Differential Role of Proline-Rich Tyrosine Kinase 2 and Focal Adhesion Kinase in Determining Glioblastoma Migration and Proliferation

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Abstract
The propensity of malignant gliomas to invade surrounding brain tissue contributes to poor clinical outcome. Integrin-mediated adhesion to extracellular matrix regulates the migration and proliferation of many cell types, but its role in glioma progression is undefined. We investigated the role of the cytoplasmic tyrosine kinases FAK and Pyk2, potential integrin effectors, in the phenotypic determination of four different human glioblastoma cell lines. While FAK expression was similar between the four cell lines, increased FAK activity correlated with high proliferation and low migratory rates. In contrast, Pyk2 activity was significantly increased in migratory cell lines and depressed in proliferative cell lines. Overexpression of Pyk2 stimulated migration, whereas FAK overexpression inhibited cell migration and stimulated cellular proliferation. These data suggest that FAK and Pyk2 function as important signaling effectors in gliomas and indicate that their differential regulation may be determining factors in the temporal development of proliferative or migrational phenotypes.

Introduction
Malignant gliomas are a leading cause of CNS tumor-related death and current therapy is of limited effectiveness. Glioblastomas are rapidly growing and are highly resistant to chemotherapeutic agents. In addition to chemotherapeutic approaches, current treatment options include surgical resection, immunotherapy, and radiation but all have largely been ineffective in advancing life expectancy beyond 1 year (1, 2). Pediatric gliomas portend a particularly abysmal prognosis (3). Curative resection of diffuse astrocytomas and glioblastomas is limited by their highly invasive behavior. Malignant cells from the primary tumor demonstrate a propensity to migrate into the surrounding brain tissue, giving rise to secondary tumors that develop at the resection margins from which subsequent migration can arise. Thus, both proliferation and migration are integral to the severity of this disease and greater insights into the molecular mechanisms that regulate these phenotypes are important for improved clinical outcome.

Integrins are a widely dispersed family of adhesion receptors interposed between the extracellular matrix (ECM) outside cells and the force-generating contractile systems inside cells that are uniquely situated to respond to molecular as well as mechanical stimuli by indirectly activating intracellular signaling pathways that play important roles in adhesion, migration, and cellular growth (4–6). The prototypical effector protein coupling integrin-matrix interactions with signaling events is the focal adhesion kinase (FAK). FAK is a Mr, 125,000 non-receptor tyrosine kinase that is rapidly phosphorylated following integrin engagement with concomitant increases in its kinase activity (7–10). These phosphorylated tyrosine residues provide high-affinity acceptor sites for other signaling effectors (Src, PI 3-kinase, structural proteins (talin, paxillin), and adaptor molecules (Grb2, p130 Cas) that are thought to mediate FAK’s role in integrin-dependent cytoskeletal organization, migration, survival, and proliferation (11). Activation of FAK can also occur by non-integrin pathways including a number of growth factors, bioactive lipids, and neuropeptides (7). Cellular regulation of FAK activity is not completely understood. FAK is inhibited by the FAK related non-kinase (FRNK), a COOH-terminal alternatively spliced variant of FAK that functions as an endogenous regulator (12, 13), as well as by PTEN, a tumor suppressor gene that directly associates with FAK and reduces its phosphorylation (14, 15). Interestingly, PTEN is mutated at very high frequency in glioblastomas (16, 17).

Substantial evidence supports a role for FAK in cell migration both in vitro and in vivo (11). FAK expression is increased in migrating cells during the process of wound repair and in a number of invasive tumors (18, 19). Direct evidence for a role of FAK in cell migration has been provided by studies with FAK null mice (20). Fibroblasts cultured from FAK null mice exhibit an increased number of focal adhesions but fail to migrate. Reconstitution of FAK in these cells promotes focal adhesion turnover and restores cell migration (21).
A recent report extended the association between FAK and invasion in glioblastoma cells *in vitro*, where investigators observed down-regulation of tyrosine phosphorylation of FAK in migrationally impaired glioblastoma cells following treatment with cerivastatin, a HMG-CoA reductase inhibitor (22). In addition to its central role in cell migration, increased FAK activity has also been correlated with increased cell proliferation and cell cycle progression which could contribute to tumor progression (23, 24).

