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Phosphotyrosine signaling analysis of site-specific mutations on EGFRvIII identifies determinants governing glioblastoma cell growth†

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To evaluate the role of individual EGFR phosphorylation sites in activating components of the cellular signaling network we have performed a mass spectrometry-based analysis of the phosphotyrosine network downstream of site-specific EGFRvIII mutants, enabling quantification of network-level effects of site-specific point mutations. Mutation at Y845, Y1068 or Y1148 resulted in diminished receptor phosphorylation, while mutation at Y1173 led to increased phosphorylation on multiple EGFRvIII residues. Altered phosphorylation at the receptor was recapitulated in downstream signaling network activation levels, with Y1173F mutation leading to increased phosphorylation throughout the network. Computational modeling of GBM cell growth as a function of network phosphorylation levels highlights the Erk pathway as crucial for regulating EGFRvIII-driven U87MG GBM cell behavior, with the unexpected finding that Erk1/2 is negatively correlated to GBM cell growth. Genetic manipulation of this pathway supports the model, demonstrating that EGFRvIII-expressing U87MG GBM cells are sensitive to Erk activation levels. Additionally, we developed a model describing glioblastoma cell growth based on a reduced set of phosphoproteins, which represent potential candidates for future development as therapeutic targets for EGFRvIII-positive glioblastoma patients.

Introduction

Protein tyrosine phosphorylation is the central mechanism by which receptor tyrosine kinases (RTKs) initiate and propagate critical downstream signaling networks regulating cellular behavior. However, the linkage between specific phosphorylation sites and in vivo signaling network activation has yet to be elucidated. The functional role of tyrosine residues on the cytoplasmic tail of the Epidermal Growth Factor Receptor (EGFR) has previously been interrogated either directly by utilizing tyrosine to phenylalanine (Y→F) site-directed mutants in vivo or indirectly via in vitro methods. To date, in vivo studies have been limited to phenotypic characterization of point mutations, while in vitro methods often rely on measuring interactions between EGFR phosphopeptide surrogates and potential downstream substrates such as individual proteins or domains, crude cell lysate and more recently, large scale protein domain binding experiments. While these in vitro approaches are capable of identifying receptor–protein interactions and measuring important biophysical parameters such as binding constants, they are conducted under conditions which result in the loss of cellular network information, including regulatory feedback loops that occur downstream of receptor activation, protein localization, and pathway compensatory mechanisms. Previous studies that have attempted to associate in vivo phenotypic data from Y→F mutants with in vitro binding measurements have often overlooked the point that signaling networks are dynamic entities that have evolved mechanisms to adapt to changes in network structure and utilization that may occur upon point mutation of the EGFR receptor.

To address these deficiencies and to complement the data obtained from previous studies, we have employed EGFRvIII, a constitutively active variant of EGFR, as a model system for probing the effects of site-specific tyrosine phosphorylation on intracellular signaling networks. EGFRvIII is expressed in a subset of glioblastoma tumors (GBM, WHO grade IV) and is correlated with poor patient prognosis. A previous investigation of the in vivo biological consequences of EGFRvIII mutation determined that Y→F mutations on Y1068, Y1148 and Y1173 of EGFRvIII each resulted in a dramatic decrease in intracranial tumor volume, implicating these sites as critical for tumorigenicity. However, the signaling networks associated with this loss in tumorigenic potential were not explored. In this
study, we build on the previous work by utilizing an unbiased mass spectrometric (MS) approach to determine the global phosphotyrosine network effects of six site specific (Y → F) mutations on the EGFRvIII receptor.

Here we show that mutation of any of four phosphorylation sites on the receptor results in a significant change in phosphorylation on most of the other eight sites on the receptor relative to intact EGFRvIII, suggesting intriguing feedback connectivity among the receptor phosphorylation sites. Altered phosphorylation of these sites is functionally significant, as indicated by the effect on tyrosine phosphorylation levels of critical EGFR downstream signaling network components. To identify key sites within these altered networks which regulate cell growth, computational modeling of GBM cell growth as a function of network phosphorylation levels was performed. This analysis identified the Erk pathway as a crucial signaling process regulating EGFRvIII-driven GBM cell behavior, with the surprising finding that Erk1/2 phosphorylation is negatively correlated with cell growth. Genetic manipulation of this pathway supported this finding and demonstrated that EGFRvIII-expressing GBM cells are sensitive to Erk activation levels. Finally, a phosphoproteomic data-driven computational model was developed that is capable of describing GBM cell growth based on a reduced set of molecular determinants.

