Signaling and Regulation

Suppression of G-protein–Coupled Receptor Kinase 3 Expression Is a Feature of Classical GBM That Is Required for Maximal Growth

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Abstract

G-protein–coupled receptor kinases (GRK) regulate the function of G-protein–coupled receptors (GPCR). Previously, we found that GPCR (CXCR4)-mediated astrocytoma growth was dependent upon abnormally sustained CXCR4 signaling and was correlated with decreased GRK-mediated receptor phosphorylation. As CXCR4 has also been implicated in the stimulation of high-grade glioma growth, we sought to determine whether dysregulation of GRK expression and/or function might also be present in high-grade gliomas. In an analysis of data from The Cancer Genome Atlas, we found that GRK3 expression is frequently decreased in glioblastoma (GBM) of the classical subtype, which possesses signature amplification or mutational activation of the epidermal growth factor (EGF) receptor. We tested the correlation between GRK3 expression and GBM subtypes, as well as the relationship between the activation of the EGF and other growth factor receptor pathways and GRK expression. In analyses of primary GBM tissue and RNA specimens, we found that GRK3 expression is correlated with established criteria for GBM subtyping including expression of EGF receptor, platelet-derived growth factor receptor (PDGFR)α, NF1, PTEN, CDKN2A, and neurofilament. We also found that established drivers of gliomagenesis, the EGF, PDGF, and TGF-β pathways, all regulate GRK expression. Coculture experiments, designed to mimic critical interactions between tumor and brain microvascular endothelial cells, showed that specifically increasing GRK3 expression reduced the trophic effect of endothelial cells on tumor cells. Together, these experiments show that GRK3 is a negative regulator of cell growth whose expression is preferentially reduced in GBM of the classical subtype as a consequence of activity in primary gliomagenic pathways. Mol Cancer Res; 10(1); 156–66. ©2011 AACR.

Introduction

G-protein–coupled receptors (GPCR) are known to regulate virtually all aspects of cell biology and comprise the single largest family of targets for current pharmaceuticals (reviewed in ref. 1). While their involvement in cellular proliferation, survival, migration, as well as angiogenesis and inflammation are active areas of research, their role in oncogenesis and their value as therapeutic targets in cancers remains to be fully defined (reviewed in refs. 2, 3). Interestingly, functionally significant mutation, amplification, or deletion of GPCR genes rarely occurs in cancer. While activating mutations of GPCRs that endow transforming activity are described, they are infrequent with limited significance to cancer biology beyond the proof of concept that dysregulated GPCR signaling can drive tumorigenesis (4–8).

Mechanisms of aberrant GPCR action more commonly arise through receptor overexpression and/or paracrine activation. Consistent with ligand-dependent roles of GPCRs in cancer, competitive antagonists of GPCR–ligand binding, such as the CXCR4 antagonists AMD3100 and AMD3465, have exhibited significant antitumor activity in preclinical models of brain tumors (9–11). However, a simple correlation between receptor binding and GPCR function in cancer is called into question by the multiple levels of counter-regulation that normally oppose sustained GPCR ligand activation. Counter-regulatory mechanisms include the inherent GTPase activity of the heterotrimeric Gα subunits, the GTPase activating activity of regulators of G-protein signaling (RGS) proteins, and the multistage process of receptor desensitization (reviewed in ref. 12).
These mechanisms of counter-regulation normally buffer against the aberrant or sustained activation that might occur in the presence of constant ligand stimulation. This feature of GPCR signaling raises the question of whether the contribution of GPCRs to cancer biology might require both increased receptor ligation and alterations in counter-regulatory mechanisms such as desensitization.

G-protein receptor desensitization is initiated by the phosphorylation of ligand-occupied receptor by G-protein receptor kinases (GRK; ref. 13). The phosphorylated receptor is bound by arrestins, resulting in its uncoupling from heterotrimeric G-proteins and subsequent internalization. GRKs comprise a family of serine/threonine kinases with 7 members (GRK1–7) that are distinguished into 3 subfamilies (14). GRK1 and 7 are the visual GRKs with expression primarily restricted to photoreceptors where they modulate opsin signaling. GRK2 and 3 share an important regulatory domain that facilitates their dynamic recruitment to the membrane by PIP2 and Gβγ (15). GRK4–6 are more tonically localized to the inner surface of the plasma membrane by covalent lipid modification or via lipid-binding domains. GRK2, 3, 5, and 6 are expressed in nearly all tissues, whereas GRK4 exhibits more limited expression.

