JNK mediates insulin-like growth factor binding protein 2/ integrin α5-dependent glioma cell migration

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Abstract. We have previously shown that a molecular interaction between insulin-like growth factor binding protein 2 (IGFBP2) and integrin α5 is necessary for the enhancement of cell migration in IGFBP2-overexpressing gliomas. In the present study, we examined the mechanism through which the IGFBP2/integrin α5 interaction mediates enhanced glioma cell migration. Although both ERK and JNK MAP kinases were activated, JNK was specifically involved in IGFBP2-mediated migration as shown by inhibitor analysis of IGFBP2-overexpressing cells. Because gliomas are solid tumors that require contact with a surface (e.g., other cells, extracellular matrix) for migration, we used the extracellular matrix (ECM) protein fibronectin, which is the sole ligand of the α5ß1 integrin receptor, to show that integrin α5 is an important mediator of JNK activation. In addition, we found the IGFBP2/integrin α5 pathway to be activated in a significantly shorter interval in cells seeded onto fibronectin-coated surfaces compared to cells seeded onto plastic alone. The activation of JNK was downstream of the IGFBP2/integrin α5 interaction, as shown by α5 knockdown experiments using IGFBP2-overexpressing cells. Based on these data we propose that the interaction between IGFBP2 and integrin α5 accelerates cell adhesion, and this, in turn, enhances JNK-mediated glioma cell migration.

Introduction

Gliomas constitute the fourth leading cause of cancer death among patients 40-50 years of age (1). Although the highest grade of glioma, glioblastoma multiforme (GBM), rarely disseminates beyond the central nervous system (CNS), it is extremely invasive within the CNS. The high mortality rate associated with GBM is attributed to resistance to chemotherapy and radiotherapy, and to the tumor's highly invasive nature, which limits the effectiveness of surgical resection (2). The lack of efficacy of standard treatments in GBM patients, with the attendant dismally short median survival of only 8-12 months (3,4), underscores the need for a better understanding of the glioma invasion process, which is a prerequisite for the development of therapies directed towards blocking this process.

Insulin-like growth factor binding proteins (IGFBPs), as part of the insulin-like growth factor (IGF) system, are important control elements for essential cellular processes such as proliferation, differentiation, apoptosis and migration (5,6). IGFBP2 is overexpressed in a wide spectrum of cancers, including prostate carcinoma, synovial sarcoma, neuroblastoma, colon adenocarcinoma, adrenocortical carcinoma, lung adenocarcinoma, Wilms' tumor and hepatoblastoma (7-14). Since our first reports characterizing IGFBP2 overexpression in GBM (15,16), studies have revealed the key roles played by this molecule in the regulation of several glioma pathophysiologic processes, including angiogenesis, cell division and cell migration, and invasion (17-23).

Under normal physiological conditions, IGFBP2 is predominantly expressed in highly proliferative fetal tissues that exhibit extensive cell movement and tissue remodeling (24). Within the central nervous system, IGFBP2 is expressed in fetal glial cells, with significantly decreased expression after birth (25). IGFBP2 is thus an important cell movement protein during development. Moreover, recent data from our laboratory has demonstrated that IGFBP2 exerts an oncogenic effect in platelet-derived growth factor (PDGF)-driven glioma development and progression (26).

In many cancers, the process of invasion involves adherence of tumor cells to the extracellular matrix (ECM), degradation of matrix components, and subsequent movement of the cell body. Signaling pathways involved in the initiation of cellular movement are triggered by a variety of anchorage-dependent signals. It is widely acknowledged that cell adhesion to the ECM plays a vital role in the signal transduction cascades necessary for cell survival (27,28). The integrin family of extracellular matrix receptors mediates cell attachment to ECM molecules. Integrins comprise a large family of heterodimeric proteins which, upon ligand binding, transduce signals from the ECM through a variety of protein kinases and adaptor

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molecules, including Src-family kinases (29,30). Signaling may occur via several different pathways, the best characterized of which are the mitogen activated protein kinase (MAPK) cascades.

MAPK family members are involved in a wide variety of important cellular processes, including cell growth, development, proliferation, differentiation, survival and innate immunity (31). The c-Jun NH_2-terminal kinase (JNK) represents one subgroup of MAP kinases that are activated primarily by cytokines and exposure to environmental stress. The JNK pathway and molecules associated with it, both upstream (MEK kinase 1 and MKK4), as well as downstream [paxillin (32), Spir (33), DCX (34), MAP1B and MAP2 (35)], have been implicated in the regulation of cell migration in a broad range of cell types and developmental systems (36-39).

We have previously shown that in GBM, IGFBP2 interacts with integrin α5 through a specific IGFBP2 RGD-binding domain, and that this interaction is necessary for promoting IGFBP2-mediated cell motility (17). However, the molecular mechanism involved in IGFBP2-mediated glioma cell migration remains unknown, and elucidation of this pathway would provide key insight into potential alternative therapeutic strategies for GBM. Here, we address the specific signaling events involved in IGFBP2-induced glioma cell migration, and demonstrate that IGFBP2/integrin α5 interaction promotes migration through JNK activation in glioma cells.

Materials and methods

Cell lines. The construction of SNB19 human GBM cells overexpressing IGFBP2, the IGFBP2/RGE mutant construct, and the vector control have been previously described (17). Briefly, SNB19 human GBM cells overexpressing IGFBP2 had been previously transfected with a pcDNA3 expression vector encoding IGFBP2 cDNA and a neo-selectable marker using FuGENE6 reagent (Roche Diagnostics Corporation, Indianapolis, IN). SNB19 cells transfected with an empty pcDNA3 expression vector served as a vector control cell line. New SNB19 cells were transfected with a pcDNA3 expression vector encoding D306E-IGFBP2 cDNA and a neo-selectable marker using Nucleofector™ (Amaxa, Gaithersburg, MD) using Solution T and program T-20. Transfected cells were subsequently selected in the presence of G418 (1,750 μg/ml) to establish the IGFBP2-overexpressing stable clones. These cells were maintained in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum in a humidified incubator containing 5% CO_2 at 37˚C.