Results

Cell lines and experimental strategy

To examine how cellular phosphotyrosine-mediated networks are modulated upon the loss of specific tyrosine residues in the cytoplasmic tail of EGFRvIII, we utilized a series of U87MG glioblastoma (GBM) cell lines that were previously engineered to express tyrosine (Y) to phenylalanine (F) EGFRvIII mutants. These mutants are depicted in Fig. 1A and consist of four cell lines expressing single mutations at Y845F, Y1068F, Y11148F and Y11173F, one cell line expressing a double mutation at Y1068F and Y11173F (denoted as DY2) and a final cell line expressing a triple mutation at Y1068F, Y11148F and Y11173F (denoted as DY3). As a control, a previously described cell line expressing the intact EGFRvIII receptor at 2 × 10^6 copies (U87MG-EGFRvIII) was employed.

To minimize confounding cell signaling events associated with growth factors commonly found in serum in cell culture media, the seven cell lines were serum-starved for 24 hours prior to cell lysis and sample preparation. As outlined in Fig. 1B, lysates from the seven cell lines were labeled with stable isotopes in 2 different sets of experiments. Each set consists of four cell lines with one cell line (U87MG-EGFRvIII) being used as an overcontrolling control for the integration of the two datasets. Peptides from the seven samples were stable isotope labeled, mixed, and tyrosine phosphorylated peptides were immunoprecipitated with a mixture of pan-specific anti-phosphotyrosine antibodies. Following immunoprecipitation, phosphorylated peptides were further enriched by immobilized metal affinity chromatography (IMAC) and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). In total, quantitative phosphorylation profiles were generated for two biological replicates with a total of 132 phosphorylation sites on 98 proteins across the seven cell lines (Table S1, ESI†).

Quantitative effects of EGFRvIII site-specific mutations on receptor phosphorylation and downstream signaling networks

In this analysis, we have mapped and quantified nine phosphorylation sites on the EGFRvIII/EGFR receptor, eight on tyrosine residues and one on a serine residue (Fig. 2A). As a consequence of the endogenous levels of wildtype EGFR (2 × 10^5 receptors) expressed in the U87MG cells (note that EGFRvIII is typically found in the context of wtEGFR in EGFRvIII-expressing glioblastomas), Fig. 2A represents an integrated phosphorylation response of both the EGFRvIII and EGFR receptors. Strikingly, mutation of any single phosphorylation site alters the phosphorylation on most, if not all, other sites on the receptor, with Y845F, Y1068F, and Y11173F demonstrating the largest effects. Single mutation of Y845F resulted in at least a 3-fold decrease in the phosphorylation levels for each of the intact tyrosine/serine residues compared to the control. This general decrease in receptor phosphorylation levels suggests that phosphorylation at Y845 may be required for the optimal activation of the EGFRvIII receptor, consistent with its location in the activation loop of the receptor. Receptor phosphorylation was also strongly negatively affected by single mutations at Y1068F and Y11148F, with each of these mutations causing a decrease in phosphorylation on all remaining phosphorylation sites. The effect of the Y1068F mutation was especially strong, such that the decrease in phosphorylation for many of the sites was greater than that detected for the Y845F mutation. Surprisingly, mutation of Y11173F displayed a dramatically different response compared to mutation of the other three sites, with Y11173F mutation leading to all other sites on the receptor increasing in phosphorylation by at least 1.5-fold relative to the control cells. The DY2 double mutation combines two mutations with opposing effects, as Y11173F led to increased phosphorylation while Y1068F gave a strong decrease in phosphorylation. In this combination, the effect of the Y11173F mutation dominates but is diminished, with all remaining sites displaying increased phosphorylation relative to control, albeit to a lesser extent than in the Y11173F cells. The addition of Y11148F, a third point mutation in the receptor, in the DY3 triple mutant abrogated the effect of Y11173F mutation, leading to decreased phosphorylation of the remaining sites on the receptor relative to control cells.

