Loss of Collapsin Response Mediator Protein1, as Detected by iTRAQ Analysis, Promotes Invasion of Human Gliomas Expressing Mutant EGFRvIII

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Abstract
Glioblastoma multiforme (GBM) is the most common and lethal primary human brain tumor. GBMs are characterized by a variety of genetic alterations, among which oncogenic mutations of epidermal growth factor receptor (EGFRvIII) is the most common. GBMs harboring EGFRvIII have increased proliferation and invasive characteristics versus those expressing wild-type (wt) EGFR. To identify the molecular basis of this increased tumorigenic phenotype, we used iTRAQ-labeling differential proteomic analysis. Among several differentially expressed proteins, we selected CRMP1, a protein implicated in cellular invasion that was markedly decreased in GBMs expressing EGFRvIII, for further study. The differential expression of CRMP1 was confirmed in a panel of human GBM cell lines and operative specimens that express wtEGFR or mutant EGFRvIII by quantitative real-time PCR, Western blot, and immunohistochemical analysis. In human GBM samples, decreased expression of CRMP1 correlated with EGFRvIII positivity. Knockdown of CRMP1 by siRNA resulted in increased invasion of wtEGFR expressing human GBM cells (U87 and U373) to those found in isogenic GBM cells. Exogenous expression of EGFRvIII in these wtEGFR-expressing GBM cells promoted their ability to invade and was accompanied by decreased expression of CRMP1. Rescuing CRMP1 expression decreased invasion of the EGFRvIII-expressing GBM cells by tilting the balance between Rac and Rho. Collectively, these results show that the loss of CRMP1 contribute to the increased invasive phenotype of human GBMs expressing mutant EGFRvIII. [Cancer Res 2009;69(22):8545–54]

Introduction
Glioblastoma multiforme (GBM) is the most common, aggressive, and incurable central nervous system malignancy, largely due to its highly invasive phenotype. Episomal amplifications in the epidermal growth factor receptor (EGFR) gene are prevalent in approximately half of GBMs, but not in lower grade gliomas (1–3). Among these GBMs, approximately half have an intragenic deletion of exons 2 to 7 of the wild-type (wt) EGFR gene, resulting in an in-frame deletion of 267 amino acid residues from the extra-cellular domain of the 180-kDa wtEGFR. This mutant EGFR, termed ΔEGFR, de2-7 EGFR, or most commonly EGFRvIII (4–7), results in a truncated ~140-kDa constitutively activated EGFRvIII, with an identical intracellular domain to wtEGFR (3, 4, 8). Preclinical studies have shown that GBMs that harbor EGFRvIII have an in vitro and in vivo growth advantage, compared with GBMs that just overexpress wtEGFR (9). Clinical studies suggest that the ~25% of all GBM patients whose tumors express both EGFRvIII and wtEGFR have a shorter interval to relapse and decreased survival rate, compared with GBMs with only wtEGFR (2, 10).

Much of the aggressive phenotype of GBMs expressing EGFRvIII is a result of increased invasion. A large number of molecules whose interactions and coregulations promote or inhibit GBM migration have been proposed, but it remains an ongoing major area of research focus in neurooncology (11–13). The mechanism(s) for the increased invasive phenotype by GBM cells expressing EGFRvIII is not clear, and not entirely explained by the ligand-independent constitutive basal activation of EGFRvIII for the following reasons: First, the basal activation of EGFRvIII has been noted to be <10% of EGFR- or transforming growth factor-α–stimulated wtEGFR found in GBMs growing in vivo (14). Second, the major intracellular tyrosine autophosphorylation sites, and the major signaling pathways they activate, are identical between EGFRvIII and ligand-stimulated wtEGFR (15). This would suggest that additional mechanism(s), as reflected by differences in the proteome between GBMs, which do or do not express EGFRvIII, likely exists, and these differences lead to the difference in the invasion and malignancy potential of GBM.