As the GPCR family contains more than 800 members and only 4 GRKs (2, 3, 5, 6) are widely expressed, individual GRKs must modulate the signaling of many receptors. In addition, individual GPCRs appear to be regulated by multiple GRKs. As an example, GRK2, 3, and 6 phosphorylate CXCR4 (16–22). Individual phosphorylation events can display site specificity, kinetic differences, and differential impact on downstream mediators. Thus, in a heterologous system, phosphorylation of CXCR4 by GRK2 and 6 and recruitment of arrestin-3 more potently regulated calcium flux than phosphorylation by GRK3 and 6, which recruit arrestin-2 and regulate extracellular signal-regulated kinase (ERK) activation (23). Therefore, despite the potential for extensive redundancy, nonoverlapping GRK function in regulating specific GPCR activities exists. In fact, alterations in specific GRK expression or function or mutation of GPCRs that interfere with desensitization are recognized as the mechanistic basis for diseases such as hypertension, rheumatoid arthritis, mental illness, and immunodeficiency (reviewed in ref. 24).

Among the GPCRs frequently implicated in cancer biology is CXCR4 (25). CXCR4 is highly expressed in brain tumors of multiple lineages, and its level of expression has prognostic significance for some brain tumors, including astrocytomas (gliomas; refs. 26–28). In a model of preneoplastic astrocytes involving complete loss of neurofibromin, we found that treatment with the CXCR4 ligand, CXCL12, resulted in an abnormal survival response (22). This abnormal survival response was dependent upon sustained CXCR4 signaling, suggestive of diminished receptor desensitization. The complete loss of neurofibromin resulted in an increase in ERK activation, and the alteration in CXCR4 function was correlated with increased ERK-dependent phosphorylation and inhibition of GRK2 and diminished ligand-induced phosphorylation (desensitization) of CXCR4.

These data suggested that changes in GRK expression or activity might promote oncogenic GPCR function. These data further suggested that the mechanism of altered GRK activity could be downstream of primary oncogenic events such as those that activate the RAS-MAP kinase pathway. On the basis of these results, we hypothesized that GRK expression may be altered in glioma (astrocytoma) and contribute to neoplastic growth. To address this hypothesis, we examined GRK expression and function in astrocytomas and the relationship between changes in specific GRK expression and common oncogenic events in gliomagenesis.

Materials and Methods

All chemicals and reagents were from Sigma-Aldrich, unless otherwise noted.

The Cancer Genome Atlas data analysis

Gene expression data for the original 202 profiled glioblastoma (GBM) specimens (File: TCGA_unified_CORE_ClNC840.txt; Cancer Cell) were obtained at: http://tcga-data.nci.nih.gov/docs/publications/gbm_exp/. The sample set was divided into 4 molecular subclasses of GBM on the basis of signature genetic alterations: (i) classical, (ii) mesenchymal, (iii) proneural, and (iv) neural (29, 30). Subtype-characteristic centroid profiles were obtained by averaging the subset of genes defining each GBM subtype. We then explored the relationship between the 4 centroid profiles with each GRK gene expression profile by pairwise scatter plots and Pearson correlation coefficients (Supplementary Fig. S1). GRK gene expression was tested between subjects of different subtypes by 2-sample t test in a pairwise manner, and Bonferroni correction was applied to adjust the resulting raw P values.

Human tissues

All human specimens were obtained and analyzed in accordance with an Institutional Review Board–approved protocol for human studies. Astrocytoma tissue sections (WHO grades I–IV) were obtained from archived paraffin-embedded blocks provided by the Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO. Additional GBM tissue microarray slides (GL806a) were obtained from US Biomax, Inc. RNA specimens were obtained from archived GBM specimens from the Siteman Cancer Center Tissue Procurement Core, Washington University School of Medicine. Additional human astrocyte RNA specimens were obtained from Clontech Laboratories, Origene, Ambion, and Biochain.

Immunohistochemistry

Immunohistochemical analyses, except for IDH1 analysis, were conducted as previously described (31). Antibodies, their sources, and details of their use are presented in Supplementary Table S1. IDH1 mutational analysis was conducted with a Benchmark XT automated tissue staining system (Ventana Medical Systems, Inc.) using a validated protocol. Endogenous peroxidase activity was blocked by
H<sub>2</sub>O<sub>2</sub> and antigen was retrieved using CC1 reagent (Ventana Medical Systems). Mutant IDH1 (IDH1-R132H) was detected with a specific mouse monoclonal antibody (clone H09, 1:500; Dianova), Ventana signal amplification kit and Ventana UltraView diaminobenzidine chromogen treatment (Ventana Medical Systems). Slides were counterstained with hematoxylin.

**FISH**

FISH for epidermal growth factor (EGF) receptor amplification was conducted on deparaffinized GBM tissue microarray (GL806a; US Biomax) as described (32). Briefly, after antigen retrieval in citrate buffer, pH 5.8, tissue was digested with pepsin and equilibrated in 2X saline-sodium citrate (SSC). Vysis premixed probe sets for CEP7 and EGFR were denatured and hybridized to the GBM specimens according to the manufacturer’s instructions (Abbott). After washing in 2X SSC, the tissue microarray was counterstained with 4,6-diamidino-2-phenylindole (DAPI).