Global analysis of the tyrosine phosphorylation data across the six mutant cell lines revealed similar trends to their receptor phosphorylation profiles, with a large proportion of tyrosine phosphorylation sites downstream of the Y11173F and DY2 mutants exhibiting increased phosphorylation levels relative to control while the remaining mutants (Y845F, Y1068F, Y11148F and DY3) portrayed an overall decrease in network phosphorylation (Fig. 2B, full hierarchical cluster with protein names and phosphorylation sites can be found in Fig. S1, ESI†). To determine how each of the EGFRvIII site-specific mutants modulated specific signaling pathways,
we mapped the phosphoproteomic data onto the canonical EGFR signaling network (Fig. 3). Interestingly, signaling nodes including SHC (Y317), PLC-γ (Y1253), the activation site on STAT3 (Y704/705), a docking site on GAB-1 (Y689), and multiple phosphorylation sites on PI3K were deficient in signal activation in the Y845F, Y1068F, Y1148F and DY3 mutants compared to intact EGFRvIII, while other sites, such as the SFK autophosphorylation site Z (Y420) and Erk1/2 activation loop phosphorylation sites (T185/Y187 and T202/Y204), were significantly upregulated in several of these cell lines.

For the Y1173F and DY2 cells, phosphorylation levels of the nodes in the canonical EGFR signaling network tended to reflect the increase in phosphorylation seen at the receptor level, including a stronger increase in the Y1173F cells relative to the DY2 cells. It is interesting to note that increased phosphorylation in these cells also includes the Src and Erk phosphorylation sites. In fact, we initially suspected that the Src and Erk pathways may be activated in selected cell lines to compensate for the loss of signal from the receptor, but their activation in the Y1173F and T202/Y204), were significantly upregulated in several of these cell lines.

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Partial least squares regression reveals an anti-correlation between activated Erk1/2 and glioma cell growth

To explore the phenotypic relevance of signaling network adaptations to various point mutations in the receptor, cell growth curves were measured for each cell line (Fig. 4A). Contrary to our initial expectations, cell growth among the mutant cell lines appeared to be uncorrelated with tyrosine phosphorylation levels in these cell lines. In fact, despite two of the cell lines displaying increased tyrosine phosphorylation and 4 of the cell lines displaying decreased tyrosine phosphorylation relative to cells expressing intact EGFRvIII, the in vitro growth profiles for five of the six mutant cell lines (with the exception of Y1068F, discussed in more detail below) were significantly slower relative to intact EGFRvIII. This divergence between tyrosine phosphorylation levels and cell growth rates implies that cell growth is not simply driven by total tyrosine phosphorylation levels. Instead, the phosphorylation level of selected protein components in the context of the overall network may determine U87MG glioblastoma cell growth.

To explore this unexpected divergence further and identify the key regulatory nodes governing cell growth rates, partial

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**Fig. 1** Mutant EGFRvIII receptors and experimental strategy. (A) Six site-directed mutants of EGFRvIII were examined in this phosphoproteomic analysis. Y = tyrosine residue and F = mutation of an existing tyrosine residue to phenylalanine. EC = extracellular region, TM = transmembrane domain, KN = kinase domain and CT = cytoplasmic tail. (B) Outline of MS-based experimental strategy. Samples were labeled with iTRAQ isobaric reagent in two sets of experiments, each consisting of 3 mutant cell lines and U87MG-EGFRvIII. U87MG-EGFRvIII (label 114) was used as a normalization point to integrate both data sets together.

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Note that the peptide containing this phosphorylation site is found in multiple different Src family kinases. Here we have used the numbering scheme for Fyn, as another peptide from Fyn or Yes, was also detected in the analysis.
least squares regression (PLSR) analysis was used to visualize the relationship between phosphorylation levels of the phosphosites in the MS dataset and cell growth across the mutant EGFRvIII cell lines. For this analysis, relative phosphorylation levels of each site were modeled as the underlying, independent-variable block, while quantified GBM cell growth rate was used as the dependent variable. This approach allowed for the identification of phosphosites that were significantly associated with increased or decreased cell growth.