A second member of the FAK subfamily of non-receptor tyrosine kinases is known variously as the proline-rich tyrosine kinase (Pyk2), cell-adhesion kinase β (CAKβ), related-adhesion focal tyrosine kinase (RAFTK), calcium-activation dependent tyrosine kinase (CADTK), and FAK2 (25–29). Pyk2 has a conserved domain structure and significant sequence homology (~43% overall, 61% kinase domain) to FAK. Pyk2 shares a number of functionally important residues with FAK including an NH₂-terminal domain) to FAK. Pyk2 has a conserved domain structure and significant sequence homology (~43% overall, 61% kinase domain) to FAK. Pyk2 shares a number of functionally important residues with FAK including an NH₂-terminal autophosphorylation site (Y402), a COOH-terminal tyrosine residue (Y881) that serves as a binding site for the adapter protein Grb2, proline-rich sequences capable of mediating interaction with SH3 containing proteins including p130Cas and Graf, and a focal adhesion targeting sequence (26). Although Pyk2 can interact with many of the same proteins as FAK, the cellular significance of these interactions are not as well defined.

Despite their similarity, Pyk2 and FAK display a number of significant differences. Pyk2 has a more limited tissue expression than FAK and, in particular, is highly enriched in the CNS (25, 27, 28). Developmental expression is also different. Pyk2 is barely detectable during early CNS development, whereas FAK is the predominant focal adhesion kinase. In the mature brain, however, expression of Pyk2 exceeds that of FAK and is primarily concentrated in the neurons of the forebrain region, but has also been found in glia in some pathological conditions (30, 31). Regulation of Pyk2 activity and its cellular roles also differs from FAK. FAK is primarily activated following integrin-mediated adhesion to ECM but is also activated in response to a number of stimuli including growth factors, neuroptides, and v-Src transformation (32–35). In contrast, Pyk2 is activated primarily in response to stimuli that increase intracellular Ca²⁺ (26, 27, 29, 36) although adhesion-dependent phosphorylation of Pyk2 has been described. Overexpression of Pyk2 in some cell lines induces apoptosis (37) while expression of FAK protects cells from apoptosis which may result from loss of attachment to ECM (38, 39). Thus, it has been proposed that Pyk2 and FAK may function in an antagonistic manner in cells that express both kinases (40). Interestingly, Pyk2 is capable of compensating for some, but not all, FAK-regulated functions in cells lacking FAK expression (41–43).

The relative activation states of diverse signaling pathways involved in cell proliferation, migration, and survival/death are likely to prove to be key determinants of the aggressiveness of malignant cells and tumor invasion. In the current study, we sought to evaluate FAK and Pyk2 in the context of the proliferative and migratory potential of glioblastoma cell lines. Our results support a differential role for the two focal adhesion kinases in determining the phenotype of glioblastoma cell lines, where Pyk2 overexpression induced migration and FAK overexpression induced proliferation.

**Results**

**Differential Expression and Activity of Pyk2 and FAK in Glioblastoma Cell Lines**

Elevated expression levels and activity of the cytoplasmic tyrosine kinase FAK have been observed in a number of human tumors and is associated with increased potential for tumor invasion (44). Therefore, we sought to determine the expression level and relative activity of FAK in glioblastoma cell lines known to have different migration behavior *in vitro*. Immunoblotting of total cell lysates indicated that the total amount of FAK protein between glioblastoma cell lines was similar (Fig. 1). Although the total amount of FAK was similar between the four cell lines, the activity level of FAK varied between the cell lines as assessed by blotting with phospho-specific FAK pY397 antibodies. FAK activity was highest in U118 and G112 cells and approximately half as much in the SF767 and T98G cell lines.

In contrast to the relatively equivalent expression of FAK between cell lines, there was a marked difference in both the expression level and activity of Pyk2 (Fig. 1). The SF767 cell line exhibited both the highest total expression level and highest phosphorylation level of Pyk2, which was 10-fold greater compared to the G112 cell line which expressed significantly less total or active Pyk2 protein. The T98G cell line expressed lower amounts of total Pyk2 than the SF767 cell line; however, a significant proportion of that was phosphorylated. Interestingly, the U118 cell line expressed the largest amount of mRNA (6-fold compared to SF767, data not shown) and the second largest amount of Pyk2 total protein; however, there was little reactivity with the phospho-specific pY402 antibody indicating that the majority of Pyk2 was not active in this line.