Differential proteomic analysis between less invasive and highly invasive cancer cells have already led to identification of several proteins/gene implicated in invasion and metastasis in several human cancers, including NM23, AKT1, and KiSS-1 (16–18). The development of novel mass spectrometry (MS)–based technologies, such as iTRAQ-labeling, yields great promise in our ability to interrogate differences in the proteome between pathologic and normal tissues (19–21). In the present investigation, we used preclinical in vitro and in vivo human GBM models that are well characterized for their expression profiles of wtEGFR and EGFRvIII. The difference in the proteome was analyzed by liquid chromatography-tandem MS (LC-MS/MS) with iTRAQ-labeling, with our interest in proteins that are related to invasion. Our initial focus is on Collapsin Response Mediator Protein 1 (CRMP1), which was markedly decreased in GBM tumors expressing EGFRvIII as determined by iTRAQ analysis, and has not been implicated previously in glioma biology. CRMP1 was previously implicated as a negative regulator of cellular invasion in lung cancer models (22, 23). Expression of CRMP1 was related to tumor stage, lymph node metastasis, and...
patient survival. Recently, Wu and colleagues (24) showed the role of cyclooxygenase-2 in regulating the expression of CRMP1 via reciprocal regulation of Sp1 and CAAT/enhancer binding protein α. Involvement of NF-kB was also reported recently (25) to negatively regulate CRMP1 gene in lung adenocarcinoma cells, thereby promoting invasion.

The above data from lung carcinoma and the decreased expression found by our iTRAQ differential labeling expression on GBMs expressing EGFRvIII are both suggestive of an “invasion suppressor” role of CRMP1. To further characterize CRMP1 in malignant glioma invasion, we found decreased CRMP1 expression at the RNA and protein levels in isogenic human GBM cells that differ only in their EGFRvIII expression profile and a larger set of human GBM operative specimens. Alteration of CRMP1 expression modulated the ability of human GBM cells to invade in in vitro assays. Mechanistically, decreased CRMP1 expression shifted the balance between activation of Rho- and Rac-GTPases to promote the invasive phenotype of human GBMs that express EGFRvIII.

Materials and Methods

GBM specimens and cell lines. Flash-frozen human GBM operative samples and corresponding paraffin sections were obtained from the University of Toronto Nervous System Tumor bank, in accordance with Research Ethics Board guidelines and consent. Human GBM s.c. explants, verified by our neuropathology colleague (SC) before implantation into Nod-Scid mice and also between serial passages, were obtained as per institutional consent and guidelines.

The established human glioma cell lines U373 and U373S (American Type Culture Collection) were maintained in DMEM containing 10% fetal bovine serum (FBS) at 37°C, 5%CO2 in a humidified chamber. EGFRvIII was cloned into the tet-inducible TET-OFF vector (pTRE, Clontech) and cotransfected with the regulatory plasmid (pTA) into human U87 and U373 astrocytoma cells. Clones expressing the pTRE-EGFRvIII (U87vIII, U373vIII) or the empty pTRE vector (U87T, U373P) were grown in DMEM supplemented with 1% tet-approved FBS (Clontech).

For iTRAQ analysis, four frozen GBM explants, with two each expressing wtEGFR and EGFRvIII, were immersed in 0.5 mL PBS containing protease inhibitors [1 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μmol/L leupeptin, 1 μg/mL aprotinin, and 1 μmol/L pepstatin] and mechanically homogenized at 30,000 rpm using a Polytron PT 1300D handheld homogenizer (Brinkmann). The samples were stored in aliquots at −70°C until used for further processing. Two hundred micrograms of each of the four GBM lysates were digested and labeled with the following iTRAQ labels: wtEGFR, 114 and 116 labels; EGFRvIII, 115 and 117 labels. Samples were processed by offline two-dimensional LC-MS/MS analysis as described previously (19, 20). After separation by strong cation exchange (SCX), each fraction was dried and resuspended in 10 μL of 0.1% formic acid. SCX fractions 6 to 30 were then sequentially analyzed by LC-MS/MS. Each fraction was analyzed in duplicate. One microliter of each of the resuspended SCX fractions was loaded onto the reverse phase precolumn. A binary gradient, comprising aqueous solutions of 5% acetonitrile plus 0.1% formic acid (solvent A) and 95% acetonitrile plus 0.1% formic acid (solvent B), was used for the reverse phase nanoflow separation as described below.

Time (min) 0 10 15 125 145 150 160 162 188
% Solvent B 5.0 5.0 15.0 35.0 60.0 80.0 80.0 5.0 5.0

In the above timetable, the sample was first loaded onto the reverse phase precolumn at 0 min, where it was desalted by passage of solvent A at a flow rate of 25 μL/min for 4 min. At the 4th minute, the precolumn was switched inline with the analytic column until the 177th min, at which time the precolumn was switched out of line. Data were acquired under the information-dependent acquisition mode, with dynamic exclusion set to exclude any m/z values that had been picked for MS/MS scan in the previous 30 s. This last condition was intended to minimize the number of repeated analyses of any particular peptide, thus maximizing the number of unique peptides identified during the course of an LC separation. The information-dependent acquisition script was also set to pick peaks nearest to a threshold setting of 12 counts in the MS scan on every fourth cycle, ensuring also the identification of low-abundance peptides.