**PCR**

The cDNA was synthesized from 100 ng of GBM and human astrocyte RNA using iScript RTase. Specific transcripts were amplified using the Power SYBR GREEN PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. Primers for each mRNA (Supplementary Table S2) were obtained from Integrated DNA Technologies and used at 300 nmol/L. Samples were run in triplicate with a corresponding human astrocyte RNA using iScript RTase. Specimen. PCR and data collection were done using the BioRad MiniOpticon Real Time PCR machine and Opticon Monitor 3 Software from BioRad. Relative transcript copy number for each transcript and corresponding β-actin or GAPDH were calculated using the ΔΔC<sub>T</sub> method. The relative expression value for each transcript was normalized to its corresponding β-actin or GAPDH. Data are presented as the GBM expression relative to human astrocyte expression.

**Cell culture**

Primary human astrocytes and primary human brain microvascular endothelial cells (HBMEC) were obtained from ScienCell Research Laboratories. Each was grown in specific media as suggested by supplier. Glial cell lines included U87MG (American Type Culture Collection), LN308, LN827, and LN428. The latter three were a kind gift from Erwin van Meir (Winship Cancer Center, Emory University, Atlanta GA; ref. 33). U87 cells were engineered to express EGFR or EGFRvIII as described (34). All glioma cell lines were grown in Dulbecco’s Modified Eagle’s Media (DMEM) and used within 6 months of their initial culture. Culture media was supplemented with FBS and contained penicillin/streptomycin (Cellgro).

**Growth factor and drug treatment**

For experiments using growth factor and drug treatments, cells were cultured in serum-free media for 24 hours prior to treatments. Control cells were maintained in serum-free conditions alone. Final concentrations of growth factors were as follows: CXCL12, 1 μg/mL (PeproTech), EGF, 10 ng/mL (R&DD Systems Inc.), platelet-derived growth factor (PDGF), 10 ng/mL (R&DD Systems), and TGF-β1, 2 ng/mL (R&DD Systems Inc.). Inhibition of EGFR was accomplished using PD153035 at 0.1 and 10 μmol/L (EMD Chemicals). The lower molarity (0.1 μmol/L) blocked autophosphorylation of EGFR, whereas the higher molarity (10 μmol/L) also abrogated phosphorylation of ERK and AKT (35). Experiments were repeated a minimum of 3 times.

**Western blot analysis**

Western blot analysis was conducted as previously described (22). Quantification was carried out with the use of Image J freeware from the NIH (Bethesda, MD). Observed molecular weights for the GRKs differed little from the predicted weights found in the Swiss Protein Database online (http://www.uniprot.org/).

**GRK overexpression**

GRK2 and 3 transgenes were cloned into a lentiviral packaging vector also encoding mCherry fluorescent protein (36). Virus encoding each GRK was produced at The Hope Center of Washington University Viral Vectors Core. U87MG and LN428 cells were infected with virus for each GRK and selected by fluorescence-activated cell sorting as previously described (37). GRK expression was verified by Western blot analysis.

**GBM cell line–human brain microendothelial cell coculture**

Cocultures of human brain microvascular cells and GBM cells were established in Matrigel as described (38, 39). Briefly, primary HBMECs were seeded onto plates coated with Matrigel extracellular matrix (BD Biosciences) and grown in EGM-2MV (Lonza). Endothelial cells added to Matrigel form networks mimicking a vascular bed within 24 hours. After 24 hours, either U87 or LN428 were added to the culture plates and grown for 24 to 48 hours in serum-free DMEM (Gibco). Tumor cells were previously engineered to express a fusion protein of firefly luciferase and enhanced GFP (eGFP; ref. 37). Bioluminescence imaging was used to measure the growth effects of endothelial cells on GBM cells. Media were removed and imaging media containing Hank’s balanced salt solution (HBSS; Cellgro) and α-Luciferin (Biosynth) were added at a final concentration of 150 μg/mL. Photon flux for each well was measured with a charge-coupled device camera (IVIS 50; Caliper; 60-second exposure; f-stop, 1; binning, 8; field of view, 15 cm; filter, open). Endothelial cells did not express luciferase and thus only the tumor cell growth was measured. Data were analyzed using Living Image/ Igor Pro software (Caliper) and expressed as total photon flux (photons/s). A standard curve for the relation between cell numbers and photon flux was determined from a serial dilution of tumor cells. Cell numbers were calculated from the standard curve.
Additional statistical methods

Box and whisker plots were used to illustrate the overall distribution of quantitative variables, whereas error bar plots were drawn to indicate mean and SEM. Two-sample t test and Wilcoxon rank-sum test were applied as appropriate to examine whether quantitative variables distinguished between 2 groups. To address the multiple testing issue, the most stringent Bonferroni correction procedure was used to adjust raw P values. All tests were two-sided unless otherwise noted. Significance was deemed at the 5% significance level. All analyses were implemented in R (http://cran.r-project.org) and GraphPad version 4.