**The Intrinsic Migratory Potential of Glioblastoma Cell Lines Correlates With the Relative Amount of Phosphorylated Pyk2**

The propensity of glioblastoma cells to assume either a migratory or a proliferative phenotype allowed us to assess the intrinsic rate of migration of each cell line against the relative amounts of the individual focal adhesion kinases. The intrinsic rates of migration between the four glioblastoma cell lines was assessed by migration assay as previously described (45) (Fig. 1). The SF767 cell line consistently displayed the highest migratory rate (mean 14.65 µm/h; 95% CI 12.03–17.27 µm/h) and the G112 cell line the least (mean 4.93 µm/h; 95% CI 4.48–5.49). The T98g (mean 14.11 µm/h; 95% CI 11.34–15.98 µm/h) and the U118 (mean 11.60 µm/h; 95% CI 9.73–13.46 µm/h) cell lines migrated with intermediate rates. The migratory phenotype correlated positively with the relative amount of phosphorylated Pyk2 rather than Pyk2 expression level. Note that the T98g cells, which express less total Pyk2 than U118 cells, but a greater active fraction, were the more migratory of the two. In contrast, cell lines with...
the highest level of phosphorylated FAK exhibited the lowest migratory rate. These results suggest that Pyk2 activity is a stronger determinant of the migratory phenotype in glioblastoma cells.

Induction of Glioblastoma Cell Migration by Overexpression of Pyk2

Previous experiments determined that phosphorylated Pyk2 correlated with increased migration of the glioblastoma cell lines tested. Next, we sought to substantiate whether the migratory phenotype could be induced in poorly migratory cell lines by overexpressing wild-type Pyk2. Replication-deficient adenoviruses encoding Pyk2 were utilized to achieve consistent and substantial recombinant protein expression in the glioblastomas. Migratory characteristics of Pyk2-overexpressing cells were compared to that of cells infected with a control adenovirus expressing β-galactosidase. Overexpression of wild-type Pyk2 significantly increased the linear migration rate for all cell lines by 82.7% (mean difference +5.97 μm/h; 95% CI of the difference +4.20 to +7.74 μm/h, \( P < 0.001 \)) (Fig. 2). The most substantial increase in migration rate (203.1%) following Pyk2 overexpression occurred in the T98g cell line (mean difference +9.50 μm/h; 95% CI of the difference +7.33 to +11.68 μm/h, \( P < 0.001 \)). Furthermore, overexpression of FRNK resulted in significant inhibition of migration of all cell lines (~61.5%)
when compared to control β-galactosidase-infected cells (mean difference across all four lines: −4.44 μm/h; 95% CI of the difference −2.91 to −5.97 μm/h, P < 0.001). This inhibition required targeting to focal adhesions because expression of the point mutant FRNK S1034, which fails to localize in focal adhesions (46), did not significantly inhibit migration rate (Fig. 3).

Expression of FAK Inhibited Glioblastoma Cell Migration

As FAK activity correlated negatively with endogenous migration rate in the glioblastoma cell lines, we examined the effect of overexpression of FAK in the SF767 and G112P cell lines. These cell lines exhibit the highest and lowest endogenous migration rates, respectively. Overexpression of wild-type FAK significantly inhibited the migration rate of both cell lines by 37.6% compared to the migration rate of the control β-galactosidase-infected cells (mean difference −3.11 μm/h; 95% CI of the difference −0.32 to −5.91 μm/h, P = 0.02) (Fig. 3). SF767 cells infected with the control virus migrated at a rate of 13.43 μm/h (95% CI 11.70–15.21 μm/h from 10 independent assays). FAK overexpression in SF767 cells resulted in a rate of 8.48 μm/h (95% CI 7.30–9.66 μm/h, P < 0.001). Control virus-infected G112 cells migrated 3.95 μm/h (95% CI 3.02–4.89 μm/h) and FAK overexpression reduced the rate to 1.84 μm/h (95% CI 0.71–2.97 μm/h, P = 0.017).