MS data were searched using ProQUANT v1.1 against the human KBMS 20041109 database containing 178,239 protein sequences (Applied Biosystems). Proteins whose unique peptides contributed to an overall score of >1.3, which corresponds to a confidence cutoff of 95%, were considered as positive hits. This led to identification of a total of 826 proteins. Proteins were selected for additional, confirmatory analyses, including immunohistochemistry, if the relative ratios of the proteins in both samples expressing EGFRvIII were >50% when compared with both samples expressing wtEGFR (Table 1). The ratios reported were automatically normalized using an “applied bias” factor, derived from the median ratio of all proteins that had been quantified from all the SCX fractions that were run in each run. This applied bias was based on the assumption that the majority of the proteins identified would not be differentially expressed between the samples being analyzed and is used to correct for any systematic errors introduced as a result of minor variations the volumes of samples used for the analysis, as well as variations in the amount of blood proteins in the initial samples.

Immunohistochemical staining for paraffin sections. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded 5-μm-thick tissue GBM sections. Sections were first deparaffinized, rehydrated, antigen retrieved in citrate buffer (pH 6), and blocked for endogenous peroxidase activity in 0.3% H2O2 in methanol for 20 min. Sections were treated with 100 μL of 10% normal human serum for 10 min to block nonspecific binding of the primary antibody. Primary antibodies, anti-human CRMP1 (Abcam; 1:500), diluted with TBS, were added to slides for 1 h at room temperature. Slides were washed twice in TBS for 5 min and incubated with biotinylated IgG secondary antibody for 30 min at room temperature. Detection of antibody was performed using Vectastain ABC reagent and 3,3′-diaminobenzidine chromogen (Vector Laboratories). Negative controls were obtained by replacing the primary antibody with an equal concentration of mouse IgG2b (Sigma).

Quantification of gene expression by real-time PCR. Two hundred nanograms of total RNA from each sample was treated with DNase 1 (0.4 units/μg RNA) according to instructions of the Message Clean kit (Gen-Hunter Corp.). Fifty nanograms of DNase I–treated total RNA was used for cDNA synthesis in a 20-μL RT reaction with oligo (dt)12-18 (Integrated DNA Technology), and SuperScript II reverse transcriptase and RNaseOUT enzyme (Invitrogen) deoxynucleotide triphosphates were maintained at 0.5 mmol/L. CRMP1-, wtEGFR-, and EGFRvIII–specific primers corresponding to the PCR targets were obtained from Integrated DNA Technology (Table 2). Unless otherwise mentioned, each reaction was done in triplicate, in three independent experiments. The mean concentration of HPRT1 was used as the control for input RNA and the average fold difference between samples was calculated using the Applied Biosystem software.

siRNA-mediated knockdown of CRMP1. U373 and U373S glioma cells at 70% confluence were transfected in OPTIMEM medium (Invitrogen) with the indicated siRNA duplexes using Lipofectamine 2000 (Invitrogen). After 4 h, the transfection medium was removed. The cells were washed twice with PBS, and then maintained in complete medium for 48 to 72 h before further experimentation. The sequences were synthesized by Qiagen. Target sequences were as follows: control, AAT TCCCGAAGTGTGTCAG; CRMP1-siRNA #1, 5′-AAGGCAGTGGCTACTGCGA-3′; and CRMP1-siRNA #2, 5′-AAGGCGATGG CCGCTTTATT-3′.

Protein extraction and western blot analysis. Whole-cell protein lysates from human GBM explants, cell lines, and frozen operative specimens were prepared in modified PLC lysis buffer supplemented with protease and phosphatase inhibitors (Calbiochem). Protein concentration was determined using the BCA assay (Pierce Chemical Co.). Protein lysates were
separated on SDS-PAGE gels (either 10% or 12.5%) and transferred onto polyvinylidene difluoride membrane (NEN Research Products/Du Pont) using a semidry transfer apparatus (Bio-Rad). Membranes were probed overnight with the following antibodies, unless otherwise stated, with CRMP1 (Abcam, 1:200), wtEGFR (1:500, 1 h at room temperature), Rho (Millipore, 1:350), Rac (Millipore, 1:1,000), FAK (Abcam, 1:1,000), p-Fak (Abcam, 1:1,000, Tyr 397), and β-actin (Sigma-Aldrich, 1:20,000). Membranes were washed the next day and incubated with appropriate horseradish peroxidase–conjugated secondary antibodies (Bio-Rad). Protein bands were visualized with Chemiluminescence Reagent Plus (PerkinElmer Las, Inc).

