Proteins and Protein Pattern Differences between Glioma Cell Lines and Glioblastoma Multiforme

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Abstract

Introduction: Research into the pathogenesis, molecular signaling, and treatment of glioblastoma multiforme (GBM) has traditionally been conducted using cell lines derived from malignant gliomas. We compared protein expression patterns between solid primary GBMs and GBM cell lines to identify proteins whose expression may be altered in cell culture.

Methods: We cultured cell lines U87, U118, U251, and A172 and used tissue-selective microdissection of eight primary GBMs to obtain pure populations of tumor cells, which we studied using two-dimensional gel electrophoresis (2DGE) and examined using differential expression software. Select protein targets expressed differentially between GBM tumors and GBM cell lines were sequenced using tandem mass spectrometry.

Results: Analysis of the primary GBM tumor samples (n = 8) and the GBM cell lines revealed reproducibly similar proteomic patterns for each group, which distinguished tumors from the cell lines. Gels contained up to 500 proteins that were consistently identified in the pH 4 to 7 range. Comparison of proteins identified in the GBM tumors and in the cell lines showed ~160 proteins that were gained and 60 proteins that were lost on culture. Using normalized intensity patterns from the 2DGE images, ANOVA tests were done and statistically significant spots were identified. Seven proteins found in the cell lines were significantly increased when compared with the GBM tumors (P < 0.05), whereas 10 proteins were significantly decreased from cell lines compared with the GBM tumors. Proteins identified included transcription factors, tumor suppressor genes, cytoskeletal proteins, and cellular metabolic proteins.

Conclusion: Global protein and proteomic differences were identified between primary GBM tumor samples and GBM cell lines. The proteins identified by 2DGE analysis elucidate some of the selection pressures of in vitro culture, help accentuate the advantages and limitations of cell culture, and may aid comprehension of gliomagenesis and enhance development of new therapeutics.

Gliomas are the most common primary brain tumors of adults, with a yearly incidence of ~25,000 cases in the United States (1). The most common form of glioma is the glioblastoma multiforme (GBM), an aggressive and malignant tumor. Despite decades of research on tumor biology and treatment, GBMs continue to have a poor prognosis, with a median survival of 1 year following aggressive surgical and adjuvant therapy. Because GBMs account for an estimated 2.5% of all cancer deaths in the United States, and treating these tumors remains a high priority for researchers and clinicians (1, 2).

One traditional approach to the study of GBMs was established in 1933 by Russell and Bland (3), who created transient, explant cultures from surgically removed tumors to study in vitro growth patterns. With the techniques available at the time, cell cultures of surgical specimens were difficult to establish and maintain. It was not until 1968 when Ponten et al. established a set of glioma cell lines, that the research community obtained a standard and unified method for studying gliomas in vitro (4). A number of cell lines derived from malignant gliomas are now commercially available and have become essential for research into the pathogenesis of GBMs (4, 5). Much of our knowledge of the molecular features of GBMs has been generated by analyzing these cell lines.

Although the use of cell lines for in vivo human disease fills a necessary gap, there is an implicit assumption that these tumor cell lines offer an accurate representation of the primary disease in humans (1, 2). Whereas discordances in the biology and behavior of tumor cell lines and primary tumors have long been recognized, until recently, it has been challenging to probe the larger realm of differences and similarities. Traditionally, researchers have focused on studying several genes or
structurally related proteins but have been unable to offer a broader perspective on the areas of concord and variance between tumors in the in vivo state and the cell lines used to model them experimentally.

To obtain a more global view of the protein expression patterns of cultured, malignant gliomas cells and primary GBMs, we used two-dimensional gel electrophoresis (2DGE) to evaluate four, commonly used malignant glioma cell lines, U87, U118, U251, and A172 (4, 5). We compared the protein expression patterns of these cell lines with GBMs that were surgically excised. We hypothesized that the differences and similarities between tumor samples and cell lines would highlight the potential areas of correspondence between the two states as well as delineate some of the selection pressures and variations that are inherent in cell culture. These findings may prove important for the use of GBM cell lines to model human gliomas and provide insight into potential, novel, or unsuspected targets for therapeutics.