Results

GRK expression is frequently altered in GBM

To determine whether GRK expression is altered in GBM, we queried gene expression data available through The Cancer Genome Atlas (http://tcga-data.nci.nih.gov/docs/publications/gbm_exp/). First, we examined the relationship between GRK expression and subtype-characteristic centroid profiles. Four subtypes of GBM can be distinguished by their pattern of gene expression: (i) Classical GBM exhibits mutation/amplification of EGFR and frequent loss of CDKN2A, (ii) Mesenchymal GBM possesses mutations/deletions of NF1, PTEN, and p53, (iii) Proneural GBM has frequent mutations of p53 and IDH1 as well as mutation/amplification of PDGFRα, and (iv) Neural GBM exhibits mutations that overlap with the other groups but is also characterized by increased expression of genes normally associated with neurons such as neurofilament (NEFL; refs. 29, 30). Several relationships between GRK expression and molecular subtypes of GBM were revealed (Supplementary Fig. S1). GRK2 and 5 expression were positively correlated ($r = 0.44$ and 0.32, respectively), and GRK4 expression negatively correlated ($r = -0.61$) with mesenchymal GBM. Levels of GRK3 were negatively correlated ($r = -0.37$) with the classical subtype, and proneural tumors exhibited increased GRK4 expression ($r = 0.6$). GRK6 exhibited no subtype-specific pattern of expression.

To assess the reliability of the correlations between GRK expression and GBM subtypes, we tested the significance of the GRK3-classical and GRK4-mesenchymal correlations with permutation tests. We generated random sets of genes from the TCGA database (minus the GRK genes) that were equal in size to the gene sets that defined each subtype (216 genes for mesenchymal and 162 genes for classical subtypes). This process was repeated 5,000 times. The centroid profile of each random-drawn gene set was calculated by averaging across the 216 or 162 genes and was correlated with the untouched GRK4 or 3 gene expression profile. The significance of the correlation coefficient between GRK4 and the mesenchymal centroid profile (which is $-0.61$) exceeded any of the correlation coefficients obtained with the random data sets ($P = 0$). The correlation coefficient between GRK3 and the classical centroid profile (which was $-0.37$) exceeded all but 183 of the 5,000 returned correlations ($P = 0.037$). Thus, like other GBM-associated genes, GRK expression is altered in GBM but may not alone uniquely distinguish between subtypes.

To directly address whether GRK expression might distinguish between GBM subtypes, we applied 2-sample t tests and examined whether the continuous gene expression of any GRK gene is differentially expressed between any two of the subtypes. The $P$ values after Bonferroni adjustment were reported to account for multiple testing (Supplementary Table S3) and box plots, where the black horizontal bar indicates the median expression level and the box represents the 25% to 75% quantile of the data, were generated to illustrate the difference in expression among the subtypes. With this analysis, we found that greater GRK2 (Fig. 1A)
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and GRK5 (Fig. 1D) expression differentiates between mesenchymal and each of the other 3 GBM subtypes [mesenchymal compared with: classical (GRK2, $P = 9.4e-05$; GRK5, $P = 0.004$), neural (GRK2, $P = 0.0025$; GRK5, $P = 2.3e-05$), and proneural (GRK2, $P = 1.5e-05$; GRK5, $P = 0.025$)]. In a similar fashion, lower GRK3 (Fig. 1B) expression distinguishes classical GBM from all other subtypes [classical compared with: mesenchymal ($P = 0.00046$), neural ($P = 0.00080$), and proneural ($P = 0.00011$)]. No exclusive relationships between individual subtypes and GRK4 and 6 expression were detected.

Relationships between changes in GRK expression and GBM were further evaluated by quantitative PCR for GRK2–6 on mRNA isolated from 30 primary GBM and 7 different normal human astrocyte specimens. GRK expression in each normal astrocyte and GBM specimen was first normalized to β-actin and then to the mean expression of the astrocyte samples. While GRK expression was variable among the GBM specimens, the majority of GBM cases exhibited altered GRK expression relative to human astrocytes (Fig. 2A). GRK3 and 4 exhibited the greatest difference in mean expression between normal astrocytes and GBMs. Expression of GRK4 was increased ($P = 0.06$) and GRK3 decreased ($P = 0.03$) compared with human astrocytes. Thus, the most consistent change observed in these analyses of primary human specimens was decreased GRK3 expression.