**Table 1.** Differential iTRAQ-labeled proteins between two EGFRvIII and two wtEGFR human GBM explants, which differ by ~1.5-fold

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Accession no.</th>
<th>EGFRvIII#1: wtEGFR#1</th>
<th>116:114 (wt2:wt1)</th>
<th>EGFRvIII#2: wtEGFR#1</th>
<th>Function</th>
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</thead>
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<tr>
<td>Cpn10</td>
<td>P61604</td>
<td>0.27</td>
<td>0.83</td>
<td>0.27</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>SPARC-like 1</td>
<td>Q14515</td>
<td>0.19</td>
<td>0.63</td>
<td>0.42</td>
<td>Neuronal migration</td>
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<tr>
<td>Transferrin</td>
<td>ISUV_E</td>
<td>0.43</td>
<td>0.73</td>
<td>0.47</td>
<td>Iron binding protein</td>
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<td>Collapsin response mediator protein-1 CRMP1</td>
<td>Q14194</td>
<td>0.34</td>
<td>0.88</td>
<td>0.59</td>
<td>Axonal guidance, invasion</td>
</tr>
<tr>
<td>Putative NF-κB activating protein</td>
<td>trm</td>
<td>Q7Z435 gb</td>
<td>AH178 01.1</td>
<td>0.08</td>
<td>0.20</td>
</tr>
<tr>
<td>3′(2′),5′-bisphosphate nucleotidase 1</td>
<td></td>
<td>0.42</td>
<td>0.82</td>
<td>0.49</td>
<td>Adhesion between cytoskeleton and plasma membrane and ECM</td>
</tr>
<tr>
<td>Glucosamine 6-sulfatase</td>
<td>P15586</td>
<td>0.24</td>
<td>0.51</td>
<td>0.32</td>
<td>Modulation of cell surface molecule and ECM</td>
</tr>
<tr>
<td>Cyclin-dependent kinase 4 inhibitor A (p16-INK4)</td>
<td>P42771</td>
<td>0.29</td>
<td>0.93</td>
<td>0.29</td>
<td>Apoptosis/cell cycle</td>
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<tr>
<td>Tetraspanin-14</td>
<td>Q8NG11</td>
<td>0.36</td>
<td>0.70</td>
<td>0.36</td>
<td>Metastasis suppressor</td>
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<tr>
<td>Barrier-to-autointegration factor (BAF)</td>
<td>075531</td>
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<td>Cell cycle</td>
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<td>Cullin homologue 1</td>
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<td>0.85</td>
<td>0.63</td>
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<td>Microtubule-associated protein 1 B (MAP 1 B)</td>
<td>P46821</td>
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<td>0.86</td>
<td>0.47</td>
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<tr>
<td>Class II bHLH protein PTF1A</td>
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<td>QRTS3 NP_006608.1</td>
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<td>0.83</td>
<td>0.23</td>
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<tr>
<td>Nestin</td>
<td></td>
<td>3.08</td>
<td>1.03</td>
<td>2.79</td>
<td>Astrocyte intermediate filament proteins</td>
</tr>
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<td>Vimentin</td>
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<td>2.24</td>
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<td>2.07</td>
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<td>0.85</td>
<td>0.80</td>
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<td>1.03</td>
<td>2.03</td>
<td>Apoptosis</td>
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</table>

**NOTE:** The ratios reported above are ProQUANT ratios of the levels of the individual proteins in the three other samples relative to that present in the sample designated wtEGFR#1. All accession numbers above without “trm,” “gb,” or “NP” prefixes are Swiss Prot accession numbers.