Materials and Methods

Clinical material. Tissues and clinical information were obtained as part of Institutional Review Board–approved studies at the NIH and Vanderbilt University Medical Center. Tumors and normal tissue were collected at surgery. A total of eight histopathologically confirmed GBMs were analyzed by 2DGE, as described below. Tissue samples were immediately snap frozen in liquid nitrogen and stored at −80°C until analyzed. Histopathology was done on each set of tissue material obtained. As part of routine clinical histopathologic processing of the tumors, H&E staining and immunohistochemistry were done to verify and characterize the tumors as glioblastoma multiforme (GBM), including staining for glial fibrillary acidic protein (GFAP), which was positive in all eight surgically resected GBMs.

Cell lines. Malignant gliomas cell lines U87, U118, U251, and A172, originally described in 1968 and 1973, were acquired from American Type Culture Collection (Manassas, VA; refs. 4, 5). Cell lines were maintained according to American Type Culture Collection recommendations at 37°C, 95% O2 and 5% CO2, in DMEM supplemented with 10% heat-inactivated FCS, 50 units/mL penicillin, 50 μg/mL streptomycin, and 1% l-glutamine. Cells that had undergone five or fewer passages after thawing were selected; all had been passed many times before being obtained by American Type Culture Collection. Cell lines were cultured, grown until −80% confluent, then trypsinized. Cells were centrifuged, washed twice in 1× PBS, and a cell pellet containing −50,000 cells was frozen at −80°C until analyzed.

Tissue microdissection and two-dimensional gel electrophoresis. Selective tissue microdissection of the eight primary surgical specimens was done as described previously (7, 8). From all tumor samples, a single 10-μm-thick section was taken and stained with H&E for histologic evaluation. A semiquantitative cell count was done on tumor-rich (>90% tumor cells) areas that were not compromised by inflammation, necrosis, or stromal or endothelial proliferation (7, 8). Subsequently, these areas were subjected to selective tumor dissection from serial sections, as previously described. We collected −50,000 cells from each sample, which were obtained from 1 to 10 consecutive sections. Procurement of normal brain or areas of inflammation, necrosis, hemorrhage, or stromal or vascular proliferation was strictly avoided. Tissue dissection was done manually on unstained sections, as previously described. We collected tumor-rich (>90% tumor cells) areas that were not compromised by necrosis, hemorrhage, or stromal or vascular proliferation. We avoided. Tissue dissection was done manually on unstained sections to avoid possible heating artifacts induced by laser-assisted technology or chemical artifacts induced by tissue staining (9, 10). Protein content was compared and found equivalent to that derived from 50,000 cultured GBM cells.

Cells were placed into 50 μL of Extraction buffer II containing 8 mol/L urea, 4% (w/v) CHAPS, 40 mmol/L Tris, 0.2% (w/v) Bio-Lyte 4/7, and 2 mmol/L tributyl phosphate (Bio-Rad, Hercules, CA), vortexed at room temperature for 10 minutes, and centrifuged in a microcentrifuge at 12,000 rpm for 10 minutes. The supernatant was combined with 200 μL of rehydration Buffer (Bio-Rad) containing 8 mol/L urea, 2% CHAPS, 50 mmol/L DTT, and 0.2% (w/v) Bio-Lyte 4/7 ampholytes, before isoelectric focussing.

The first dimension of two-dimensional electrophoresis was done on a Protean IEF System (Bio-Rad) with ReadyStrip IPG strips (pH 4/7, 11 cm; Bio-Rad) rehydrated with 185 μL of sample for 12 hours and subsequently subjected to high voltages at 20°C for electric focussing: 250 V for 20 minutes, 8,000 V for 2 hours and 30 minutes, and a final step of 2,500 V for 10 hours. IPG strips were washed in rehydration buffer I containing 6 mol/L urea, 2% SDS, 375 mmol/L Tris-HCl (pH 8.8), 20% glycerol, and 2% (w/v) DTT; and buffer II containing 6 mol/L urea, 2% SDS, 375 mmol/L Tris-HCl (pH 8.8), 20% glycerol, and 2.5% (w/v) iodoacetamide (Bio-Rad), for 10 minutes each. Criterion Precast Gels (8-16% Tris-HCl, 1.0 mm; Bio-Rad) were used for the second dimension of protein separation in a Criterion Dodeca cell (Bio-Rad) under a constant voltage of 200 V for 55 minutes. Silver Stain kit (Bio-Rad) was used to detect protein spots according to the manufacturer's instructions. Methanol and acetic acid needed for fixing gels were purchased from Sigma-Aldrich (St. Louis, MO). Samples were run in duplicate.