Inhibition of the migration rate observed following overexpression of WT FAK was not observed when cells were infected with FAK variants containing mutations that affected FAK activity. SF767 cells infected with the FAK autophosphorylation mutant Y397F had a migration rate comparable to SF767 cells infected with the control β-galactosidase (11.52 μm/h; 95% CI 9.60–13.43 μm/h versus 13.46 μm/h; 95% CI 11.70–15.21 μm/h, P = 0.17). Similar results were observed in the G112 cell line expressing either FAK Y397F or control protein (4.87 μm/h; 95% CI 3.12–523 μm/h in FAK Y397F-infected cells versus 3.95 μm/h; 95% CI 3.02–4.89 μm/h, P = 0.28 in control-infected cells). Infection of cells with a kinase dead variant of FAK (K454R) also failed to inhibit the migration rate relative to the rate in control-infected cells. These results suggest that FAK inhibition of migration rate requires FAK activity. In this regard, it is noteworthy that the G112 cell line which has the lowest migration rate exhibited an endogenous level of FAK activity that is twice as high as the endogenous activity of FAK in the SF767 cells which have the highest migration rate (Fig. 1B).

The Pyk2 related non-kinase, PRNK, is a recently described isoform of Pyk2 that encodes part of the COOH-terminal domain of Pyk2. PRNK is expressed at high levels in the brain and has been proposed to selectively down-regulate Pyk2 function (47). Therefore, we examined the effect of expression of PRNK on the migration rate of the SF767 and G112 cell lines. Interestingly, PRNK expression significantly inhibited the migration rate of SF767 cells (4.47 μm/h; 95% CI 3.32–5.63 μm/h versus 13.46 μm/h; 95% CI 11.70–15.21 μm/h, in control cells; P < 0.001) while PRNK expression in the G112 cell line did not significantly alter migration rate relative to control-infected cells (4.37 μm/h; 95% CI 2.85–5.89 versus 3.95 μm/h; 95% CI 3.03–4.89, P = 0.68).

Pyk2 and FAK Are Differentially Localized in the Proliferating (Core) and Migratory (Rim) Phenotypes

In the radial migration assay, cells can be divided into two populations: a proliferating core population and a migratory population at the rim of the cell dispersion (48). The subcellular localization of FAK and Pyk2 in the core and migratory rim SF767 cells was examined with immunofluorescence and laser scanning confocal imaging. Cells in the core region of the migration assay displayed heavy FAK staining throughout the cytoplasm and also at the cell membrane (Fig. 4). Cells in the migrating rim population were evident by their membrane ruffling and were much larger and more flattened than the core cells. Overall intensity of FAK immunofluorescence in the migratory rim cells was reduced compared to cells in the core; furthermore, the preponderance of signal was localized in the perinuclear region. In contrast to FAK, Pyk2 immunofluorescence was markedly higher in the migrating rim cells than the core cells. Pyk2 distribution in the core cells localized to the perinuclear region while the rim cells displayed an abundant cytoplasmic staining.

SF767 cells, which possess the highest level of activated Pyk2, exhibited the highest migration rate. Overexpression of FAK in these cells led to a decrease in cell migration. Therefore, we examined if the inhibition of migration induced by FAK overexpression was accompanied by an increase in cell proliferation. The percentage of the total infected cell population with cell nuclei expressing significant quantities of Ki67, a nuclear protein expressed prominently in proliferating cells, was determined for SF767 cells infected with FAK, Pyk2, or a control β-galactosidase encoding adenoviruses (Fig. 5 and Table 1). The reduction of the migration rate observed in the SF767 cells following FAK expression was accompanied by a significant increase in the percentage of proliferating cells compared to control (64.3% versus 29.9%, P < 0.001). In particular, the number of proliferating cells in the invasive rim...
The population increased significantly compared to control. Conversely, overexpression of Pyk2 in SF767 cells significantly decreased the percentage of proliferating cells compared to control-infected cells (7.1% versus 29.9%, $P < 0.001$). This reduction was particularly apparent in the core population that normally exhibits significant proliferation as is observed in the control treated cells.