**Table 2.** List of primer sequences used in this study

<table>
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<tr>
<th>SL #</th>
<th>Gene</th>
<th>Primer sequence</th>
<th>Tm</th>
<th>Product size (bp)</th>
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</thead>
<tbody>
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<td>1</td>
<td>CRMP1</td>
<td>GCAGTCCCAAAACCCCTTACA GAGGATGCTTCTCTGCAAC</td>
<td>60</td>
<td>154</td>
</tr>
<tr>
<td>2</td>
<td>EGFR</td>
<td>ATG CGA CCC TCC GGGACG GAG TAT GTG TGA AGG AGT</td>
<td>60</td>
<td>1153 (ForWt) 352 (For VIII)</td>
</tr>
<tr>
<td>3</td>
<td>EGFR-WT</td>
<td>TGT CGA TGG ACT TCC AGA AC ATT GGG AC A GCT TGG ATC A</td>
<td>60</td>
<td>62</td>
</tr>
<tr>
<td>4</td>
<td>EGFR-VIII</td>
<td>GAG TGG GTC TCT GGA GGA AAA G CCA CAG GCT CCG AGG CAC</td>
<td>60</td>
<td>72</td>
</tr>
<tr>
<td>5</td>
<td>HPRT1</td>
<td>ATGCTGAGGATTTGAAAG CTCCCAATCTTCTCATTACA</td>
<td>60</td>
<td>93</td>
</tr>
<tr>
<td>6</td>
<td>β-ACTIN</td>
<td>GATGAGATTGGCATGGCCTT</td>
<td>60</td>
<td>100</td>
</tr>
</tbody>
</table>

**Plasmids and transfection.** Human CRMP1 was obtained from OR-Feomes collection (26, 27) and cloned into a Gateway entry vector pDONR223 (Invitrogen). A recombination reaction was performed to transfer the inserts from these entry vectors into destination vectors to generate pcDNA3.2-Myc-DEST-CRMP1, which were used to transform Escherichia coli DH10B-R cells. The empty vector plasmid used as a control was prepared by removing the Gateway recombinational region between SacI and ApaI fragment from pcDNA3.2-Myc-DEST-CRMP1. Transient transfections were done in U87-vIII glioma cell lines by FuGENE 6 transfection reagent (Roche Diagnostics) following the protocol of the manufacturer. Cells were transfected with the following plasmids: pcDNA3.2-Myc-DEST-CRMP1, empty vector, or pDONR223-CRMP1.
Figure 1. CRMP1 peptide: Tandem mass spectrum and corresponding iTRAQ reporter ions. A, tandem mass spectrum identifying a peptide from CRMP 1. B, iTRAQ reporter ions for the CRMP1 peptide shown in A.
transfected with empty plasmid vector were used as controls. Transfected cells were cultured for an additional 48 h before use.

GTPase pull-down assays. Rac-GTP and Rho-GTP were pulled down by the use of lysates containing 2 mg protein and GST-PBD (Rac-binding domain of PAK1) and GST-Rhotekin BD—conjugated (Rho-binding domain of Rhotekin) beads (28, 29). In short, cells were lysed with lysis buffer [25 mmol HEPES (pH 7.5), 150 mmol NaCl, 1% NP40, 10% glycerol, 10 mmol MgCl₂, 1 mmol/L EDTA, and protease inhibitors] and then incubated for 1 h at 4°C with glutathione-Sepharose beads bound to 20 μg of GST-PBD or GST-Rhotekin BD. The beads were then washed thrice in lysis buffer, resuspended, and boiled in sample buffer (1×), and the supernatants were subjected to SDS-PAGE followed by immunoblotting with the appropriate antibodies, as described above.

Migration assay. Migration assays were performed using gelatin-coated polycarbonate filters (pore size, 8 μm) of transwells separating the upper and lower chamber of 24-well plates (Costar). Chemotaxis medium (DMEM with 0.5% bovine serum albumin and 10 mmol/L HEPES) was added to the bottom chamber. Parental and CRMP1-transfected U87 and U373 cells (0.5 × 10⁶/well) were placed in the upper chamber. The plates were incubated at 37°C overnight. Viable cells in the lower chamber were collected and counted.

Invasion assay. Invasive potential was measured by using an 8-μm pore, 24-well format Matrigel-coated transwell chamber assay (Becton Dickinson Discovery Labware). For the CRMP1 siRNA and transient overexpression experiments, cells were transfected and were harvested 48 h after transfection. After harvesting, cells were washed and resuspended in serum-free medium. Cell concentration was measured and adjusted to 2 × 10⁵ cells/mL. Sixty thousand cells (0.3 mL) were added to the top of each hydrated chamber, with 1 mL medium plus 10% FBS used as chemoattractant in the bottom well plate. After 22 h of incubation, cells were processed according to the manufacturer’s protocol. The number of invading cells was quantified by counting them in at least six random fields (200×).

Statistical analysis. The unpaired Student’s t test was applied for comparison between two groups; one-way ANOVA test with post hoc Tukey-Kramer multiple comparisons was used for comparison among multiple groups.