The chemical inhibitors SB60025 and PP2, and the inactive analog PP3 were obtained from Calbiochem (San Diego, CA), solubilized in DMSO, and used at the various concentrations described in the figures. For inhibitor analysis, cells were serum-starved overnight prior to the addition of the inhibitors for 1 h.

Co-immunoprecipitation and Western blotting. The co-immunoprecipitation performed to check protein-protein interaction, cells were detached by manual scraping, collected by centrifugation, and lysed. Cell lysates underwent 1 h of preclearing incubation with agarose-conjugated goat immunoglobulin G (IgG AC; Santa Cruz Biotechnology, Inc.) at 4˚C. Precleared supernatants were then incubated with anti-IGFBP2 antibody and protein G agarose at 4˚C overnight. Agarose beads were washed three times in cold phosphate-buffered saline (PBS), after which the precipitates were boiled for 5 min in sodium dodecyl sulfate (SDS) loading buffer, loaded onto 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels, electroblotted, and immunoblotted with anti-integrin α5 antibody. Production of the IGFBP2 and D306E-IGFBP2 proteins was measured by Western blotting.

For immunoblot analyses, cells were detached by manual scraping, collected by centrifugation, and lysed. The lysates were boiled for 5 min in SDS loading buffer, loaded onto 10% SDS-PAGE gels, electroblotted onto Hybond ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ), blocked in 5% bovine serum albumin in Tris buffered saline containing 0.1% Tween-20, and probed with the following primary antibodies: anti-JNK polyclonal antibody (Cell Signaling, Waltham, MA), anti-phospho-JNK monoclonal antibody (Cell Signaling), anti-IGFBP2 polyclonal antibody C-18 (Santa Cruz Biotechnology, Inc.), anti-actin polyclonal antibody I-19 (Santa Cruz Biotechnology, Inc.), and anti-integrin α5 monoclonal antibody (BD Biosciences Pharmingen, San Diego, CA). Proteins were detected with an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Piscataway, NJ).

In vitro migration and wound healing assays. Cell migration rates were determined using Boyden migration chambers, specifically, 24-well Biocoat Control Insert chambers (BD Biosciences Pharmingen) with an 8-μm pore polycarbonate filter. The lower compartment contained either 0.5 ml of Dulbecco's modified Eagle's medium/F12 medium with 2% fetal bovine serum as the chemoattractant, or serum-free Dulbecco's modified Eagle's medium/F12 medium as a control. In this assay, $2x10^4$ cells/well were placed in triplicate wells in the upper compartments and incubated for 2 h at 37˚C in a humidified incubator with 5% CO_2. After incubation, the cells that had passed through the filter into the lower wells were fixed, stained, and counted under a microscope. Assays were performed in triplicate. Inhibitor studies were performed by adding given concentrations of inhibitors to both chambers. The differences in the cell migration rates between control and experimental cell lines were analyzed using a two-tailed Student's t-test.

For the wound-healing assays, cells were grown in 6-well plates to 100% confluence. Inhibitors were added at various concentrations 1 h before introducing a small linear scratch into the middle of the well using a 10 μl pipette tip. Cells were allowed to continue growing at 37˚C in a humidified incubator with 5% CO_2. Cells were only removed from the incubator at designated time points (0 and 24 h), at which times photographs were taken under a microscope.

JNK kinase assay. Total cell lysates were prepared and JNK activity was determined using a JNK assay kit according to the manufacturer's instructions (Calbiochem, San Diego, CA). Briefly, JNK was immunoprecipitated using a JNK-specific antibody from the total cell lysate and incubated with a c-Jun/ATP mixture in a kinase reaction. The products resulting from this reaction were then electrophoresed on a

12% SDS-PAGE gel and immunoblotted with a phospho-c-Jun specific antibody.

siRNA knock down of integrin α5. Integrin α5 siRNA (Santa Cruz Biotechnology, Santa Cruz) was transiently transfected into SNB19 cells using the siPORT NeoFX transfection agent from the Silencer® siRNA Transfection II kit (Ambion Inc., Austin, TX). A siRNA concentration of 60 nM and a seeding cell count of $2.3x10^6$ cells/transfection well was used. Glyceraldehyde-3-phosphate dehydrogenase siRNA and a scrambled sequence siRNA from Ambion were used as positive and negative controls. Cells were incubated in the transfection reagent at 37˚C in a humidified incubator with 5% CO_2 for 72 h. Cells were then harvested for protein expression changes and assayed via Western blotting.

Fibronectin and laminin activation experiments. To study the effects of the ECM proteins fibronectin and laminin on the IGFBP2/integrin α5 pathway, cells were first grown on uncoated plastic 150 mm cell culture dishes in normal growth medium, under normal growth conditions, until approximately 75% confluency was reached. Cells were then trypsinized and re-seeded onto either new uncoated plastic 150 mm cell culture dishes, fibronectin-coated 150 mm cell culture dishes (BD Biosciences Pharmingen), or laminin-coated 150 mm cell culture dishes (BD Biosciences Pharmingen). The re-seeded cells were grown in serum-free medium (because of soluble ECM proteins present in serum) under normal growth conditions. Re-seeded cells were allowed to grow for approximately 4 h, after which cells were harvested and lysates prepared for further experiments. For cell morphology comparison, microscopy pictures were taken of the cells immediately (within 4 h of cell seeding) in order to capture the maximal effects of the presence of the pre-coated ECM proteins.