To more closely examine the relationship between GRK3 expression and specific genetic changes in GBMs, we conducted quantitative PCR–based subtyping according to the following schema: (i) classical GBM = increased EGFR receptor and decreased CDKN2A expression, (ii) mesenchymal GBM = decreased PTEN and NF1 expression, (iii) neural GBM = increased neurofilament expression and, (iv) proneural GBM = increased PDGFR expression. While each of these genetic changes can occur in any GBM subtype, they do so with distinctly different frequencies (30). The mRNA for each of the above subtype markers was measured in 22 GBM specimens and normalized to the level of expression in human astrocytes. Subtype assignments were made blinded to GRK3 expression and based on the reported frequency that each change (e.g., increased EGFR expression) occurs in each GBM subtype. Seventeen of the 22 samples could be subtyped according to this schema but 5 specimens exhibited nonspecific patterns of gene expression (Supplementary Table S4). While each subtype contained at least one specimen with low GRK3 expression, only in the classical subtype, did all specimens exhibit low levels of GRK3 (Fig. 2B). Similar to the TCGA analysis, mean GRK3 expression was lowest in the classical and highest in the proneural subtype.

Prognostically significant GBM subtyping has also been based on the concept of primary and secondary disease. Primary GBMs account for approximately 90% of all GBM cases (40) and most commonly occur with rapid onset of symptoms and amplification of EGFR (41). Secondary GBM are those tumors with a prior history of lower grade astrocytomas or more protracted clinical courses prior to diagnosis. They more often possess p53 mutation and, recently, have also been shown to be distinguished from primary GBMs by frequent mutation in isocitrate dehydrogenase (IDH1- R132H; refs. 42–45). IDH1 mutation also most commonly occurs in proneural GBM (30). Given the differences in GRK3 expression in classical and proneural tumors, we wondered whether GRK3 expression would correlate at the protein level with EGFR amplification and IDH1 mutation. We obtained a GBM tissue microarray with 35 GBM specimens. FISH for EGFR amplification and immunohistochemical detection of IDH1 mutation were conducted as described in Materials and Methods. Five of the 35 GBM cases exhibited clear amplification of EGFR (Fig. 3A and B), whereas four of the 35 cases exhibited IDH1 mutation (Fig. 3C and D). None of the cases possessed both EGFR amplification and IDH1 mutation. A broad range of GRK3 expression was evident in the tissue microarray. As illustrated in Fig. 3E and F, and consistent with data presented above, cases with IDH1 mutation (secondary, proneural GBM) exhibited greater GRK3 expression than those with EGFR amplification (primary, classical GBM). In all, 4 of the 5 EGFR-amplified cases showed low levels of GRK3 expression, whereas all of the cases with IDH1 mutation exhibited moderate to high levels of GRK3 expression. Together, the above data strongly suggest that GRK expression differs between molecular subtypes of GBM and that GRK3 expression is decreased in tumors of the classical subtype.

To assess whether GRK expression might also differ as a function of tumor grade, we conducted immunohistochemical analyses of GRK expression in 19 specimens representing all 4 grades of astrocytoma (46). The intensity of specific GRK isoenzyme expression appeared consistent across all tumor grades (I–IV; Table 1). Interestingly, GRK localization within tumor tissue was isoenzyme specific. While GRKs 2, 4, and 5 exhibited diffuse expression throughout

![Figure 2](image.png)

**Figure 2.** GBM subtype-specific expression of GRK3 mRNA. A, expression of GRKs in 30 primary GBM specimens relative to 7 normal human astrocyte (HA) specimens was measured by quantitative PCR. For each GRK, expression in GBM was normalized to the mean astrocyte expression. Presented are the box and whiskers plots of these results. *, $P < 0.05$ as determined by Wilcoxon rank-sum test for comparisons between GRK expression in GBM and HA specimens. B, seventeen GBM specimens were subtyped on the basis of quantitative PCR measures of (i) EGFR and CDKN2A (classical, C), (ii) PTEN and NF1 (mesenchymal, M), and (iii) NEFL (neural, N) and PDGFRα (proneural, P). GRK3 expression was quantified and is plotted as a function of subtype.
This hypothesis, we consequence of increased EGF receptor activation. To test decreased expression of GRK3 in classical GBM was a of GPCRs (29, 30, 47). Therefore, we hypothesized that the p53 or IDH1 and with evidence for increased contributions amplification of the EGF receptor without mutation of IDH1. Reduced GRK3 expression might not require increased EGFR expression in vitro.

To test whether EGF pathway activation could regulate GRK expression in GBM cell lines, we compared GRK expression in U87MG cells engineered to overexpress EGFR or the constitutively active truncated form of EGFR, EGFRvIII. Increased EGF pathway activation had significant effects on GRK expression. Notably, GRK2 levels were significantly reduced in cells with overexpression of either EGFR or EGFRvIII (Fig. 4C). Expression of GRK3 was similarly reduced in U87-EGFRvIII, but not in U87-EGFR.