Results

Differential proteome between wtEGFR and EGFRvIII GBM explants as detected by iTRAQ. iTRAQ-labeling LC-MS/MS analyses led to the identification of ~800 proteins. Most of these proteins did not exhibit differential expression between the two pairs
of samples. Those that show expression changes in the EGFRvIII samples larger than 1.5-fold relative to the wtEGFR-expressing GBM samples are summarized in Table 1, along with cytoplasmic actin, a housekeeping control protein that is not expected to show differential expression. Out of this list of proteins, the CRMP1 (Fig. 1) showed decreased expression in both EGFRvIII-expressing GBMs, and was picked for further analysis and verification by Western analysis and reverse transcription-PCR (RT-PCR) analysis. We chose CRMP1 for our initial studies as this protein had been implicated in cellular invasion—our prime biological query as to differences between GBMs, which express EGFRvIII versus those which only express wtEGFR. Figure 1 shows tandem MS spectra and iTRAQ reporter-ion windows, demonstrating differential expression of CRMP1. As shown below, confirmatory biochemical tests performed on the same samples corroborated the iTRAQ findings of differential expression.

**Verification of CRMP differential expression in human GBMs.** The four human GBM explants used for the iTRAQ analysis were used for verification of the iTRAQ results that CRMP1 was decreased in EGFRvIII GBMs (Fig. 2). Western blot analysis (Fig. 2A) and RNA level as shown by RT-PCR analyses (Fig. 2B) confirmed decreased CRMP1 expressions in the two EGFRvIII-expressing GBM explants. This decrease in CRMP1 expression was confirmed at the immunohistochemical level (Fig. 2C).

For additional verification, we examined isogenic human U87 and U373 GBM cell lines that only differ in their EGFRvIII expression profile (Fig. 2D). Similar to the GBM explants, expression of EGFRvIII in U87 and U373 cells resulted in decreased CRMP1 both at the RNA and protein levels.

Lastly, we examined a panel of human GBM operative specimens (n = 10), by RT-PCR and quantitative RT PCR (Fig. 3). In the human GBM samples, 9 of 10 expressed CRMP1 to varying extent by qRT-PCR analysis (Fig. 3A). Levels of CRMP1 expression was quantified by qRT-PCR analysis, with the same samples also checked for the expression of wtEGFR and EGFRvIII. Levels of CRMP1 correlated inversely to expression of EGFRvIII, with lower expression in those GBMs that expressed high levels of EGFRvIII, compared with those GBMs that express mainly wtEGFR (Fig. 3B). This inverse correlation was statistically significant (P <.05) as analyzed by ANOVA. These RNA results were also reflected by immunohistochemical analysis (Fig. 3C). Of interest, CRMP1 expression was abundant in the normal region of the brain from a surgery for a GBM that expressed EGFRvIII (Fig. 3C). Negative controls (antibody omission or nonimmune similar antibody subclass) were uniformly negative (data not shown).

**Functional significance of differential expression of CRMP1.** To determine if CRMP1 contributes to the migration of glioma cells, transwell migration assays were performed. CRMP1 expression level in siRNA-transfected cells were determined by Western blot analysis. siRNA #1 at 60 nm concentration showed significant level of CRMP1 knockdown (~70%) in both the cell lines and were selected for further studies. Figure 4B shows that following
knockdown of CRMP1 in U87 and U373 cells, migration from the upper chamber to the lower was increased ∼75% (P < 0.01) and ∼60% (P < 0.01), respectively. In fact, CRMP1 knockdown parental cells migrated at almost the same rate as U87-vIII and U373-vIII cells. Differences in invasion was analyzed by modified Boyden chamber assay and also showed statistically significant (P < 0.01) increase in invasion of U87 and U373 cells with CRMP1 knockdown, compared with parental cells (Fig. 4C). Similar to migration, the CRMP1 knockdown cells had a similar invasion rate to U87-vIII and U373-vIII cells, respectively. Cells treated with scrambled control siRNA showed no significant difference from parental cells in either migration or invasion assays.

To further confirm the role of CRMP1, we rescued the expression of CRMP1 in U87-vIII and U373-vIII cells, as both these cell line express lower amounts of CRMP1 compare to parentals (Figs. 2D and 5A). Overexpression of CRMP1 in U87-vIII and U373-vIII cells resulted in a ∼35% and ∼38% reduction (P < 0.01) in invasion compared with the empty vector controls (Fig. 5B).