Results

ERK and JNK are phosphorylated in IGFBP2-overexpressing cells. ERK 1/2 and JNK have been shown to play a role in integrin-mediated migration-associated signaling pathways (40,41). Hence we examined the effect of wild-type and mutant (RGE) IGFBP2 overexpression in SNB19 cells on these signaling molecules by Western blot analysis. Both ERK and JNK were phosphorylated to a higher degree in the wild-type IGFBP2 overexpressors compared to the vector control (Fig. 1A and B, see untreated samples). The IGFBP2/RGE mutant overexpressors showed a lower level of JNK phosphorylation compared to wild-type IGFBP2-overexpressing cells, indicative of JNK being downstream of the IGFBP2/integrin mediated signaling cascade. ERK phosphorylation in the RGE mutant IGFBP2 overexpressors, however, was very similar to that of wild-type IGFBP2-overexpressing cells. Consistent with the changes in JNK phosphorylation seen in IGFBP2-overexpressing cells, JNK kinase activity was enhanced in IGFBP2-overexpressing cells, and down-regulated in the RGE mutant overexpressors (Fig. 1C). In addition, a dose-dependent inhibition of both ERK and JNK phosphorylation in IGFBP2 overexpressors produced by chemical inhibitors of those molecules (PD98059 and SP600125, respectively), was also seen.

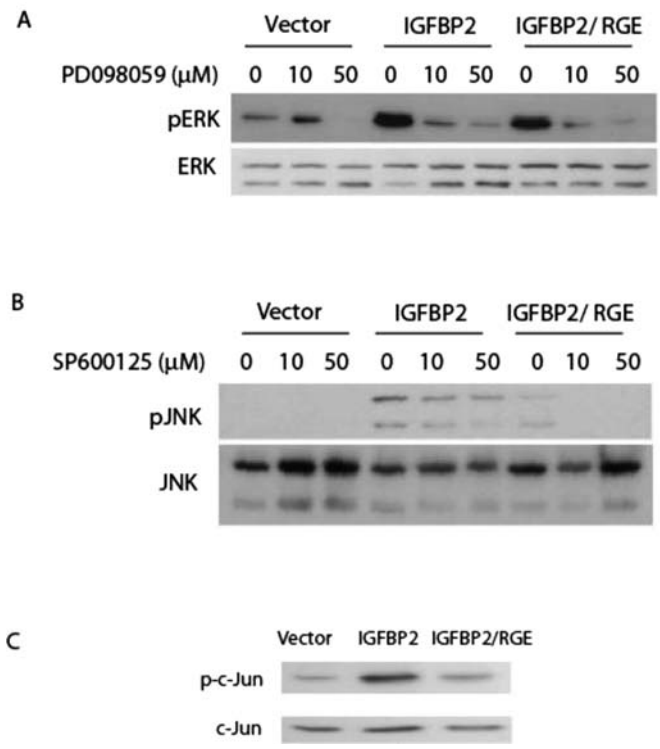

Figure 1. MAPK phosphorylation and activity are enhanced in IGFBP2 overexpressors. SNB19 glioma parental cells and SNB19 cells overexpressing either IGFBP2 or the IGFBP2/RGE mutant were serum-starved overnight prior to the addition of the given concentrations of inhibitors for 1 h, lysed, and subjected to immunoblot analysis for (A) phospho-ERK1/2 and (B) phospho-JNK. (C) An *in vitro* JNK kinase assay followed by Western blot analysis for c-Jun.

ERK does not affect IGFBP2-mediated migration, while inhibition of JNK results in reduced migration of IGFBP2 overexpressors. To determine which of the MAPK pathways may be responsible for IGFBP2-mediated migration in SNB19 cells, we used chemical inhibitors of the three major MAPK pathways, ERK, JNK and p38. PD098059, an ERK inhibitor, had no effect on migration as determined by a wound healing assay (Fig. 2A). Similarly, SB203580, a p38 inhibitor, produced no change in the migratory potential of IGFBP2 overexpressors (Fig. 2B). However, the JNK inhibitor SP600125 dramatically inhibited cell migration in IGFBP2 overexpressors without exerting as much of an effect on cells overexpressing the RGE mutant (Fig. 3A and B), suggesting that the JNK pathway is specifically linked to an IGFBP2-integrin signaling cascade that mediates migration.

Activation of the IGFBP2/integrin α5 pathway follows cell surface attachment. Because solid tumors often require cell adhesion to a surface prior to invasion (i.e., non-hematogenous invasion), physical contact with either the ECM or with other cells would precede expenditure of cellular energy towards mobility. Hence we investigated whether the IGFBP2/integrin α5 pathway is active in glioma cells prior to cell attachment. In order to determine whether cell attachment is necessary for activation of the IGFBP2/integrin α5 pathway, previously seeded IGFBP2-overexpressing SNB19 cells were trypsinized and allowed to re-seed onto the cell culture dish. Immunoprecipitation was used to assay the interaction between