Image and statistical analysis. High-resolution digital images were acquired for each sample gel and were analyzed using Progenesis Workstation software, according to the manufacturer's protocol (Non-Linear Dynamics, Durham, NC). Protein spots were identified by using both the automatic spot detection algorithm and manual detection as available in the software. Individual spot volumes were normalized against total spot volumes for a given gel. Averages for each cell line and for the GBM samples were then compared by their normalized volume using one-way ANOVA between-group test. One-way ANOVA compares the effect of an independent factor on the system of interest. Two normalized averages for each spot, GBM cell line and GBM tumors, were tested for the hypothesis that there is no statistical significance in the mean of the variable (normalized volumes) between the groups. Only statistically significant spots (P < 0.05) that were in common between the two averages were selected for further evaluation. Differential expression between groups was also measured using at least a 2-fold increase or decrease between averaged gels. Spots that showed evidence of saturation were not included for further analysis.

In-gel digestion. Statistically significant (P < 0.05) protein spots of interest were excised from the gel, placed into clean, 0.5-mL Eppendorf tubes, and stored at −20°C. Silver-stained gels were destained according to the method of Blum et al. (11). After destaining, individual protein gel spots were subjected to reduction and alkylation followed by in situ digestion with trypsin. The resultant peptide mixtures were recovered by sequential extraction, dried to near completion in a vacuum centrifuge, and diluted to 4 mL final volume in 2% CH3CN, 0.1% HCO2H, 0.01% trifluoroacetic acid, as previously described (12).

Mass spectrometry. Peptides from in-gel digests were analyzed by Nano-Spray liquid chromatography tandem mass spectrometry (MS/MS) technology. A capillary high-performance liquid chromatography system (LC Packings, Inc., San Francisco, CA) was interfaced to a QSTAR mass spectrometer (Applied Biosystems, Foster City, CA). Reversed phase high-performance liquid chromatography was carried out using a Fusiaca column (0.075 × 150 mm; packed with PepMap C18, 5 μm, 100Å, LC Packings, Inc., Dionex Company). The high-performance liquid chromatography system was operated at −200 mL minutes. Mobile phase A was H2O/CH3CN/HCO2H/trifluoroacetic acid (98:2:0.1:0.01), whereas mobile phase B was H2O/CH3CN/HCO2H/trifluoroacetic acid (20:80:0.1:0.01). The chromatograph was developed using a linear gradient from 5% B to 40% B over 20 minutes. Then, a second linear gradient was applied from 40% B to 95% B in 5 minutes. The gradient was held at 95% B for 10 minutes. At the end of the period, the gradient was returned to 5% B in 3 minutes and was held for 10 minutes for column equilibration before next injection. The
QSTAR was set to iteratively acquire a Positive TOF MS scan at 1 second accumulation time between 400 and 1,700 m/z followed by information-dependent acquisition. MS/MS scans at 5 seconds accumulation time between 50 and 2,500 m/z of the three most abundant ions from the preceding MS scan. An enhanced, all-collision energy for collision-induced dissociation was set to 16.7% at 30, 75, 150, 300, and 600 amu and to 16.5% at 1,200 amu. The information-dependent acquisition experiment surveys ions, which exceeds 10 counts with charge state from 2 to 5. The former target ions were excluded for 45 seconds.