Modulation of RhoA/Rac and FAK activation by CRMP1. It has been previously recognized that EGFR activation contributes to cell motility in a mechanism involving RhoA/Rac pathway (30). Compared with parental U87s, in U87-vIII cells, Rac1-GTP levels were elevated, whereas Rho-GTP was decreased in U87-vIII cells (Fig. 5C). CRMP1 overexpression in these U87-vIII cells decreased Rac1-GTP and increased RhoA activity, compared with the U87-vIII empty vector transfected controls (Fig. 5C).

We next examined the effects of CRMP1 on U87 cell morphology and the status of focal adhesion kinase (FAK) turnover by monitoring FAK activation. U87-vIII cells had statistically high levels of active phosphorylated FAK (P-FAK Y397), compared with parental U87 cells (Fig. 5D). CRMP1 overexpression in these U87-vIII cells resulted in decrease of P-FAK, to levels approaching parental U87 cells. Typical phase-contrast images of control and CRMP1 expression–modulated cells illustrate marked changes in morphology (Fig. 5E). Parental U87 cells showed small cell bodies with short protrusions, whereas U87-vIII cells also had small cell bodies but with long extensions. In contrast, U87-vIII cells overexpressing CRMP1 exhibited flat morphology and very little or no protrusions (Fig. 5E). Interestingly knockdown of CRMP1 in parental U87 cells showed elongated protrusion, similar morphology to U87-vIII cells.

**Figure 4.** Functional significance of CRMP1 expression. A. siRNA-mediated knockdown of CRMP1 resulted in ∼70% downregulation of CRMP1 protein expression in parental (i) U87 and (ii) U373 cells. Numbers below each Western blot represent densitometry values. B. change in migration after siRNA-mediated knockdown of CRMP1 in (i) U87 cells and (ii) U373 cells. Knockdown of CRMP1 resulted in increased migration of U87 and U373 cells, which approached values observed by U87-vIII and U373-vIII cells. Columns, mean from three independent experiments done in triplicate; bars, SEM; *, P < 0.01. C. invasion potential of the (i) parental U87, U87-vIII, and U87 with CRMP1 knockdown cells, and (ii) parental U373, U373-vIII, and U373 with CRMP1 knockdown cells using modified Boyden chamber assay. Knockdown of CRMP1 resulted in increased invasion of U87 and U373 cells, which approached values observed by U87-vIII and U373-vIII cells. Columns, mean from three independent experiments done in triplicate; bars, SEM; *, P < 0.01.
To understand the consequences of molecular variations of EGFR status on tumor invasion and growth, we used two well-characterized GBM subtypes differing in their expression profile for EGFRvIII and wtEGFR for identification of differentially expressed proteins by iTRAQ analysis. The results of the EGFRvIII versus wtEGFR iTRAQ experiments, which yielded differential expression of CRMP1, a protein implicated in lung cancer invasion, were further validated at the mRNA and protein levels. Five members of the CRMP gene family (CRMP1–5), encoding closely related 60- to 66-kDa proteins, have been cloned and are transcriptionally differentially regulated (31–36). The best characterized function of these proteins remains the repulsive guidance of nerve axons, but the molecular mechanism remains uncharacterized (34). CRMP2 is associated with neurofibrillary tangles in patients with Alzheimer’s disease (37). CRMP3 and CRMP5 are recognized by autoantibodies from patients with small-cell lung cancers who develop paraneoplastic neurologic syndrome (38, 39). Our focus was on CRMP1 because it was implicated as a suppressor of invasion in lung cancer (23–25), the rate-limiting biological property of human malignant gliomas.

We found expression of both CRMP1 mRNA, and protein was inversely associated with the migratory and invasive activity of two human GBM cell lines, with knockdown of CRMP1 increasing in vitro invasive activity (Fig. 4B and C). In addition, we found lower CRMP1 mRNA and protein expression in nearly 50% of GBM specimens examined (Fig. 3A and B). This negative regulation of EGFRvIII on CRMP1 is at the transcript and subsequent protein level and not genomic alterations in CRMP1 chromosomal locus (chromosome location 4:5,822,492–5,894). Future experiments will focus on establishing if an association exists between CRMP1 expression and advanced disease, early postoperative recurrence, and survival of patients with GBM.