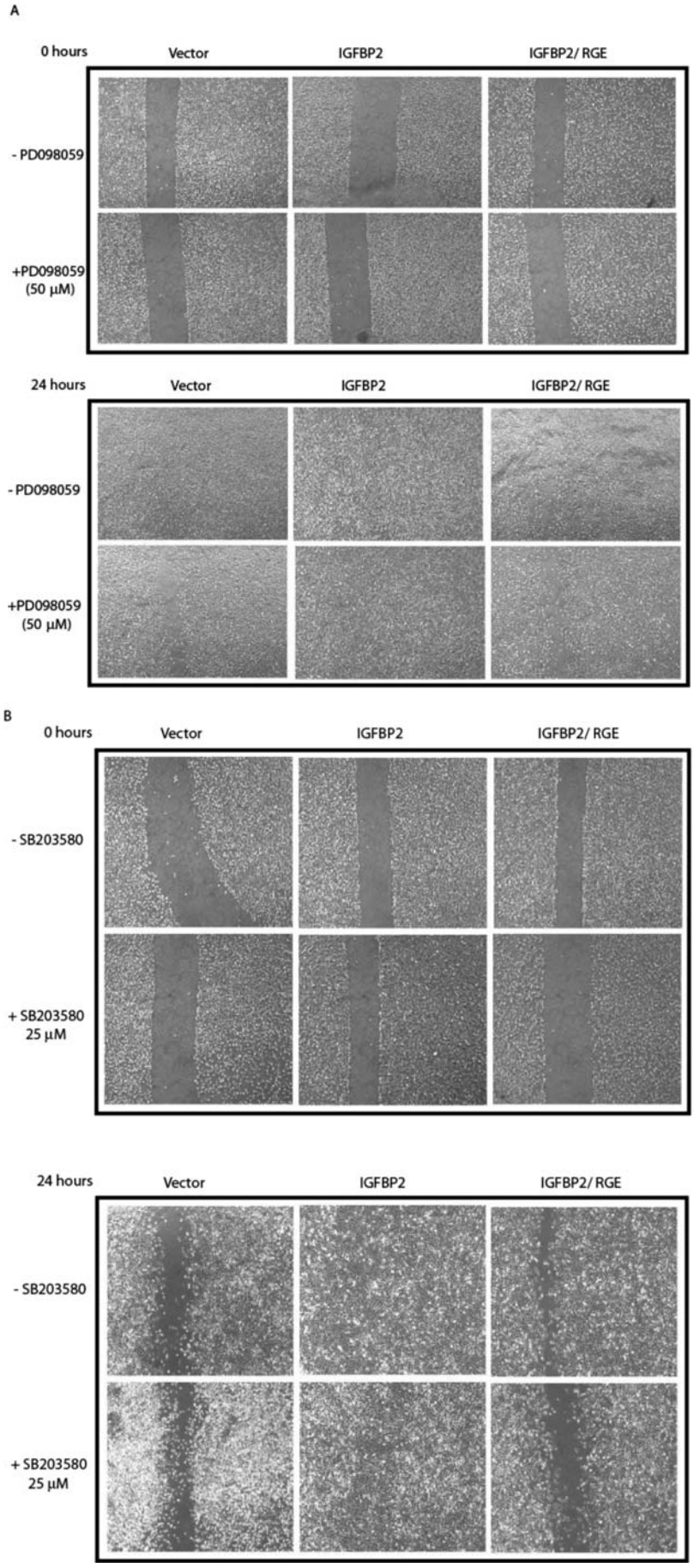

Figure 2. ERK kinase inhibitor PD98059 and p38 inhibitor SB203580 have no effect on IGFBP2-mediated migration. Cells were grown in 6-well plates until they reached 100% confluence. Cells treated with DMSO (-Inhibitor) were used as the control. Inhibitor analysis was done by adding (A) PD98059 (50 μM) or (B) SB203580 (25 μM) 1 h before introducing a small linear scratch into the middle of the well and pictures were taken at designated time points (0 and 24 h).

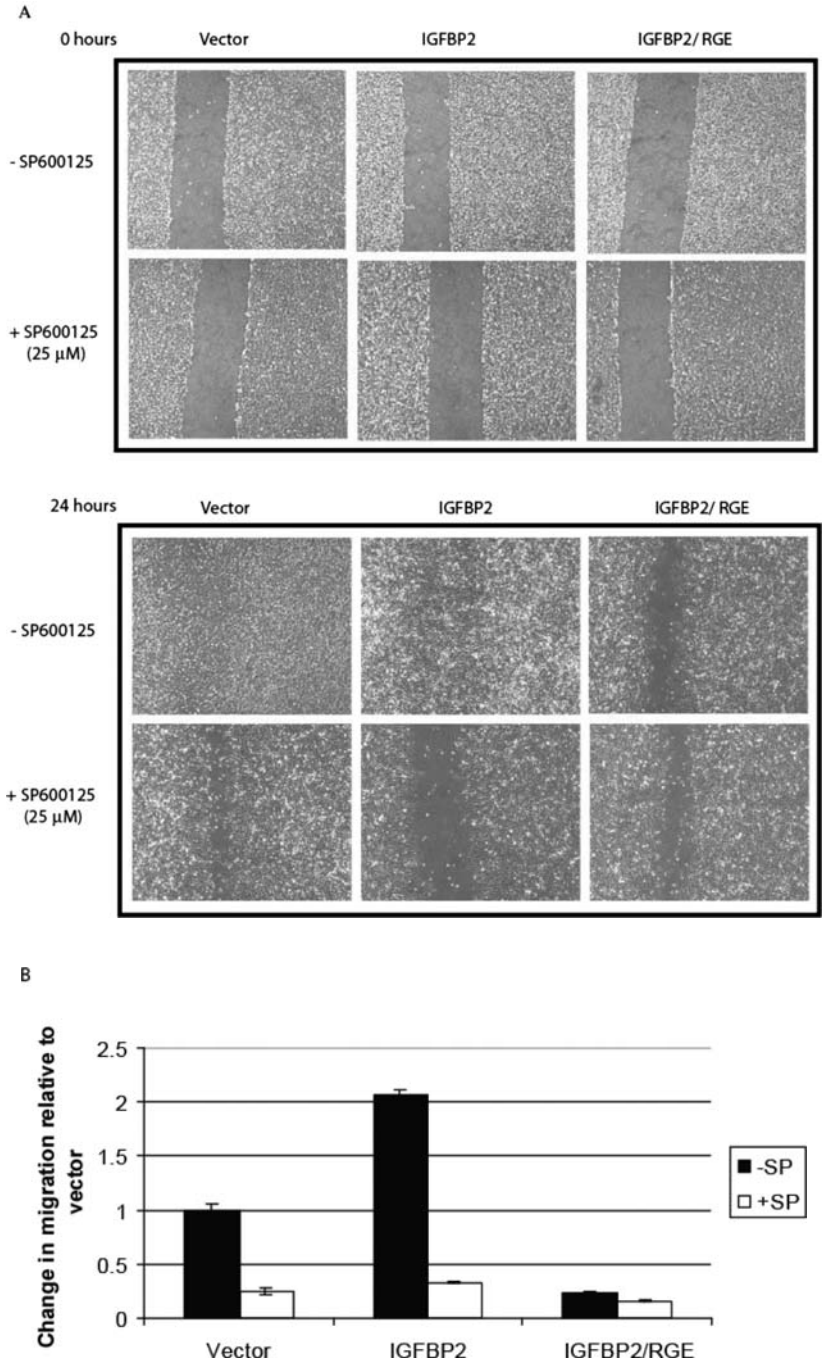

Figure 3. JNK kinase inhibitor SP600125 inhibits IGFBP2-mediated migration. (A) Cells were grown in 6-well plates until they reached 100% confluence. DMSO (-SP600125) or SP600125 (25 μM) was added 1 h before introducing a small linear scratch into the middle of the well, and photographs were taken at designated time points (0 and 24 h). (B) Cell migration rates were determined using a Boyden migration chamber and DMEM/F12 with 2% fetal bovine serum as the chemoattractant. Assays were performed in triplicate and analyzed using a two-tailed Student's t-test. Inhibitor analysis was done by adding either DMSO (-SP) or 25 μM SP600125 (+SP) to both the chambers.