Table 1. Proteins uniquely expressed in tumors or cell lines

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Protein ID#</th>
<th>Mascot score</th>
<th>Gene locus</th>
<th>OMIM#</th>
<th>Potential biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins that are specific to surgically resected GBMs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ryanodine receptor 2</td>
<td>Q92736</td>
<td>53.2</td>
<td>1q32.1-q43</td>
<td>180902</td>
<td>A calcium-release channel that is a major source of intracellular calcium.</td>
</tr>
<tr>
<td>GFAP</td>
<td>P14136</td>
<td>173.0</td>
<td>77q21</td>
<td>137780</td>
<td>An intermediate-filament protein that is highly specific for cells of astroglial lineage. Used to identify astrocytomas.</td>
</tr>
<tr>
<td>Sp2 transcription factor (Sp2)</td>
<td>gi</td>
<td>13543512</td>
<td>41.3</td>
<td>NA</td>
<td>601801</td>
</tr>
<tr>
<td>DRP-2</td>
<td>Q16555</td>
<td>123.0</td>
<td>8p21</td>
<td>602463</td>
<td>Originally identified in brain; found in all tissues except liver. Preferentially binds tubulin dimers, which are involved in cell division.</td>
</tr>
<tr>
<td>Large proline-rich protein BAT-2</td>
<td>P48634</td>
<td>60.5</td>
<td>6p21.3</td>
<td>142580</td>
<td>Also known as “HLA-B-associated transcript 2.” It contains an ubiquitin-like domain.</td>
</tr>
<tr>
<td>Unknown protein</td>
<td>gi</td>
<td>12804743</td>
<td>60.3</td>
<td>NA</td>
<td>N/A</td>
</tr>
<tr>
<td>Proteins that are specific to malignant gliomas in culture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL receptor–related protein 1</td>
<td>Q07954</td>
<td>58.9</td>
<td>12q13.1-q13.3</td>
<td>107770</td>
<td>Close homology to LDL receptor. Identical to α-2-macroglobulin and is involved in degradation of secreted amyloid precursor protein. Also seems to modulate tissue plasminogen activator’s stimulation of matrix metalloproteinase 9 secretion.</td>
</tr>
<tr>
<td>Protein disulfide isomerase precursor</td>
<td>P07237</td>
<td>94.6</td>
<td>NA</td>
<td>N/A</td>
<td>Also known as “cellular thyroid hormone binding protein.” Found on nuclear envelope; receptor is activated and levels are down-regulated, by binding thyroid hormone.</td>
</tr>
<tr>
<td>Calcyclin (S100A6)</td>
<td>P06703</td>
<td>84.1</td>
<td>1q21</td>
<td>114110</td>
<td>Calcyclin binds calcium and like S100 may serve as a substrate for tyrosine kinase(s)</td>
</tr>
<tr>
<td>α-Actin 3</td>
<td>P12718</td>
<td>134.0</td>
<td>2p13.1</td>
<td>102545</td>
<td>Now known as “enteric γ-2 actin.” Actin and its subunits are important components of cytoskeletal systems of nonmuscle cells</td>
</tr>
</tbody>
</table>

(Continued on the following page)
based, in part, on MOWSE scoring algorithms, which take into account the nonuniform distribution of peptide molecular weights. The nonuniform distribution is a function of the protease(s) used to digest the protein under study and incorporates amino acid sequence and composition qualifiers. The Mascot ion score ("Mascot score"; see Table 1) for a MS/MS match is based on the absolute probability ($P$) that the observed match between the experimental data and the database sequence is a random event: score $= -10 \times \log_{10}(P)$. The threshold for significance is chosen to be $P < 0.05$. Because the Mascot score is a logarithmic function, a score increase of 10 represents an order of magnitude in absolute probability. The SwissProt-Trmbl database was searched using Homo sapiens as a taxonomic restrictor (14).

Whereas it is conceptually possible to identify a protein based on a single peptide, we considered single peptide identification insufficient for protein identification due to the "golden match standard." Briefly, it is possible that a peptide sequence is deemed correct (i.e., the top match) based on statistical and/or subjective criteria and not actually be correct due to incompleteness of genomic databases (15). For this reason, we considered only proteins identified by MS/MS matches to at least two individual peptides.