**Discussion**

The tendency of gliomas to invade surrounding brain tissue contributes significantly to a poor clinical prognosis for affected patients. This invasive behavior reduces the efficacy of current surgical, radiological, and chemotherapeutic treatment modalities. Although the molecular mechanisms that regulate the migration of gliomas from primary tumor sites have not been defined, interactions with ECM are likely to play an important role in this process. In this report, we examined the roles of two focal adhesion kinases, FAK and Pyk2, with regard to their contribution to the migratory or proliferative phenotypes of glioblastoma cells in vitro. The major findings of this work are as follows: (a) The migratory potential of glioblastoma cells correlated with the level of activated Pyk2, but not the total amount of Pyk2 protein; (b) glioma cell lines possessing the slowest absolute migration expressed high levels of FAK activity; (c) overexpression of Pyk2 stimulated the migratory phenotype, whereas FAK overexpression inhibited migration of glioma cell lines; (d) Pyk2 and FAK localization appeared to differ in proliferative versus migrating cells; (e) proliferation of these cell lines was potentiated by FAK expression and decreased by Pyk2 overexpression. These data indicate that FAK and Pyk2 may function as important signaling effectors in gliomas and suggest that their differential regulation may be determining factors in the temporal development of proliferative or migrational phenotypes.

Integrins are the predominant cellular receptors for ECM and play an integral role in cell migration. A role for integrin-matrix interactions in glioma invasion has been supported by previous studies demonstrating that glioma cell migration in vitro is inhibited by antih1 integrin antibodies or inhibitory peptides (49, 50). The disparate migratory behavior of the glioblastoma cell lines used in the current study did not appear to be due to differences in integrin expression as flow cytometric analysis revealed equivalent expression of $\beta_1$ integrin on the fastest and slowest migrating cell lines (data not shown). Instead, dissimilarity of the migratory behavior between the cell lines may result from inherent differences in the remodeling of cell-ECM contacts. Integrin ligation and receptor clustering is accompanied by a rapid increase in the phosphorylation of FAK which has a well-defined role in the regulation of focal contact formation (20, 51). As such, FAK serves as a proximal mediator of integrin effects on cell spreading, migration, and proliferation in many different cell types (6, 7, 52). Integrin-dependent Pyk2 activation has been described for a number of cell types (53–55); however, the role of Pyk2 in many of these processes is uncertain and is likely influenced both by cell type and whether FAK is co-expressed in the cell because they may interact and even oppose one another.

FAK activity is often associated with enhanced cell spreading and migration (11, 56) although the mechanism for this promotion remains unknown. Moreover, FAK levels are elevated in many cancers and associated with increased...
Unexpectedly, the intrinsic migratory rates of the glioblastoma cell lines used in this study correlated positively with Pyk2 activity rather than FAK activity. SF767 cells, the most inherently migratory cell line, expressed significant amounts of Pyk2 protein and much of this was phosphorylated. The least migratory of the four cell lines, G112, expressed little total or activated Pyk2. FAK expression and activity contrasted sharply with the effect of Pyk2 activity on migration. Total FAK protein expression was relatively constant between the four cell lines used in the present study; however, FAK activity in the more migratory SF767 cells and T98g cells was approximately 50% of that observed in the less migratory U118 and G112 cells. The basis for the difference observed between total protein and activity for both Pyk2 and FAK in each of the four cell lines is not known. However, it may be due to dissimilar activity of cell specific elements that regulate kinase activity in the different cell lines. These results suggest that the dynamic balance between Pyk2 and FAK activity appears to be an important factor in regulating the migratory behavior of glioblastoma cells in vitro.