The mechanism(s) of how CRMP1 acts to suppress invasion is of importance. Recent studies showed that members of the CRMP family of proteins mediate semaphorin3A-PlexinA1 signal transduction...
cascade (31), as semaphorin/collapsin families might control the movement of cells (40). The Rho GTPase family, which regulates the actin cytoskeleton, has been shown to be involved in processes of neurite outgrowth following semaphorin signaling (41). Some direct connections between CRMPs and Rho protein signaling have been implicated with CRMP-2 in combination with active Rho or Rac GTPases effect, a cyclical switch in signaling promoting dynamic shape change (42, 43). In the present study, parental U87 cells have higher levels of CRMP1 with associated increased levels of activated Rho-A compared with activated Rac1 (Fig. 5C). In comparison, highly invasive U87-EGFRvIII–transfected cells had lower levels of CRMP1 expression, with resultant increase in activated Rac-1. We believe this shift causes actin reorganization governed by Rac-1 to become dominant, with resultant formation of membrane ruffles and lamellipodia, and increased invasion (Fig. 5E). Furthermore, reintroduction of CRMP1 in U87-EGFRvIII cells caused a significant decrease in Rac-1 activity, while at the same time, it increased the activity of Rhoa (Fig. 5C), resulting in formation of focal adhesions (44). Together, these two GTPases enable the dynamic process of detachment of cells from the extracellular matrix (ECM) and their reattachment to it, which are essential for cell migration (45). Studies have shown that activation of one of these proteins can abolish the actin organization governed by the other, suggestive of a morphologic antagonism between Rac-1 and Rha (46–48).

The turnover of focal adhesions, regulated by activation of FAK, is important for cellular movement. FAK activation (Fig. 5D) was modulated by CRMP1 and correlated with morphologic changes of the U87 cells (Fig. 5E) along with its changes in invasive and migratory behavior (Fig. 4). Overexpression of CRMP1 in U87vIII cells, which express high levels of activated phospho-FAK Y397, resulted in decreased P-FAK toward levels exhibited by parental U87 cells (Fig. 5D). The modulation of CRMP1 expression was accompanied by alterations in cell morphology. Knockdown of CRMP1 by siRNA caused the U87 cells to become longer and slender compared with the parental U87 with small cell bodies and short protrusion (Fig. 5E, i and ii), similar to the morphologic features of U87-EGFRvIII cells with decreased endogenous CRMP1 expression (Fig. 5E, iii). However, U87-EGFRvIII cells overexpressing CRMP1 became flatter with large cell bodies, devoid of any protrusion (Fig. 5E, iv). The significance of these morphologic changes have implications for cell motility (45). Once the actin is organized into stress fibers, and focal adhesions are assembled, the cells flatten and become attached to the ECM. Cells in motion need to assemble and disassemble actin structures to progress by alternating between attachment to and detachment from the ECM (45).

Overall, our observations are in support of the thesis that loss of CRMP1 in GBMs, which express EGFRvIII, is involved in their more aggressive tumor phenotype. Mechanistically, this involves increased relative activation of Rac-GTPase and inhibition of Rho-GTPase. Alteration of these GTPases results in increased activation of FAK, thereby modifying the morphology of glioma cells and increasing their migratory and invasive characteristics. Our study also is an early demonstration of how differential MS analysis of well-defined tumor sets can shed light into relevant alterations in their proteome. In addition to CRMP1, the iTRAQ analysis between GBMs that do and do not express EGFRvIII also yielded several other differentially upregulated proteins, including the intermediate filaments nestin and vimentin (Table 1). These structural proteins have been implicated as positive regulators of GBM cell motility and metastasis. They are in support of the recognized increased invasiveness of EGFRvIII–expressing GBM cells, analogous to the focus of our work on loss of CRMP1 and increased invasion in EGFRvIII GBM cells. In addition, overexpression of these proteins may also play a role in epithelial-mesenchymal transition resulting from expression of EGFRvIII. Epithelial-mesenchymal transition is defined as switching of polarized epithelial cells to a migratory fibroblastic phenotype and is implicated in the progression and metastasis of various cancers. Loss of epithelial proteins and/or the acquisition of mesenchymal proteins are associated with poorly differentiated histology, advanced stage, and poor outcome, all attributes of EGFRvIII–expressing GBMs. Verification of the MS findings in human specimens followed by functional and mechanistic studies can contribute much to the understanding of the biological differences between the relevant tumor sets and hopefully elucidate novel biological targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 5/14/09; revised 7/29/09; accepted 8/3/09; published OnlineFirst 11/10/09.

Grant support: The study was financed in part by grants from National Cancer Institute Canada and Neurofibromatosis Society of Ontario (A. Guha).

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J. Mukherjee is thankful to American Brain Tumor Association and Ministry of Research and Innovation, Gov. of Ontario for personnel funding.

References


Loss of Collapsin Response Mediator Protein1, as Detected by iTRAQ Analysis, Promotes Invasion of Human Gliomas Expressing Mutant EGFRvIII

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