### Table 1. Proteins uniquely expressed in tumors or cell lines (Cont’d)

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Protein ID#</th>
<th>Mascot score</th>
<th>Gene locus</th>
<th>OMIM#</th>
<th>Potential biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate kinase, M1 isozyme</td>
<td>P14618</td>
<td>43.8</td>
<td>NA</td>
<td>N/A</td>
<td>One component of the intermediate metabolism pathways. Also called &quot;Cytosolic thyroid hormone-binding protein.&quot; Functions as a kinase and binding protein.</td>
</tr>
<tr>
<td>Macrophage capping protein (actin regulatory protein)</td>
<td>P40121</td>
<td>237.0</td>
<td>2cen-q24</td>
<td>153615</td>
<td>Also called &quot;capping protein, gelsolin-like.&quot; The gelsolin family of proteins helps to modify actin structures in response to external signals, which permits rapid changes in shape during movement.</td>
</tr>
<tr>
<td>Glutathione S-transferase P</td>
<td>P09211</td>
<td>44.3</td>
<td>11q13</td>
<td>134660</td>
<td>One member of a family of enzymes that are critical to detoxification pathways.</td>
</tr>
<tr>
<td>Protein kinase A anchor protein 9</td>
<td>Q99996</td>
<td>57.5</td>
<td>7q21-q22</td>
<td>604001</td>
<td>Also known as &quot;yotiao.&quot; It appears to function as a scaffolding protein that assemblies several protein kinases and phosphatases on centrosomes.</td>
</tr>
<tr>
<td>Calgizzarin (S100A11)</td>
<td>P31949</td>
<td>46.6</td>
<td>1q21</td>
<td>603114</td>
<td>Calgizzarin is one of the S100 family of calcium-binding proteins. Overexpressed in other tumors, such as colorectal cancer.</td>
</tr>
<tr>
<td>Annexin I</td>
<td>gi</td>
<td>442631</td>
<td>493.0</td>
<td>9q11-q22</td>
<td>151690</td>
</tr>
<tr>
<td>DNA polymerase epsilon, catalytic subunit A</td>
<td>Q07864</td>
<td>39.5</td>
<td>12q24.3</td>
<td>174762</td>
<td>One of four nuclear DNA polymerases in eukaryotes. It is involved in DNA repair and possibly in replication of chromosomal DNA. OMIM # 174762. Gene locus 12q24.3.</td>
</tr>
<tr>
<td>Ubiquitin-protein ligase EDD</td>
<td>O95071</td>
<td>55.1</td>
<td>8q22.3</td>
<td>608413</td>
<td>The human orthologue of the Drosophila tumor suppressor gene &quot;hyperplastic discs.&quot; EDD is frequently over-expressed in breast and ovarian cancer. Expression can be induced by progesterone.</td>
</tr>
</tbody>
</table>

**NOTE:** 1: primary or de novo GBM; 2: secondary or progression GBM. LOD score is defined in the methods. A score of $\geq 37$ equals a $P < 0.05$. Protein ID #, protein identification number in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). OMIM (http://www.ncbi.nlm.nih.gov). Additional references on protein function can be found in the OMIM online database, with cross-references to citations available in PubMed (http://www.ncbi.nlm.nih.gov; accessed 2/7/2005). Gene locus refers to human chromosome location, if known. Abbreviations: NA, not available or not identified; OMIM, Online Mendelian Inheritance in Man; LDL, low-density lipoprotein.
study, we required a minimum of two unique peptides at disparate sites within a protein to be the minimum for a positive identification. In other words, the protein sequence was identified only after mass spectrometric analysis of two or more unique peptides, each of whose probability scores met or exceeded the threshold (P < 0.05) for statistical significance. Proteins and associations with normal and diseases states were checked and verified on the National Center for Biotechnology Information protein and Online Mendelian Inheritance in Man web sites (accessed 10/10/2004; ref. 16).

**Protein verification.** Antibodies for GFAP (1:500 dilution), S100A6 (1:500 dilution), and actin (1:500 dilution) were obtained from Chemicon (Temecula, CA), DAKO USA (Carpinteria CA), and Sigma (St. Louis, MO), respectively. Western blotting and immunohistochemistry were done using standard techniques (8).

**Results**

**Homogenous proteomic patterns.** Analysis of the primary GBM tumor samples revealed reproducibly similar proteomic patterns (Fig. 1). These basic type-specific patterns were observed in all eight of the tumor samples used for 2DGE. Similarly, a reproducible 2DGE pattern was identified in the four GBM cell lines (Fig. 1). The homogenous patterns within the two groups allowed for further comparison of the two proteomes.