Consistent with our initial findings for the intrinsic migratory behavior, overexpression of exogenous Pyk2 significantly increased migration in all cell lines tested compared to cells infected with a control virus substantiating a positive role for Pyk2 in cell migration. Surprisingly, overexpression of FAK inhibited migration in all cell lines; this is in stark contrast to the effect of FAK overexpression in many other cell types. A potential explanation for this effect is that FAK expression suppressed the activity of endogenous Pyk2 as has been previously reported (57). The mechanism of the inhibition of Pyk2 induced by FAK remains to be determined although this effect required FAK kinase activity. It is noteworthy that

**Table 1. FAK and Pyk2 Exert Antagonistic Effects on Cell Proliferation**

<table>
<thead>
<tr>
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<th>Mean Percentage Cells With Ki67 Positive Stained Nuclei (95% CI)</th>
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<tr>
<td>β-Galactosidase</td>
<td>29.9 (24.9–34.9)</td>
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<tr>
<td>PYK2</td>
<td>7.1* (3.3–10.8)</td>
</tr>
<tr>
<td>FAK</td>
<td>64.3* (59.9–68.6)</td>
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Note: The percentage of proliferating SF767 cells was assessed after infection with adenoviruses expressing a control protein (β-galactosidase), Pyk2, or FAK. Values represent the means ± 95% CIs.

*P < 0.001 versus β-galactosidase.
overexpression of FAK did not completely inhibit migration of the highly motile SF767 cells that express high levels of activated Pyk2. This could suggest that Pyk2 interaction with intracellular mediators that do not interact with FAK or interact less efficiently might be essential for Pyk2 to regulate cellular locomotion.

The clinical progression of glioblastoma is characterized by multifocal, proliferative masses; this speaks to a pathological process whereby there is a regulated temporal inverse relationship between migration and proliferation. In the present study, cell lines with high endogenous proliferation rate exhibited low migratory activity. The high proliferative rate of cells was accompanied by increased FAK activity as compared to cells with a highly migratory phenotype. This is consistent with previous studies positively linking FAK activity to cell cycle progression (24, 40). Endogenous FAK activity was decreased in the highly migratory cell lines concomitant with significantly increased Pyk2 activity, suggesting that the commitment to migrate or proliferate in these cell lines may be differentially regulated by the activity of each kinase. Indeed, overexpression of FAK in the highly migratory SF767 cells down-regulated their migration and significantly increased their proliferation, whereas overexpression of Pyk2 decreased cell proliferation and induced migration. While Pyk2 activity has been positively linked to migration in cells that lack FAK expression (43), this activity has not been well characterized in cells that express both kinases. Interestingly, Pyk2 overexpression in some cell types has been linked to apoptosis (37). We failed to observe apoptosis in any of the cell lines following Pyk2 overexpression perhaps due to the accompanying expression of FAK. The basis for the regulation of these kinases in vivo that dictates proliferation or migration remains to be determined but could be due to clues from their microenvironment that differentially lead to activation of one kinase over the other.

The subcellular localization of Pyk2 and FAK might be important in understanding the basis of the function of Pyk2 distinct from FAK. FAK localization was predominantly cytoplasmic in proliferating cells with punctate areas located at the periphery, presumably at the cell membrane, suggesting integration in focal contacts. Overall, FAK staining was decreased in migrating rim cells, and the location of FAK became predominantly perinuclear. Pyk2 localization differed from FAK in that non-motile, proliferating cells possessed a weak, mostly perinuclear signal. Migrating cells, however, were observed to have more intense cytoplasmic staining that extended out to the cell’s periphery. Pyk2 staining resembled that observed in monocytes where it plays a critical role in monocyte spreading and motility (43). The observed difference in the distribution of FAK and Pyk2 may be intimately involved in the behavioral switching observed in the overexpression experiments. For example, PSGAP (Pleckstrin Homology and SH3 domain containing rhoGAP protein) is a recently described GTPase that is distributed in cell periphery regions of fibroblasts (58). Pyk2 binding to PSGAP activates CDC42 and RhoA by inhibiting PSGAP stimulation of their intrinsic GTPase activity. Interestingly, although FAK can associate with PSGAP, it does not induce CDC42 activation via inhibition of PSGAP. Similarly, the endogenous regulator of both FAK and Pyk2, FIP200 (FAK-interacting protein of Mr, 200,000), exhibits a predominately diffuse cytoplasmic distribution (59) consistent with that observed for Pyk2 in glioblastoma cells in vitro. FIP200 can also inhibit FAK activity; however, it has been described to only partially localize with FAK in focal contacts in fibroblasts (60). Therefore, while the expression and localization of potential inhibitors such as PSGAP or FIP200 in glioblastomas is unknown, differences in the subcellular localization between FAK and Pyk2 would be expected to influence protein-protein interactions with such regulators as well as downstream signaling effectors regulating proliferation or cytoskeletal reorganization integral to cell migration.