Fig. 2  EGFRvIII/EGFR receptor and network phosphorylation. (A) Relative quantification of EGFRvIII/EGFR phosphorylation sites across the mutant cell lines. Phosphorylation levels were normalized relative to the U87MG-EGFRvIII (control) cell line with error bars representing phosphorylation sites that appeared in both biological replicate analyses. (B) Heat map of 132 phosphorylation sites that were quantified in the phosphoproteomic analysis. Phosphorylation levels were normalized relative to the U87MG-EGFRvIII control cell line.

Fig. 3  Effect of site-specific EGFRvIII mutations on phosphorylation sites within the EGFR network. Visualization of the fold change in phosphorylation levels in the canonical EGFR signaling network as a function of EGFRvIII site-specific tyrosine residue mutants. Phosphorylation levels are normalized to that of the U87MG-EGFRvIII control cell line.
constants were modeled as the dependent variable block (calculation of GBM cell growth rate constants from the growth curves is described in Supplementary Methods, ESI†). Two principle components were sufficient to capture ~90% variance in growth rate constants among the cell lines (Fig. S2, ESI†), defining a 2-dimensional space for projection of both growth rate constants and phosphosite vectors. Projection of growth and those phosphosites that contributed most to principle component axes revealed correlative relationships between phosphosites and growth (Fig. 4B). Most saliently, the PLSR-space quadrant negatively correlated (opposite) to growth was enriched for both singly- and doubly-phosphorylated forms of Erk1 and Erk2. Indeed, dramatic fold-changes in the doubly-phosphorylated, active forms of MAP kinases Erk1 and Erk2 correlated negatively with growth (see Fig. 4A and 5A), leading us to the non-intuitive hypothesis that further increasing Erk1 and Erk2 activity may inhibit cell growth in U87-EGFRvIII cells.

The active forms of Erk1 and Erk2 exhibit a distinct profile from the majority of canonical EGFR signaling nodes (Fig. 3),
in which the slower growing mutants (Y845F, Y1148F, Y1173F, DY2 and DY3) demonstrate an up to 4-fold increase in Erk phosphorylation, while the faster growing Y1068F line has both Erk1 and Erk2 phosphorylation on par with the control. The Y1068 residue of both EGFRvIII and wtEGFR has previously been found to interact with the Grb2 adaptor protein and is a direct activator of Erk1/2 via the Sos and Ras pathways. It is plausible that inactivation of this site on EGFRvIII prevents recruitment of Grb2 to the receptor, resulting in a decrease in activation of Erk1/2 compared to the other Y → F mutants.

**Modulation of the Erk1/2 activation state in U87MG-EGFRvIII cells decreases cell viability**

To test the hypothesis that Erk activation negatively regulates cell growth, U87MG-EGFRvIII cells were stably transfected to express a constitutively active (CA) form of MEK, in which mutation of two serine residues into aspartate converts MEK into a constitutively active protein. MEK is the upstream kinase activator of Erk1/2; overexpression of CA-MEK in the U87MG-EGFRvIII cells therefore results in hyperactivation of Erk1/2 (Fig. 5B). As suggested by the PLSR analysis, increased Erk1/2 activation in the CA-MEK/U87MG-EGFRvIII cells resulted in a 25% decrease in the number of viable cells (Fig. 5C). We also set out to determine if the Erk pathway was required for U87MG-EGFRvIII cell growth. U87MG-EGFRvIII cells were transfected with a dominant-negative (DN) mutant of MEK, leading to a reduction in active Erk1/2 (Fig. 5B). Interestingly, decreasing Erk activation led to a 20% decrease in the number of viable cells (Fig. 5C) relative to control U87MG-EGFRvIII cells, similar results were obtained following MEK inhibition with U0126 (Fig. S3, ESI). Taken together, these experiments suggest that GBM cells expressing EGFRvIII are sensitive to modulation of the Erk pathway, and that increasing or decreasing normal Erk activation levels results in a reduction in the number of viable cells.