Levels of GRK4–6 were not altered by changes in EGF receptor expression. These findings lend additional support to the hypothesis that the EGF pathway can regulate GRK expression, especially the closely related isoenzymes GRK2 and 3. Importantly, this subfamily of GRKs has previously been implicated in astrocytoma biology (22) and therefore, subsequent evaluations focused on GRK2 and 3 only.

These findings raised the possibility that low levels of GRK expression in U87 cells might reflect basal activation of the EGF receptor pathway. To address this possibility, we treated U87, U87-EGFR, and U87-EGFRvIII cells with the potent EGFR inhibitor PD153035 or vehicle control (dimethyl sulfoxide) in the presence and absence of EGFR (Supplementary Fig. S3). PD153035 eliminated phosphorylation of EGF receptor (data not shown) as well as baseline and EGF-stimulated phosphorylation of ERK and AKT.

PD153035 treatment had no effect on GRK3 expression. Thus, in U87 cells, EGFR activation can regulate, but it is not required for regulation of GRK3 expression.

To further evaluate the regulation of GRK expression by pathways commonly activated in GBMs, we treated normal human astrocytes with EGF, PDGF, TGF-β, or CXCL12. Contrary to the effect of overexpression or mutational activation of the EGF receptor in U87 cells, treatment of astrocytes with EGF increased GRK2 expression to a small degree (Fig. 4D). Astrocyte GRK3 expression, however, was reduced by EGF treatment. Similar to EGF treatment, treatment with PDGF or TGF-β resulted in increased GRK2 but tended to reduce GRK3 expression (Fig. 4D). The CXCR4 ligand, CXCL12, had no effect on GRK2 expression.

GRK expression is regulated by growth factors

If reduced GRK3 expression has mechanistic significance in GBM, it is important to understand how its expression is reduced. The classical subtype of GBM is characterized by the amplification of the EGF receptor without mutation of p53 or IDH1 and with evidence for increased contributions of GPCRs (29, 30, 47). Therefore, we hypothesized that the decreased expression of GRK3 in classical GBM was a consequence of increased EGF receptor activation. To test this hypothesis, we first characterized 4 commonly used GBM cell lines with regard to their EGFR expression. We measured EGFR expression relative to normal human astrocytes in U87MG, LN308, LN428, and LN827 GBM cells. None of the cell lines exhibited significant alterations in EGFR levels compared with astrocytes (Fig. 4A). We next conducted Western blot analyses for GRK expression and found that the cell lines were generally similar to the primary GBM specimens, exhibiting significantly reduced expression of GRK3 compared with human astrocytes and frequent elevation of GRK4 (Fig. 4B). The cell lines differed dramatically from the primary GBM specimens in the universal elevation of GRK6 expression. These findings suggested that whereas decreased GRK3 expression was common to both GBM tissue and established GBM cell lines, the mechanisms for regulating expression of GRK3 and other GRKs might differ in vivo and in vitro. Whereas a strong correlation exists for increased EGFR and decreased GRK3 in GBM tissue, reduced GRK3 expression might not require increased EGFR expression in vitro.

Figure 3. GBM subtype-specific expression of GRK3 protein: a GBM tissue microarray was evaluated for EGF receptor amplification, IDH1 mutation, and GRK3 expression. A, an example of normal FISH analysis for EGFR. Red, the EGFR gene; green, chromosome 7 centromeric sequences (CEP7 probe). B, an example of abnormal FISH for EGFR revealing EGFR amplification. C, positive staining for IDH1 mutation (IDH1 positive, brown). D, absence of IDH1 mutation. E, GRK3 expression is diffusely expressed within an IDH1-positive tumor specimen (red). Nuclei are stained blue with 4′,6-diamidino-2-phenylindole (DAPI). F, absence of GRK3 expression in a tumor specimen with EGFR amplification. Scale bars, 50 μm (A–D) and 100 μm (E and F).
expression but consistently suppressed GRK3 expression by a small amount.

Together the above data suggest that regulation of GRK expression differs between primary cells or tumor tissue and established GBM cell lines. These findings may also point to differences in GRK expression in transformed versus normal cells. While the regulation of some GRKs, such as 2 and 6, appeared to be context dependent, GRK3 expression was commonly decreased by gliomagenic pathways at the mRNA and protein levels, in GBM tissue, established GBM cell lines, and normal human astrocytes.

