expression library by autologous serum screening, and RTN4, CLIP2 and HCLS1 from a pilocytic astrocytoma expression library also by autologous screening.

The identification of antibodies in pooled tumor patient sera does not necessarily mean that the endogenous antigens are also traceable in the tumor tissue of individual patients. Therefore, we compared the protein lists obtained from SEREX analysis and proteomic analysis with respect to overlapping proteins. Table 3 shows that five of the 13 antigens identified by SEREX were also found in the proteomic analysis of individual tumor tissue. This proves that autoantigens identified indirectly over the patients' serum can be actually found in the tumor. Moreover, as we can assume that the autoantigens must be present at a concentration high enough to provoke a humoral immune response which is also high enough to be detectable by our analytical method, the results suggest that not all antigens found in the pooled sera of different patients are actually present in an individual tumor tissue. As we will discuss in the following paragraph, the semi-top-down method elaborated in this work now allows for a more targeted screening for the tumor antigens in a larger collection of tissue samples.

Table 4 demonstrates the advantage of the semi-top-down approach compared to the shotgun approach with respect to the targeted analysis of tumor antigens in individual tissue samples. The protein reticulon 4 is readily identified by both employed methods, but shows two distinct retention windows only in the first dimension of the semi-top-down approach. In the bottom-up method, the peptides are distributed over 13 fractions. Therefore, it is not necessary to analyze the entire set of fractions obtained from the first dimension in further studies employing the semi-top-down approach, which enables the targeted analysis of a large number of tumor samples in significantly less time.

Conclusions

With this study, we deliver the most complex protein data set for glioblastoma multiforme from direct tissue analysis

available so far, including 2660 proteins with significant peptide hits in total and 1401 validated protein hits with at least two unique peptide identifications. The brain tumor proteome is represented in the pool of both employed analytical strategies by proteins covering a broad p*I* and mass range. It is further shown that, in spite of the known heterogeneity of the glioblastoma multiforme tumor tissues, a high percentage of proteins previously identified for this kind of tumor is corroborated in this study.

Furthermore, it is shown that semi-top-down analysis of a human glioblastoma multiforme tissue employing ion-pair reversed-phase chromatography and mass spectrometry represents a feasible approach to tumor tissue analysis and that the protein identification results complement those of the commonly used bottom-up approach. The advantage of the semi-top-down approach compared to the bottom-up strategy rests within the elution of the intact proteins in only few or even a single fraction of the first-dimension separation. Thus, the semi-top-down approach enables a targeted analysis in studies aiming at the characterization of tumor proteins that are eventually suitable as tumor biomarkers. The approach is proven useful for (i) a subset of membrane proteins that are potential targets for new diagnostic tools and for (ii) autoantigens immunogenic in patients with glioblastoma multiforme. In further studies, these antigens will be microprepared by the semi-top-down approach and analyzed with respect to expression levels and post-translational modifications to elucidate potential differences of different tumor grades.

Abbreviations: 2D-GE, two-dimensional gel electrophoresis; DIGE, differential gel electrophoresis; glu^1-fib, [Glu^1]-fibrinopeptide B; HBPP, Human Brain Proteome Project; HUPO, Human Proteome Organization; IP-RP-HPLC, ion-pair reversed-phase high-performance liquid chromatography; MS, mass spectrometry; SEREX, serological identification of antigens by recombinant expression screening; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TBP, tributylphosphine; WHO, World Health Organization.

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Supporting Information Available: Complete protein lists of the bottom-up and semi-top-down, as well as the merged and validated protein list. Moreover, the Mascot generic files, containing all spectral information utilized in the database searching of bottom-up and semi-top-down analysis are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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