**Experimental validation of glioma cell growth model hypothesis**

To provide additional experimental support for our finding that Erk phosphorylation negatively regulates growth in this glioma cell line, we generated stable transfectants of CA-MEK in the six mutant cell lines (Fig. S4A, ESI). When grown under serum-starvation conditions, CA-MEK mutant cells demonstrated varying viability profiles. The slowest growing cell lines had no significant response to hyperactivation of Erk1/2 (Y1173F, DY2, DY3), while the rest of the EGFRvIII mutants showed sensitivity to increased Erk1/2 phosphorylation (Y1068F, Y1148F and Y845F) (Fig. S4B, ESI) relative to the control. To visualize the CA-MEK cell viability measurements in relation to cell line growth rates and phosphorylation levels, we used PLSR analysis once again. Growth rate and CA-MEK cell viability measurements were modeled as dependent variables, while phosphosites constituted the independent variable block (Fig. 6A). Cell viability under CA-MEK clustered proximal to Erk1/2 phosphorylation and varied inversely with growth rate.
Phosphosite determinants of growth

We also used PLSR to develop models of GBM cell line growth as a function of phosphorylation state. To determine which phosphosites were most crucial to modeling growth, a variable importance for projection (VIP) score was calculated for each phosphosite. Without loss in model fitness or prediction, we constructed a “reduced” PLSR model consisting of the thirteen phosphosites with highest VIP score (Fig. 6B and C). The “reduced” model ($R^2 = 0.9$, $Q^2 = 0.51$) is built on three principle components, and shows predictive improvement over the “full” model ($R^2 = 0.92$, $Q^2 = 0.17$), which includes all 132 phosphosites in the MS dataset (Fig. S5, ESI†).

Discussion

Receptor and network alterations in response to EGFRvIII site-specific mutations

This study is, to our knowledge, the first comprehensive evaluation of the contribution of EGFRvIII tyrosine phosphorylation sites to intracellular downstream signaling networks. We have demonstrated that EGFRvIII/EGFR phosphorylation profiles fall into two broad categories in response to mutation of critical receptor tyrosine residues (Fig. 2A). Mutation at Y845 on EGFRvIII exemplifies the first category, in which a single point mutant leads to a decrease in receptor phosphorylation at multiple other sites, causing a general decrease in overall tyrosine phosphorylation throughout most of the cellular signaling network. It has previously been demonstrated that the Src proto-oncogene phosphorylates EGFR on Y845 and is critical for cell cycle progression and proliferation. Accordingly, mutation of the Y845 site on EGFRvIII led to a 1.5-fold decrease in exponential cell growth compared to intact EGFRvIII. The growth defect is accompanied by a decrease in the levels of all 8 phosphorylation sites that were identified on the EGFRvIII receptor, and a concurrent drop in the phosphorylation levels of downstream signaling components in the EGFR network. Single mutation of Y1068F or Y1148F also falls in this category, as does the DY3 triple mutation of Y1173F, Y1068F, and Y1148F. It is interesting to note that the effect of the Y1068F mutation appears to be equal or greater than that seen for the Y845F mutation, as phosphorylation on almost all sites on the receptor is actually lower for the
Y1068F mutation compared to the Y845F mutation. The mechanism by which mutation of Y1068F or Y1148F leads to decreased receptor phosphorylation remains to be investigated, but may involve loss of binding of cytoplasmic tyrosine kinases such as Src or Abl, or could be due to a conformational change in the C-terminal region. Although the mechanism has yet to be established, this finding highlights the importance of studying the function of receptor tyrosine phosphosites within the context of the cell. Analysis of the functional roles of post-translational modifications in an in vitro assay cannot fully recapitulate the biological milieu in which receptors normally function and may have obscured the apparent connectivity between Y845/Y1068/ Y1148 and other receptor phosphosites that we have uncovered in this study.