The level of GRK3 expression regulates growth

Changes in GRK3 expression have the potential to alter signaling and function of GRK targets including GPCRs, receptor tyrosine kinases, and multiple additional components of the GRK interactome (reviewed in ref. 48). As a consequence, changes in GRK expression might directly

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**Table 1. GRK expression in astrocytoma**

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>n</th>
<th>GRK2 Range</th>
<th>GRK3 Range</th>
<th>GRK4 Range</th>
<th>GRK5 Range</th>
<th>GRK6 Range</th>
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<tr>
<td>A-I</td>
<td>6</td>
<td>1.5–3</td>
<td>1.5–3</td>
<td>2.7–1</td>
<td>2.8–2</td>
<td>0.8–1</td>
</tr>
<tr>
<td>A-II</td>
<td>3</td>
<td>1.0–1</td>
<td>0.7–1</td>
<td>2.3–1–3</td>
<td>2.3–2</td>
<td>1.0–1</td>
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<tr>
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<td>1.6–1–2</td>
<td>1.0–2</td>
<td>2.4–1–4</td>
<td>2.0–1–3</td>
<td>0.8–0–1</td>
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<tr>
<td>A-IV</td>
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<td>0.8–1</td>
<td>3.4–3–4</td>
<td>2.4–2</td>
<td>1.0–1</td>
</tr>
</tbody>
</table>

NOTE: Immunohistochemical scores indicate the percentage of tumor cells positive for each GRK: 1 = 0%–25%, 2 = 26%–50%, 3 = 51%–75%, and 4 = 76%–100%.

aGRK scores are for vascular structures.

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**Figure 4.** Growth factor regulation of GRK expression. A, EGFR expression was examined in human astrocytes (HA) and GBM cell lines by quantitative PCR. B, GRK expression in GBM cell lines U87, LN308, LN428, and LN827 was measured by Western blot and normalized to GRK expression in normal human astrocytes. C, GRK expression in U87 cells engineered to overexpress EGFR (U87-EGFR) or the constitutively active truncated form of EGFR (EGFRvIII). Expression levels for GRK were normalized for each GRK to expression in U87 cells. D, GRK2 and 3 expression in normal human astrocytes treated with growth factors as indicated. Expression for each GRK was normalized to GRK expression in astrocytes grown in serum-free media (SF) alone. In each case, the means and SEM of 3 independent experiments are presented. *P < 0.05; **P < 0.005 as determined by 2-tailed t test comparing expression for each specific GRK. Though not denoted by asterisks, GRK4 and 6 expression (B) in each of the GBM cell lines is significantly different from GRK4 and 6 expression in HAs.
regulate astroglial growth. To determine whether reduced GRK3 expression was necessary for maximal GBM cell growth, we compared the effect of increased GRK2 or 3 expression on cell growth in vitro. U87 and LN428 cells were engineered to overexpress GRK2 or 3 (Fig. 5A). Overexpression of GRK3 significantly reduced growth of both U87 and LN428 cells (Fig. 5B). Overexpression of GRK2 also reduced cell growth albeit not to the extent observed for GRK3. These data indicate that GRK2 and 3 can exert a cell autonomous effect on growth.

To further evaluate the growth effects of GRK overexpression, we used a coculture model of the GBM perivascular niche (PVN). This model was designed to mimic the critical growth-promoting interactions that occur between GBM cells and microvascular endothelial cells (38, 39, 49). CXCL12 and its GPCR, CXCR4, are implicated as mediators of the interaction between brain tumor cells and endothelial cells (50), and therefore, we hypothesized that changes in GRK expression would alter tumor cell behavior in this coculture model of the PVN. Parental U87 or LN428 cells, or GRK2 and GRK3 overexpression variants of both cell lines, were grown in coculture with HBMECs. HBMECs were first cultured in Matrigel resulting in the formation of capillary-like lattices (Fig. 5C). Addition of U87 or 428 cells to these HBMEC networks resulted in the colocalization of the U87 or 428 cells with the endothelial cells (Fig. 5D). In this configuration, HBMECs promoted increased U87 and LN428 growth compared with the growth of these cells when they were plated alone (Fig. 5E). Overexpression of GRK2 had no significant effect on the HBMEC-stimulated growth of U87 or 428 cells. In contrast, GRK3 overexpression significantly reduced HBMEC-stimulated U87 and LN428 growth. Similar to what was seen in U87 monoculture, GRK3 overexpression resulted in an approximately 50% reduction in U87 cell number. Interestingly, the effect of GRK3 overexpression on LN428 growth was much greater in coculture, where it completely abrogated the HBMEC effect on growth. Thus, in addition to cell autonomous effects on growth, GRK3 may also function in regulating growth responses to the tumor microenvironment. Together these data indicate that GRKs possess growth-inhibitory properties and that maximal growth of GBM, especially of the classical subtype, may require suppression of GRK3 expression.

**Discussion**

In the current study, we found that altered expression of GRKs is a characteristic feature of GBMs. Changes in GRK expression were apparent in the multidimensional genomic data sets available through the TCGA as well as in PCR validation and immunohistochemical analyses of primary GBM specimen. While several relationships between GRK expression and specific subtypes of GBM were suggested by
the analysis, the most consistent association, evident in primary GBM tissue specimens, established GBM cell lines, and normal human astrocytes, was between low levels of GRK3 and EGFR activation. Decreased GRK3 expression was functionally significant as low levels of expression were necessary for maximal growth of 2 different GBM cell lines assayed as monoluculture as well as in a coculture model of the perivascular space. Strikingly, while there were multiple circumstances in which GRK2, a closely related GRK family member exhibited similar activity to GRK3, GRK3 possessed greater potency in growth regulation.