In summary, the present study presents evidence that two focal adhesion kinases, FAK and Pyk2, may function as important mediators of the proliferative or migratory phenotypes of glioblastomas, respectively. These observed differences might be related to localization to different cellular compartments or likely to changes in kinase activity. Identification of the critical downstream effectors of Pyk2 and FAK in these cells and determining the molecular basis for the regulation of Pyk2 and FAK activity is central to understanding how these kinases exert their effects on glioma cell phenotype. In addition, ongoing studies seek to corroborate the present findings by examining the activities of these two kinases in primary human tumor samples. As both invasion and proliferation are important components of malignant glioma, these data support a potential role for inhibitors of these kinases in improving the clinical prognosis in this disease.

Materials and Methods

Antibodies and Other Reagents

Affinity purified polyclonal antibodies to the COOH-terminal portion of FAK were obtained from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibody to the COOH-terminal portion of Pyk2 was obtained from Transduction Laboratories (Lexington, KY). Site- and phospho-specific polyclonal antibodies to FAK pY397 and Pyk2 pY402 were obtained from Biosource International (Camarillo, CA). Affinity purified antibody to the HA epitope tag was obtained from Upstate Biotechnology. FITC- and Cy5-conjugated secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Rhodamine phallolidin was obtained from Molecular Probes (Eugene, OR). Human placental laminin was obtained from Life Technologies, Inc. (Rockville, MD).

Cell Culture

Four established human malignant glioblastoma cell lines T98g (ATCC CRL 1690), SF767, U118, and G112 (61), were routinely passaged in DMEM containing 10% fetal bovine serum, 1% nonessential amino acids, 2 mM glutamine, 100 units/ml penicillin, and 10 μg/ml streptomycin. Where indicated, culture plates or coverslips were coated with purified ECM solutions (10 μg/ml) for 1 h at 37°C, then rinsed before addition of cells. For adenoviral infections, 1.5 × 10⁵ cells were added to each well of a six-well plate and cultured for 24 h before infection.
Adenovirus Production and Infection

Coding sequence for human FRNK was amplified from cDNA prepared from WI-38 human lung fibroblasts (ATCC CCL-75). Coding sequence for the PRNK was isolated by RT-PCR (Titan System, Roche Biochemicals, Indianapolis, IN) of total RNA isolated from rat hippocampus. The PRNK coding sequence was cloned into pcDNA3.1 in frame with an NH2-terminal FLAG epitope. Sequences of FRNK and PRNK were verified by DNA sequencing. HA-tagged sequences encoding wild-type murine FAK, FAK Y397F, and FRNK-S1034 in the vector pcDNA3.1 were generously provided by David Schlaepfer (The Scripps Research Institute). A cDNA clone encoding full-length rat Pyk2 in pBluescript was a generous gift from Dr. T. Sasaki (Cancer Research Institute, Sapporo, Japan). The Pyk2 insert was subcloned into pcDNA3 in frame with an NH2-terminal 2× HA epitope. Coding sequences of all cDNAs were excised and subcloned into the adenoviral shuttle vector pShuttle-CMV to prepare recombinant E1-deleted adenoviruses using the Ad-Easy system as described (61). Viruses were propagated in 293 cells (ATCC CRL 1573), cloned, isolated, and titered. Cells were infected at matched multiplicity of infection (MOI) ranging from 10 to 20.

Immunoblotting

Cells were washed in cold PBS and lysed by addition of 1 ml ice-cold RIPA buffer [150 mM NaCl, 10 mM Tris (pH 7.2), 1 mM EGTA, 1 mM orthovanadate, 1% Triton X-100, 1% Na deoxycholate, 1% SDS, 100 μM leupeptin, 5 IU/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride] and incubated on ice for 30 min. Lysates were clarified by centrifugation at 16,000 g for 10 min at 4°C. Protein content of the lysate was determined using the BCA assay (Pierce, Rockford, IL). For immunoblotting, equal amounts of protein (10–20 μg) were electrophoresed on 8–16% gradient SDS-PAGE gels (Novex, Carlsbad, CA) and transferred to nitrocellulose. Immunoblotting of transferred proteins was performed with the appropriate antibodies at the indicated concentrations for 1 h at room temperature and was visualized by enhanced chemiluminescence (Perkin Elmer Life Sciences, Boston, MA). Signals were quantified by densitometry utilizing Kodak 1D Image Analysis Software.