The second category of EGFRvIII mutants exhibited an increase in receptor phosphorylation in response to site mutation, as seen for the single mutation at Y1173F and the double mutation at Y1173F/Y1068F. While the mechanism underlying this increase is also still undefined, it is plausible that mutation of Y1173 results in the loss of binding of a negative regulator of receptor phosphorylation; the SHP-1 protein tyrosine phosphatase is one potential candidate. SHP-1 is highly expressed in hematopoietic cells and a closely related isoform of the protein is expressed in epithelial cells. A previous report has shown that SHP-1 binds to wildtype EGFR primarily via the Y1173 site and that Y1173F mutation leads to a decrease in SHP-1-mediated receptor dephosphorylation. Additional studies are required to determine if loss of SHP-1 binding is the predominant mechanism driving increased phosphorylation for the Y1173F and Y1173F/Y1068F double mutation, as other feedback pathways are likely to be contributing as well.

Tyrosine 1173 is a major phosphorylation site on EGFRvIII, responsible for the activation of downstream signaling components through the recruitment of adaptor proteins such as SHC. Intriguingly, loss of Y1173 phosphorylation in the Y1173F mutant led to increased signaling of downstream EGFR canonical signaling network components, including SHC–GABI–PI3K, STAT3, and PLCγ, indicating that increased phosphorylation on the remaining sites on the receptor was more than sufficient to compensate for the loss of this phosphorylation site. Our data support redundancy in EGFRvIII phosphorylation sites in the activation of downstream signaling pathways, such that multiple tyrosine phosphorylation sites on the receptor may serve to recruit similar adaptor proteins. Such overlap in adaptor binding has been detected during in vitro EGFR phosphopeptide interaction experiments. For instance, in addition to Y1173, the adaptor protein SHC has been shown to also bind to Y974, Y1086, Y1114 and Y1148 on EGFR, all of which were shown to increase in our phosphorylation study.

Sensitivity of U87MG-EGFRvIII cells to the Erk1/2 activation levels

In a recent study of EGFRvIII signaling networks, we observed that the activation of Erk1/2 increased only slightly with increasing EGFRvIII receptor levels. In light of those findings, we asserted that the EGFRvIII receptor mediates its tumorigenic effect through the preferential utilization of other upregulated pathways (e.g. the PI3K pathway) over the MAPK pathway. Taken together, our current study would suggest that, in addition to the preferential utilization of alternative signaling pathways, the constitutive activation of EGFRvIII may be effectively repressing phosphorylation/activation of Erk1/2. Mutation of specific tyrosine phosphorylation sites on EGFRvIII appears to relieve this repression, resulting in an up to 4-fold increase in Erk1/2 phosphorylation levels (Fig. 3 and 5A). Repression of the Erk1/2 pathway by EGFRvIII appears to have functional consequences, as hyperactivation of this pathway in the context of EGFRvIII expression leads to a decrease in viable cell numbers. Consistent with our data, a previous study demonstrated that while MEK activity is 4-fold higher in EGFRvIII expressing fibroblast cells compared to the corresponding cells expressing wildtype EGFR, Erk phosphorylation levels remained invariable, even upon the addition of the Erk agonist PMA. In fact, treating EGFRvIII expressing cells with a tyrosine phosphatase inhibitor increased Erk activation, suggesting that the MAPK phosphatase family (MKPs) may be possible candidates for the negative regulation of the MAPK pathway by EGFRvIII. A similar finding was recently made in a mutant, constitutively active, K-RasG12D-driven colon tumor model where MEK activity is upregulated while phosphorylated Erk levels remain attenuated. The mechanism by which Erk1/2 hyperactivation leads to decreased cell viability remains to be determined, but Erk1/2 attenuation has been reported in the context of other constitutively active tyrosine kinases.