To characterize the proteomic pattern of the four GBM cell lines, representative images from each cell line were compared. Each two-dimensional gel contained proteins in the pH 4 to 7 range and up to 500 differentially expressed protein spots were reproducibly identified in each gel (Fig. 1). Shared proteins common to all four GBM cell lines appear as circled areas (Fig. 1). Analysis of the GBM cell lines also showed that each cell line possessed a small quantity of unique proteins when compared with the other three cell lines (data not shown). However, the number of unique proteins for each individual cell line was small (5-10 proteins) when compared with the number of shared proteins (>100 proteins).

Similarly, in the primary tumors, whereas each tumor possessed a small number of proteins that seemed expressed in one or another tumor but not all eight tumors, this quantity (10 proteins or fewer) was small and likely represents the potentially different clinical and genetic backgrounds of the patients. In fact, the homogeneity of the protein expression patterns among the tumors, as shown in Fig. 1, was a striking and consistent feature.

**Proteins gained and lost on cell culture.** Next, we investigated the proteins that were uniquely identified in the two-dimensional gels in the GBM tumor samples or in the GBM cell lines as a whole. Gels for the primary GBMs were matched, averaged, and compared with each cell line. Only proteins found consistently in every GBM tumor and not in the cell lines were recorded (Fig. 2A). Proteins unique to the cultured cell lines were analyzed with this same method; only proteins that appeared in each of the four GBM cell line gels and not in any of the GBM tumor gels were recorded (Fig. 2B). This analysis illustrates that, from an estimate sample of ~500 proteins or protein spots resolved by 2DGE under slightly basic conditions (pH 4-7), that ~160 proteins are gained and 60 proteins are lost during cell culture.

**Statistical analysis and individual protein identification.** To analyze our data quantitatively, statistical analysis was done on averages generated from the GBM tumor samples and from the four GBM cell lines. Using normalized volumes from 2DGE images, ANOVA tests were done on the two averages and common spots with statistically significant values (P < 0.05) were chosen (Fig. 2C). Differential protein expression between cell lines and GBM tumors was also done to illustrate the degree to which these statistically significant spots were either increased or decreased (Fig. 2C). Normalized volume levels with a >2-fold increase or decrease were chosen. Seven proteins found in the cell lines were significantly increased when compared with the GBM tumors. Similarly, 10 proteins were significantly decreased from cell lines to the GBM tumors.

Next, we used MS/MS techniques to isolate and sequence 19 proteins that were uniquely expressed in either the surgically excised GBMs or in the cultured malignant gliomas (Table 1) but not in both; these proteins served to distinguish the two types of proteomic patterns. The proteins were identified after mass spectrometric analysis of two or more unique peptide sequences within that protein, each of whose probability scores for a positive identification met or exceeded the threshold (P < 0.05) for statistical significance. Analysis of microdissected solid GBMs showed expression of six unique proteins that were expressed consistently in the eight GBM tumors: GFAP, ryanodine receptor 2, the transcription factor Sp2, dihydropyrimidinase-related protein-2 (DRP-2), and the large proline-rich protein BAT2. A final protein, whose function remains unknown, was distinctive to the surgically resected GBMs. In contrast, GBM cells in culture did not express those proteins but rather 13 different proteins, including several involved in cytoskeletal movement or scaffolding (calcyclin, α-actin 3, macrophage capping protein, calgizzarin, yotiao, and LASP-1); proteins involved in intermediate metabolism or detoxification (pyruvate kinase M1 isozyme and glutathione S-transferase P); DNA repair, replication, or transcription (cellular thyroid hormone binding protein, pyruvate kinase, M1 isozyme, and DNA polymerase epsilon, catalytic subunit A); secretion of matrix metalloproteinases (low-density lipoprotein receptor–related protein 1); and a putative tumor suppressor frequently overexpressed in breast and ovarian cancer (EDD). A listing of these unique, differentiating proteins, with their potential biological functions, can be found in Table 1 (16).