Migration Assay

A monolayer migration assay was used as previously described (61). Briefly, slides containing 10 individual 7-mm circular seeding areas surrounded by a hydrophobic template mask (Creative Scientific Methods Inc., Phoenix, AZ) were coated with laminin (10 μg/ml) as described (45). Cells were infected with recombinant adenoviruses at the indicated MOI and cultured 16 h before harvest. Infected cells were resuspended in DMEM containing 10% serum and seeded at a density of 2000 cells per well (internal diameter of 1 mm) of a Cell Sedimentation Manifold (Creative Scientific Methods Inc., Phoenix, AZ). After overnight incubation (16 h), the manifold was removed and an initial measurement (t0) of the diameter of the cell colony was made using an inverted microscope (Axiovert; Carl Zeiss, Thornwood, NY) and image analysis equipment (Scion Image, Frederick, MD). The change in the diameter of the cell population over time was determined at 6 and 24 h following the initial measurement. Slopes of the lines derived from the measurements (radius versus time) were used to calculate the migration rate of the cells. Linear migration from the initial seeded area at t0 was determined for at least 10 replicate samples for each infected construct. Specific migration rates were calculated by normalizing the measurements to nonspecific migration on BSA. The absolute migration and migratory rates were calculated and group means determined along with 95% CIs.

Immunofluorescence

Slides containing migrating cells were washed in PBS and fixed with freshly prepared 2% paraformaldehyde in PBS for 15 min at room temperature. Cells were then washed in PBS, followed by permeabilization in 0.5% Triton X-100 (15 min at RT). Cells were washed with PBS then pre-blocked with PBS containing 3% BSA and 1% porcine serum for 1 h. Cells were then incubated with primary antibody in blocking buffer for 1 h at room temperature. After washing three times in PBS, cells were incubated with either FITC- or Cy5-conjugated secondary antibody for 1 h at room temperature. Cells were washed three times with PBS, methyl salicylate mounting solution was added to preserve fluorochromes, and coverslips were applied. Specimens were examined using either a Nikon Microphot-FXA fluorescent microscope (Nikon, USA) or a LSM510 laser scanning confocal microscope (Carl Zeiss).

Proliferation Assay

Cell proliferation was assayed by detection of the nuclear antigen Ki67 (63). At the migration period end-point, 24 h after removal of the sedimentation manifold, cells were fixed and permeabilized for immunofluorescence. A monoclonal antibody against Ki67 (MIB-1, Immunotech, Westbrook, ME) was used at 1:50 dilution in Tris-buffered saline and incubated at room temperature for 1 h. After three washes in PBS, cells were incubated with a 1:50 dilution of a Cy3-conjugated secondary antibody for 30 min at room temperature. Cells were washed three times in PBS then incubated with a 1:100 dilution of affinity purified rabbit anti-HA antibody followed by a FITC-conjugated anti-rabbit secondary to detect the recombinant HA-tagged Pyk2 or FAK. The percentage of the FAK- or Pyk2-infected cells co-labeled with Ki67 positively stained nuclei was determined by analyzing color photomicrographs of random fields by an observer blinded to the treatment. Values were compared to the control virus-infected cells.

Statistics

Migration and proliferation data were imported to SAS (Cary, NC), checked, and cleaned. Preliminarily, distributional assumptions were tested using the Kolmogorov-Smirnov test. No data set, within cell line or adhesion kinase, deviated significantly from normality. Therefore, means and 95% CIs were chosen for statistical tests. Independent-sample t tests were used for analyses involving two samples, and ANOVA, with post-hoc Scheffe tests, for tests involving more than two samples. All tests are two-tailed, criterion level, P = 0.05.
Acknowledgment

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References


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