We have also shown that a minimal activation level of the Erk pathway is required for EGFRvIII driven cell growth, as Erk inhibition results in a decrease in cell viability (Fig. 5C). Similarly, in the K-RasG12D study, treating mice with CI-1040 (an oral inhibitor of MEK) suppressed proliferation in the colonic epithelia. We propose a model in which EGFRvIII fine-tunes the activation of the Erk1/2 pathway (Fig. 7): basal activation of Erk1/2 is required for optimal GBM cell growth, but increasing Erk1/2 phosphorylation beyond a particular threshold decreases cell viability. Our data would also imply that the sensitivity of EGFRvIII expressing tumor cells to threshold levels of Erk1/2 activation may be exploited for therapeutic purposes. Glioblastoma patients with PTEN-null, EGFRvIII-positive tumors are refractory to monotherapy with EGFR kinase inhibitors. This poor efficacy plagys a significant population of GBM patients, since ~40% of GBM patients lack the expression of functional PTEN. All of our experiments were performed in the U87MG cell line which is PTEN-null. Given the sensitivity of these EGFRvIII-expressing cells to Erk activation levels, hyperactivation of Erk, through the inhibition of negative regulators such as the MKPs, or Erk inhibition, via the use of MEK inhibitors, may be alternative treatment strategies for EGFRvIII positive, PTEN null patients.

PLSR analysis as a tool for identification of GBM cell growth determinants

In this work, PLSR enabled visualization of the multivariate phosphorylation network trends in relation to cell growth and
metabolism, the function of tyrosine phosphorylation on bind tyrosine phosphorylated proteins and alter cellular phosphoglycerate mutase Y92, and pyruvate kinase M2 are also featured in the reduced model, including enolase Y44, kinases in regulating glioma cell growth in U87-EGFRvIII. phosphorylation sites implicate a potential role for Src-family component in a reduced model of migration and proliferation (SLC38A2 Y41) has previously been identified as a key enzymes and glioblastoma cell growth is intriguing, and connection between tyrosine phosphorylation of metabolic these three proteins is still uncharacterized. However, the further functional studies need to be performed to determine EGFRvIII-driven GBM cell growth.

Experimental procedures

Cell culture, retrovirus infection, and transfection

Human glioblastoma cell line U87MG and engineered U87MG derivatives with the exception of the Y845F mutant have been previously described in ref. 8 Construction of the Y845F-EGFRvIII mutant cell line, including oligo sequences, is described in the Supplementary Methods (ESI†). Cells were cultured in DMEM with 10% fetal bovine serum, 2 mM glutamine, 100 units ml\(^{-1}\) penicillin, and 100 mg ml\(^{-1}\) streptomycin in 95% air/5% CO\(_2\) atmosphere at 37 °C. U87MG cells expressing EGFRvIII or engineered mutant receptors were selected in 400 μg ml\(^{-1}\) G418. Stable transfection of MEK mutants (a kind gift from Dr Chris Marshall, ICR) in U87MG cells is elaborated in detail in the Supplementary Methods (ESI†).

Cell lysis, protein digestion and peptide fractionation

U87MG cells were maintained in DMEM medium supplemented with 10% FBS. 1.5 × 10\(^{6}\) cells per 10 cm plate were seeded for 24 h, then washed with PBS and incubated for 24 h in serum-free media. Cells were lysed in 8 M urea as previously described.\(^9\) A small aliquot of each sample was analyzed by micro-BCA (bicinchoninic acid) to provide an approximate normalization for the amount of protein in each sample. More refined normalization occurs through quantification of non-phosphorylated proteins found in the supernatant from the immunoprecipitation (see below). For each of the two biological replicates performed, lystate from three 10 cm plates were pooled together. Cell lystate was reduced, alkylated and digested as previously described.\(^{36}\) Digested lysate were acidified to pH 3 with acetic acid and loaded onto a C18 Sep-Pak Plus cartridge (Waters). The peptides were desalted (10 ml 0.1% acetic acid) and eluted with 10 ml of a solution of 25% acetonitrile and 0.1% acetic acid. Each sample was divided into 5 aliquots and lyophilized to dryness.

iTRAQ labeling of peptides

Lyophilized peptides were subjected to labeling with iTRAQ 4-plex reagent (Applied Biosystems). Each aliquot of peptides was dissolved in 0.5 M triethylammonium bicarbonate, pH 8.5, and reacted with two tubes of iTRAQ reagent. The reagents for each of the conditions used were: iTRAQ-114 (U87MG-EGFRvIII), iTRAQ-115 (U87MG-Y1148F and U87MG-Y1173F), iTRAQ-116 (U87MG-Y1068F and U87MG-DY2) and iTRAQ-117 (U87MG-845F and U87MG-DY3). The mixture was incubated at room temperature for 50 min and then
concentrated to 30 µL. The four different isotopically labeled samples from each set (outlined in Fig. 1B) were combined and acidified with 360 µL of 0.1% acetic acid and then reduced to dryness.