All eight primary tumors, which stained immunohistochemically for GFAP (data not shown), were shown by 2DGE and liquid chromatography MS/MS to express GFAP. In contrast, none of the cell lines expressed GFAP, which is consistent with previous findings documenting loss of GFAP expression in these cultured cell lines (17, 18). To confirm the utility of this method, we validated the differential expression of one protein that segregated within each group using Western blotting (Fig. 3).

**Discussion**

In this study, we have shown that 2DGE provides a functional picture of GBM tumor cells, both in vivo and in vitro, and has several potential advantages over traditional protein-by-protein studies. Protein profiling gives a more comprehensive view of the cell’s protein compliment than is possible by individual biochemical analysis. Furthermore, protein profiling provides insight into a cell’s functional repertoire for addressing changes and selection pressures in its microenvironment. These pressures can originate from a variety of in vitro culture or in vivo tissue factors or other changes to which a tumor cell is
subjected. In addition, proteomic techniques permit a broader perspective, so that many diverse cell types, under variegated conditions and stresses, can be compared. The development of sensitive detection and identification methods, coupled with the establishment of more comprehensive human protein databases, has helped make 2DGE analysis an effective method to identify proteins involved in human disease (19–21).

Further refinements in genetic and proteomic profiling came with the development of selective tissue microdissection, which enables the procurement of pure populations of cells of interest (7, 8). We used selective tissue microdissection of primary tumor samples to confirm that GBMs and their cell lines have reproducible proteomic patterns. All four cell lines and all eight GBM tumor samples had reproducible but specific protein patterns. As suggested in previous work, this consistent proteomic-profiling pattern has diagnostic potential for GBMs and other tumors (7, 20–23).

The essential similarities among members of the four different cell lines or the surgically excised GBMs, and the significant variability between the two groups, suggests that in vitro culture exerts selection pressures that drive the GBM cell lines toward cellular homogeneity, with a consistent pattern of overexpression of some proteins and down-regulation of others. These pressures influence the phenotype of cells in culture and were documented, on a smaller scale, in gliomas and other cell lines, by several groups (6, 17, 24, 25). The changes that occur as a result of in vitro cell culture have been attributed to several factors (6, 17, 24, 25). The complex media used in cell culture are replete with metabolites, growth factors, and cytokines, among others, for which cells normally must compete in vivo. Similarly, in vivo, GBM cells are under the influence of a variety of local signals (both stimulatory and inhibitory) that they use to grow and co-opt the host environment. The in vivo state also involves capitalizing on vascular blood flow by promoting tumor angiogenesis to secure a more constant source of oxygen, often within a hypoxic microenvironment. By contrast, tumor cells in culture are grown under conditions where oxygen is unlimited and the pH is optimized. Furthermore, cell culture...
plates do not recapitulate the dynamic, three-dimensional multicellular interfaces with which tumor cells interact in vivo (6, 17, 24, 25).

These differences in environmental selection pressures may help explain the differential proteomic patterns between the GBM tumor samples and the cell lines. Our study illustrates that over 60 proteins are lost during cell culture and over 160 proteins are gained, out of the ~500 protein spots reliably identified on 2DGE over the pH range 4 to 7. It is likely that many more proteins are differentially expressed when an even larger fraction of the proteome is examined (20, 21). These proteins may have many different influences on GBM growth, resistance to current therapies, and brain invasion and migration. Further studies may yield greater insight into their role in tumorigenesis, the neoangiogenic process, and the relative insensitivity of malignant gliomas to current adjuvant treatments (1, 2, 24–26).

In addition to the large number of proteins differentially expressed between whole tumors and several commonly used GBM cell lines, we used MS/MS protein sequencing to identify a subset of proteins that were expressed in either the solid GBM tumors or the cell lines but not both (Fig. 2; Table 1). These findings offer additional insight into some of the proteins altered as tumors pass from in vivo to in vitro conditions. Six proteins expressed in all of the surgically resected tumors were sequenced (Table 1). These proteins are likely to have important roles in cell growth in several ways: by enhancing cell signaling (ryanodine receptor 2), by enhancing transcription (Sp2), or by promoting cell division (DRP-2; refs. 16, 27).