IGFBP2 and integrin α5 at varying points following trypsinization and cell seeding. We observed that the IGFBP2/integrin α5 pathway is optimally activated while cells are attached to the cell culture dish, and that this activation is a progressive event following seeding of trypsinized cells (Fig. 4A). To confirm the status of cell re-attachment after seeding, photographs were taken at corresponding time points when the IGFBP2/integrin α5 pathway activity was assayed. Cell attachment status can be estimated based on the number of detached cells (smaller, rounded cells) compared to the number of attached cells (larger, flattened cells) (Fig. 4B). Clearly, results show that IGFBP2/integrin α5 pathway activation re-achieves baseline levels following completion of cell attachment.

Fibronectin is critical to the activation of the IGFBP2/integrin α5 pathway. Because fibronectin is the only ECM substrate of the integrin α5ß1 receptor, we investigated

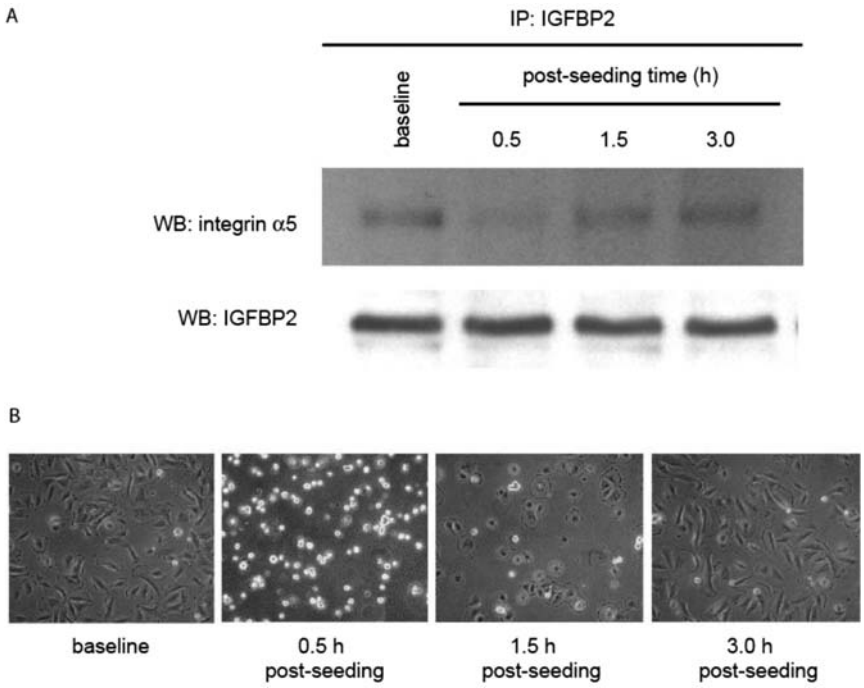

Figure 4. Activation of the IGFBP2/integrin α5 pathway is progressive following cell seeding. (A) SNB19 cells overexpressing IGFBP2 were trypsinzed and re-seeded, interaction between IGFBP2 and integrin α5 was assayed via immunoprecipitation (IP) of IGFBP2 with anti-IGFBP2 IgG at various time points following cell seeding. Subsequent Western blotting (WB) of integrin α5 and IGFBP2 was performed using their respective antibodies. (B) Photomicrographs of seeded cells over time, showing attachment of cells returning to baseline after approximately 3 h. Detached cells are small/round, while attached cells are flattened/transparent. Baseline is defined as cells seeded and grown overnight.

whether the presence of fibronectin would affect the IGFBP2/integrin α5 pathway. SNB19 cells were grown on either a plastic uncoated surface or on a fibronectin-coated surface. Cell lysates were then assayed for IGFBP2/integrin α5 interaction. Cells grown on the fibronectin-coated surface showed enhanced activity of the IGFBP2/integrin α5 pathway compared to cells grown on an uncoated plastic surface (Fig. 5A). In order to confirm that the difference in interaction between IGFBP2 and integrin α5 is not due to differences in protein expression level, we concurrently checked protein level by Western blotting. Results confirm that the enhancement of IGFBP2/integrin α5 seen on fibronectin is not due to protein level differences (Fig. 5B). Cell contact with surface-coated fibronectin is thus observed to be a significant factor in the activation of the IGFBP2/ integrin α5 pathway.

JNK activation in IGFBP2 overexpressors is downstream of the integrin α5 pathway. Since we had previously shown that IGFBP2 interacts with α5 integrin to mediate cell migration, we hypothesized that JNK activation is downstream of the integrin α5 signaling pathway. To validate this hypothesis, we used two different strategies. First, we used α5 siRNA to downregulate integrin in IGFBP2-overexpressing cells. This resulted in reduced levels of phosphorylated JNK (Fig. 6A), suggesting that α5 integrin is upstream of JNK signaling in these cells. GAPDH siRNA and a scrambled sequence siRNA were used as positive and negative controls to determine the efficiency and specificity of the transfection. Second, we plated parental SNB19 cells on the sole ligand of the α5ß1 integrin receptor, fibronectin, and compared JNK activation in these cells to those plated on uncoated tissue culture plates. An increase in JNK phosphorylation observed at 1 h in cells plated on fibronectin compared to cell lysates from the uncoated plates confirmed our hypothesis that JNK is a downstream effector of the fibronectin-integrin α5 signaling pathway (Fig. 6B).