Peptide immunoprecipitation and mass spectrometry

The combined sample was reconstituted with IP buffer (100 mM Tris, 100 mM NaCl, 1% NP-40, pH 7.4) and incubated with 20 µg of protein G Plus-agarose beads (Calbiochem) and 24 µg of anti-phosphotyrosine antibody (PY100 Cell Signaling Technology and 4G10 Millipore) for 8 h at 4 °C. The antibody–bead conjugates were then spun down for 5 min at 6000 rpm at 4 °C and the supernatant was saved. The beads were then washed prior to elution with 100 mM glycine, pH 2.5, for 30 min at room temperature. Immobilized metal affinity chromatography (IMAC) was performed to enrich for phosphorylated peptides and remove non-specifically retained non-phosphorylated peptides as previously described.37 Peptides retained on the IMAC column were eluted with 250 mM sodium phosphate (pH 8.0) and analyzed by electrospray ionization liquid chromatography tandem MS on a QStar Elite mass spectrometer (QSTAR Elite, Applied Biosystems). MS/MS spectra of the five most intense peaks with 2–5 charge states in the full MS scan were automatically acquired in information-dependent acquisition.

Phosphopeptide sequencing and quantification

MS/MS spectra were extracted and searched using MASCOT (Matrix Science). For MASCOT, data were searched against the human non-redundant protein database with trypsin specificity, 2 missed cleavages, precursor mass tolerance of 2.2 amu for the precursor ion and 0.15 for the fragment ion tolerance. Phosphorylation sites and peptide sequence assignments were validated and quantified by manual confirmation of raw MS/MS data. Peak areas of iTRAQ marker ions (m/z 114, 115, 116 and 117) were obtained and corrected according to the manufacturer’s instructions to account for isotopic overlap. The quantified data were then normalized with values from the iTRAQ marker ion peak areas of non-phosphorylated peptides in the supernatant of the immunoprecipitation (used as a loading control to account for possible variation in the starting amount of sample for each condition). Each condition was normalized against the U87MG-EGFRvIII (114 label) cell line to quantify fold change across all 7 conditions.

Details for biochemical measurements, phenotypic experiments, growth rate calculations, partial least squares regression analysis, and statistical analysis are provided in Supplementary Methods (ESI†).

Conclusion

In summary, phosphoproteomic analysis of EGFRvIII site-specific mutants has led to the discovery of network-wide cellular response to site-specific mutations on EGFRvIII. The response occurs at the level of the receptor, where mutation of any of four tyrosine phosphorylation sites leads to altered phosphorylation on the remaining receptor tyrosine phosphorylation sites, thereby implicating novel interactions among the receptor phosphorylation sites. The changes in receptor phosphorylation are propagated downstream, leading to altered phosphorylation throughout the cellular signaling network, an unexpectedly widespread response to site-specific mutations. Phenotypically, increased or decreased phosphorylation throughout much of the cellular signaling network appears to affect cell growth rate similarly, with five of the six cell lines expressing mutant forms of EGFRvIII growing significantly slower than cells expressing the intact EGFRvIII receptor. To better define the critical nodes regulating cell growth in this complex network, signaling networks and growth rates were correlated, leading to a model where EGFRvIII fine-tunes the activity of the Erk1/2 pathway to optimize cell viability. Experimentally, both hyperactivation and inhibition of the Erk pathway result in a decrease in the viability of U87-EGFRvIII cells. If these results are extensible to xenografts and tumors expressing EGFRvIII, it may be that perturbing Erk1/2 activation in these tumors, through either hyperactivation or inhibition, may lead to decreased tumor growth rates.

Abbreviations

GBM glioblastoma
EGFR epidermal growth factor receptor
LC-MS/MS liquid chromatography tandem mass spectrometry
RTK receptor tyrosine kinase
IMAC immobilized metal affinity chromatography
PLSR partial least squares regression

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The authors declare that they have no conflict of interest.

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