Several proteins identified in the GBM cell lines but not in the surgically excised tumors were also sequenced (Table 1). One unifying feature, which is likely a function of cell culture and/or part of the broader response to serum, is that 5 of the 13 unique proteins in the cultured cells are involved in cytoskeletal movement or scaffolding (caldesmon, α-actin 3, macrophage capping protein, calgizzarin, yotaio, and LASP-1; refs. 16, 28, 29). Similar results were noted in a microarray study of cultured fibroblasts, where up-regulation of genes involved in extracellular matrix synthesis, lipid metabolism, and cellular motility were distinctive features (30, 31). In addition, loss of GFAP expression in the cultured gliomas cells, which has previously been identified by several groups, was also seen here and serves as an important internal control (6, 17, 19, 32).

Finally, several of the proteins we identified in either the microdissected GBMs or in the cultured cells were not previously known to play a role in tumor formation in general (e.g., ryanodine receptor 2, BAT-2, DRP-2 in the solid GBMs) or in gliomas in particular (low-density lipoprotein receptor–related protein, which has been implicated previously in abnormal degradation of amyloid precursors in Alzheimer’s disease and EDD, the human orthologue of the Drosophila gene ‘‘hyperplastic’’ discs, which has been suggested to play a role in breast and ovarian cancers; refs. 16, 28, 33). Methods such as
ours raise the potential of identifying new or unsuspected protein targets for diagnosis and therapy both for GBMs and for other tumors (30, 31, 34–36).

One advantage of the 2DGE method, compared with other protein-based techniques such as matrix-assisted laser desorption/ionization-MS or surface-enhanced laser desorption/ionization-MS, is that the protein can be spotted, excised from the gel, and sequenced directly (20, 21). Whereas matrix-assisted laser desorption/ionization or surface-enhanced laser desorption/ionization are more tractable for running large-scale experiments, select tissue microdissection and 2DGE requires less in the way of instrumentation, cost, repairs, or personnel. There are some potential advantages with this method compared with oligonucleotide microarrays, as well. Several recent studies have highlighted the broad utility of microarrays in identifying transcriptional networks or in describing tumor subtypes, both in gliomas, in other tumors, and in normal cells such as fibroblasts in culture (30, 31, 34–46). One disadvantage of microarrays is that they require significant investments in time and equipment, can be challenging technically, and require significant statistical expertise (20, 34, 44). Like our method, microarrays may require downstream confirmation and functional validation (20, 34). It is also important to emphasize that with 2DGE methods such as this one, low-abundance proteins may be difficult to identify and special methods may be needed to isolate membrane-bound proteins or proteins that are very basic or acidic (the pH range in this study was 4–7, which is slightly basic; ref. 20).

Nonetheless, with the proteomic method used here, one obtains direct information about the protein or proteins encoded by a gene, as well as the ability to identify potential translational and post-translational modifications (20, 34). Thus, 2DGE may be a useful adjunct to microarrays and other expression technologies. Additional methods, such as difference gel electrophoresis or isotope-coded affinity tagging, may be needed to explore complex protein-protein interactions, to quantitate the differential expression of expressed at basal levels or to recognize tissue-restricted protein expression (20, 21). Subtractive experiments, in which microdissected tumor cells are compared with cultured cells to delineate certain pathways or mechanisms (e.g., signaling through receptor tyrosine kinases or understanding the process of tumor angiogenesis) may be done using this proteomic technique.

In summary, we used 2DGE and definitive MS/MS protein sequencing to identify proteomic profiles and to discover proteins that distinguish surgically excised GBMs from several malignant glioma cell lines. A relatively small number of proteins can be used to differentiate these two tumor states. Expression of several of these proteins seems to have arisen as a function of in vitro culturing. This method is simple, accurate, and reliable and may prove useful to gain a broader understanding of gliomagenesis in general as well as some of the advantages and pitfalls of in vitro modeling systems (8, 47). Additional studies are under way to extend 2DGE analysis to a larger spectrum of human gliomas as well as to identify and characterize additional known and suspected peptide/protein biomarkers, which may permit their use as diagnostic and prognostic markers, to predict response to therapy, or to identify new molecular targets for therapy.

References