Src is involved in IGFBP2-mediated cell migration in SNB19 cells. Several molecules have been implicated upstream of JNK in integrin-mediated cell migration pathways. Among the most common affectors of JNK is the Src-family of kinases. To determine if Src is activated in response to IGFBP2, we performed a Western blot analysis. Levels of phosphorylated Src at tyrosine 418 were upregulated in IGFBP2 overexpressors, suggesting that Src might be a molecule upstream of JNK that is important for cell migration (Fig. 7A). Also, to determine whether this Src activation is necessary for cell migration in the SNB19 cells, we treated the cells with PP2, an Src inhibitor, and conducted a wound healing assay using the vector control, IGFBP2 overexpressors, and IGFBP2/RGE-overexpressing cells. Cells treated with PP3, an inactive analog of PP2, were used as a control. A reduction in cellular migration in the presence of the Src inhibitor in all the different cell types was observed, suggesting that Src is likely a global regulator of migration in these cells (Fig. 7B), and although it is not specific to the IGFBP2-mediated migration pathway, it might lie upstream of some of the molecules that are specific to the pathway under study. These data were also confirmed using a Boyden chamber migration assay (Fig. 7C).

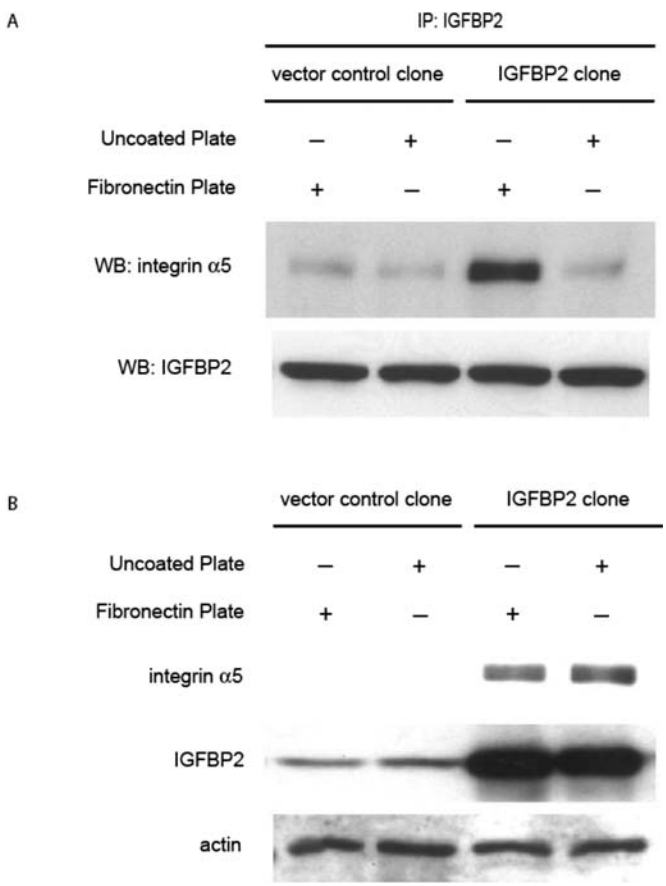

Figure 5. Activation of the IGFBP2/integrin α5 pathway is accelerated when cells are grown on fibronectin. (A) SNB19 cells overexpressing IGFBP2 and vector control clone cells were grown on either uncoated or fibronectin-coated plates. Activation status of the IGFBP2/integrin α5 pathway was assessed via immunoprecipitation (IP) with anti-IGFBP2 IgG and subsequent Western blotting (WB) of integrin α5 and IGFBP2 using their respective antibodies. (B) In order to confirm that the interaction differences between IGFBP2 and integrin α5 are not due to differential expression levels of protein, Western blotting was performed for each condition.

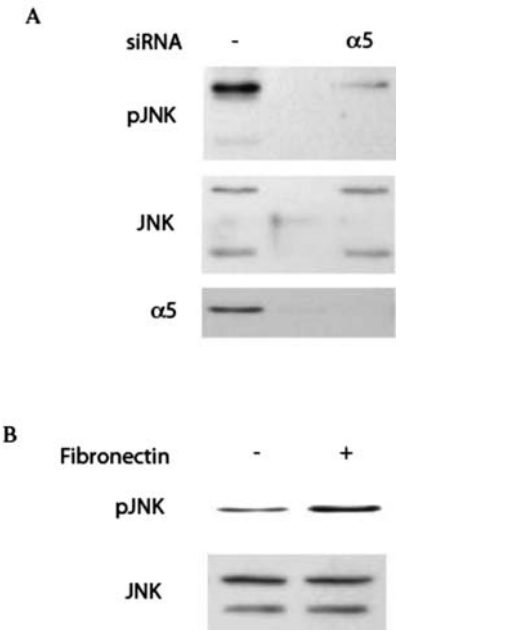

Figure 6. JNK activation is downstream of the fibronectin-integrin α5 signaling pathway. (A) siRNA (integrin α5 or scrambled negative control) was transiently transfected into SNB19 cells overexpressing IGFBP2. Lysates were then harvested 72 h later and subjected to immunoblot analysis with phospho-JNK, total JNK and integrin α5 antibodies. (B) Parental SNB19 glioma cells were were trypsinzed and re-seeded onto uncoated or fibronectin-coated plates. Lysates were harvested 1 h post-seeding and analysed by immunoblot analysis for phospho-JNK and total JNK.

Discussion

Overexpression of IGFBP2 is one of the most frequent molecular alterations that characterize GBM. Further investigation has revealed that increased cell motility in IGFBP2-overexpressing GBM is promoted through enhanced expression of a series of adhesion and migration-related genes, prominently including integrin α5, which is a cell adhesion molecule whose sole ECM ligand is fibronectin (18). Recently, we reported that IGFBP2 enhances mobility through a direct interaction with integrin α5 (17). Previous findings from our group have shown a role for IGFBP2 in ovarian cancer cell invasion (42), and more recent data from Chakrabarty *et al* have extended this observation by demonstrating that IGFBP2 activates multiple MAPK pathways, including ERK1/2, p38 and SAPK/JNK (43). Given that IGFBP2 enhances migration through its interaction with integrin α5 in glioma cells, we sought to investigate the role of MAPKs in IGFBP2-mediated cell migration.