Defining how decreased expression of GRK3 stimulates tumor growth has significance for cancer biology and experimental therapeutics. Decreased GRK3 expression could impact tumor growth through GPCR-dependent and/or GPCR-independent mechanisms. Pathologic GPCR function in other diseases frequently involves alterations in receptor desensitization as a consequence of abnormal GRK expression or activity. Canonical homologous GPCR desensitization requires the phosphorylation of ligand-occupied receptor by GRKs and subsequent arrestin binding (13). This process functionally uncouples the receptor from the activation of heterotrimeric G-proteins and shifts GPCR signaling toward arrestin-dependent pathways. Arrestins function as scaffolds for a variety of signaling molecules including Raf, MEK, ERK, ASK1, as well as non–receptor tyrosine kinases (51). Thus, loss of desensitization results in both sustained heterotrimeric G-protein signaling and diminution of arrestin-mediated signals. The potential pathologic consequences of failed desensitization are well illustrated in WHIM (Warts, Hypergammaglobulinemia, Infections, Myelokathexis) syndrome (52). Among the hallmark features of this disorder is abnormal chemotaxis of leukocytes resulting in retention in the bone marrow (Myelokathexis) and increased accumulation at sites of cutaneous papilloma virus infection (warts). Loss of CXCR4 desensitization has been identified as the molecular basis for WHIM syndrome in many cases, resulting from either truncation of C-terminal GRK phosphorylation sites on CXCR4 or loss of GRK3 activity (16, 53). In addition, GRK6 may also be required for normal CXCR4 desensitization in leukocytes (54). Similarly, altered GRK-mediated GPCR desensitization has been identified as pathogenic in several other diseases including cardiac failure (reviewed in ref. 55), bipolar disease (56), and asthma (57). To date, no cancer-promoting effect of GRK loss has been described, including in patients with WHIM syndrome.

In addition to shaping the intracellular signaling of classic 7-transmembrane domain GPCRs, GRKs, especially GRK2, have been shown to also regulate a large number of non–GPCR substrates. With regard to cancer biology, prominent among these targets are EGF and PDGF receptors, as well as Gqα, phosphoinositide 3-kinase (PI3K), MAP/ERK kinase (MEK), AKT, and GIT1 (reviewed in ref. 48). These kinase-dependent and -independent functions of GRK2 regulate cell motility, proliferation, differentiation, and apoptosis. The direction of GRK effects on these substrates can be stimulatory or inhibitory. As examples, GRK2 recruitment to ligand-bound EGFR increases ERK-1/2 activation (58), whereas GRK2-mediated phosphorylation of PDGFR reduces tyrosine kinase activity (59). Particularly interesting with regard to the growth effects observed in this study is the role that GRK2 plays in the G2–M cell-cycle checkpoint. GRK2 is normally phosphorylated by CDK2 during G2 and degraded (60). Abrogation of GRK2 phosphorylation results in its stabilization and cell-cycle delay. Thus, the effects of altered GRK2 and by homology, GRK3, on GBM and astrocyte cell growth might be related to known GRK2 effects on G2–M progression.

Among the important findings in the current study is that common genetic alterations in GBMs result in altered GRK expression. In primary GBM specimens, GRK mRNA and protein abundance was altered as compared with normal human astrocytes. These findings are consistent with known regulation of GRK expression at the mRNA and protein levels. Importantly, pathways frequently altered in cancer, such as the RAS-MAPK pathway (61, 62), the PI3K/AKT pathway, and MDM2-mediated ubiquitination (63), are known to regulate GRK transcription and protein stability. Moreover, protein kinases, such as PKC, PKA, ERK and SRC, have all been shown to regulate GRK2 activity (48). The close homology between GRK2 and 3, including at consensus phosphorylation sites, suggests that these kinases might similarly regulate GRK3 activity. Thus, primary oncogenic events resulting in autocrine, paracrine, or mutational activation of growth factor receptor pathways alters GRK expression and function, impacting on growth.

Understanding how altered GRK activity affects GBM growth has the potential for an enormous impact on cancer care. In cases of increased GRK activity, kinase inhibitors (64) as well as decoy-binding partners [i.e., ARKct (ref. 65)] could be applied. In cases of decreased GRK activity, phosphatase inhibitors to promote retained GPCR phosphorylation would be appropriate trial therapeutics. If altered GRK activity affects GBM growth secondary to dysregulated GPCR signaling, then therapeutic options to address this aspect of GBM biology could also include specific GPCR antagonists.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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References


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## Suppression of G-protein–Coupled Receptor Kinase 3 Expression Is a Feature of Classical GBM That Is Required for Maximal Growth

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