Integrin stimulation by extracellular matrix proteins such as fibronectin leads to the activation of MAPKs in several systems (44). Of the three main types of MAPKs activated by integrin signaling, JNK in particular is believed to correlate with increased migration and invasion, and this observation is consistent with our present data. Increase in both ERK and JNK phosphorylation in IGFBP2 overexpressors suggests that they are activated in response to IGFBP2. Inhibition of the JNK pathway by SP600125 decreased migration in IGFBP2 overexpressing cells. Reduced migration seen with the JNK inhibitor in vector control cells (as measured by Boyden chamber assay) might be due to the low level of endogenous IGFBP2 expression in SNB19 cells, which thus may exhibit a very low degree of JNK-dependent migration. Also, consistent with the putative importance of IGFBP2/integrin α5 interaction in glioma cell migration, JNK activity was inhibited by integrin α5 knockdown in IGFBP2-overexpressing cells, as well as in cells overexpressing the IGFBP2/RGE mutant, which can no longer bind α5. Activation of the ERK pathway, however, seems to be independent of IGFBP2/integrin α5 interaction, as ERK is activated in cells that overexpress both wild-type and RGE mutant IGFBP2.

Because of the inherent solid tumor characteristics of GBM, we hypothesized that individual GBM tumor cells require surface adhesion prior to activation of cell mobility signaling pathways. We tested this hypothesis by checking for protein interaction between IGFBP2 and integrin α5 at various time points following cell seeding. As expected, the IGFBP2/integrin α5 pathway appears to be inactivated prior to cell seeding (in suspension), and reactivated after cells have established anchorage. As with most solid tumor cells that are susceptible to anoikis, pathways identified in these

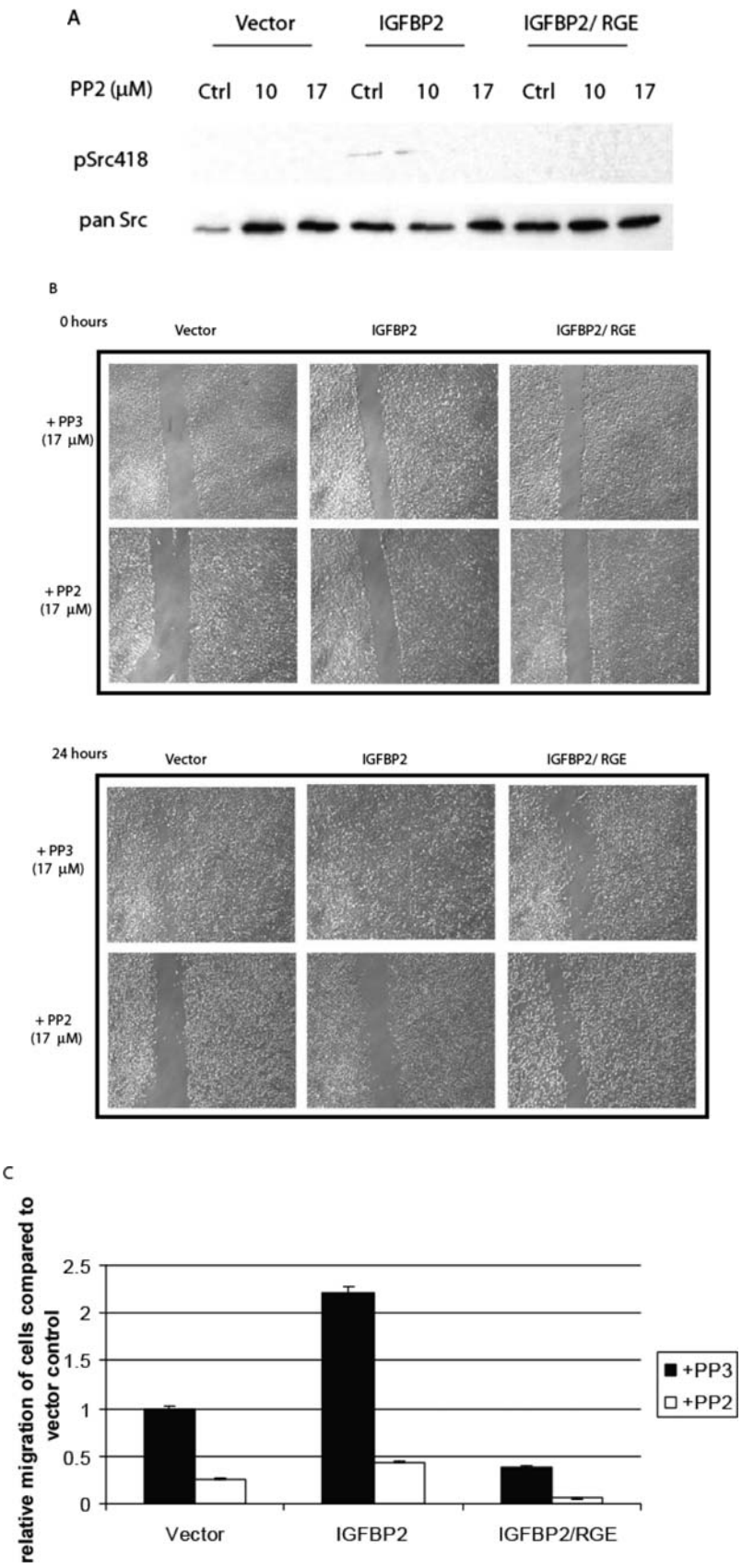

Figure 7. Role of Src in IGFBP2-JNK-mediated cell migration. (A) SNB19 glioma parental cells and SNB19 cells overexpressing either IGFBP2 or the IGFBP2/RGE mutant were serum-starved overnight prior to the addition of PP2 (17 μM) for 1 h, lysed, and subjected to immunoblot analysis for phospho-Src 418 and total c-Src. Cells treated with the same concentration of the inactive analog PP3 served as the control (Ctrl). (B) Cells were grown in 6-well plates until they reached 100% confluence. PP2 or PP3 (17 μM) was added 1 h before introducing a small linear scratch into the middle of the well and photographs were taken at designated time points (0 and 24 h). (B) Cell migration rates were determined using a Boyden migration chamber and DMEM/F12 with 2% fetal bovine serum as the chemoattractant. Assays were performed in triplicate and analyzed using a two-tailed Student's t-test. Inhibitor analysis was done by adding either PP2 or PP3 (17 μM) to both chambers.

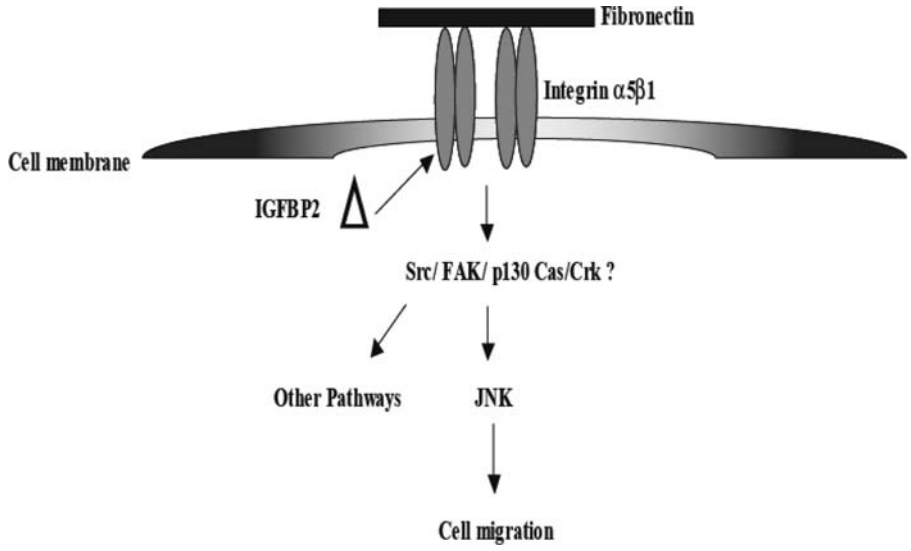

Figure 8. Schematic of the signaling pathway involved in IGFBP2-mediated glioma cell migration. IGFBP2 binds integrin α5 and enhances cell migration via JNK activation. Src activation is also involved in the signaling pathway, but is not specific to IGFBP2-mediated signaling.

tumors that are coordinately 'shut off' on suspension and 'turned on' once the cell has reattached present broad ramifications. The importance of an ECM protein, fibronectin, to the IGFBP2/integrin α5 cell mobility pathway further confirms the role of cell anchorage in the activation of this pathway. This would explain the more highly activated IGFBP2/integrin α5 pathway seen with JNK activation in cells grown on fibronectin-coated culture plates. Consistent with our hypothesis that JNK activation is downstream of the IGFBP2/integrin α5 interaction, we see a temporal sequence of IGFBP2/integrin α5 interaction occurring at 30 min post-seeding, followed by JNK activation at 1 h post-seeding. Further, we show using α5 siRNA in IGFBP2 overexpressors that the IGFBP2/integrin α5 interaction is an important step in JNK activation. Consistently, integrin α5 has been shown to be overexpressed in GBM compared to normal brain tissue, and, most recently (45), fibronectin expression in GBM has been shown to be elevated compared to the expression level seen in pilocytic astrocytoma, which is a low-grade, non-invasive glioma (46).

We initially expected fibronectin to interfere with IGFBP2/integrin α5 interaction, based on our assumption that IGFBP2's RGD site was interacting with integrin α5 at the ligand (fibronectin) binding site. Our data, however, indicate that fibronectin enhances IGFBP2/integrin α5 interaction. This effect is not due to the upregulation of protein levels of either IGFBP2 or integrin α5 by fibronectin. Ligand activation by fibronectin of integrin α5 may result in increased affinity of integrin α5 for IGFBP2 due to a conformational change. This also suggests that IGFBP2 and fibronectin probably bind integrin α5 at two completely different sites, and that the binding of these two molecules to integrin α5 is not competitive.

In a classical integrin stimulated JNK activation pathway, following cell adhesion, Src family kinases associate with focal adhesion kinase (FAK), a central focal adhesion protein associated with integrin-activated JNK pathways (47-49). Subsequently, FAK autophosphorylation occurs, activating p130cas and its association with CrkII, thereby mediating JNK activation (50). Thus, although we saw an increase in Src activation in IGFBP2 overexpressors, the fact that Src appears to be a global regulator of migration, and not IGFBP2-specific, can be explained by the fact that Src is upstream of a number of integrin-linked pathways. Recently, Hu *et al* established a functional link between angiopoietin 2 interaction with $\alpha_v\beta_1$ integrin and glioma cell invasion through the FAK/p130cas/ ERK1/2 and JNK mediated signaling pathways (51), constituting one of the first reports of integrin-mediated JNK activation in glioma cells. We have not been able to confirm the activation of FAK in our IGFBP2-overexpressing cells. One explanation could be that as FAK autoregulates its own activation, depending upon the cellular environment, this may be a transient event (30).

Integrin engagement is believed to recruit and activate the multidomain, focal adhesion protein, integrin linked kinase (ILK) (52). ILK has been shown to activate JNK in hepatic stellate cells (HSCs) isolated from rat liver (53). Data published by Koul *et al* indicate that ILK is upregulated in glioma cells plated on fibronectin, and the inhibition of ILK decreased cell proliferation and invasion (54). Unpublished data from our laboratory have shown an increase in ILK expression in IGFBP2-overexpressing cells, suggesting that IGFBP2-dependent regulation of JNK might occur via ILK. Another molecule that may be an intermediary between the integrins and JNK is TGF-ß-activated kinase (TAK1). TAK1 has previously been reported to be downstream of an integrin-mediated pathway (55) and upstream of JNK (56-58). These molecules and their role in IGFBP2-mediated migration are currently under investigation.

In summary, our data indicate that JNK regulates IGFBP2-mediated glioma cell migration and is a downstream event of the IGFBP2 interaction with integrin α5 (Fig. 8). Targeting this pathway may provide a novel therapeutic approach to diffuse gliomas.

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