An Interaction between Insulin-like Growth Factor-binding Protein 2 (IGFBP2) and Integrin α5 Is Essential for IGFBP2-induced Cell Mobility*

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In the study we report here, we tested the hypothesis that insulin-like growth factor-binding protein 2 (IGFBP2) promotes cell mobility through its interaction with integrin α5. Our previous microarray studies showed that IGFBP2 activates the expression of integrin α5. In addition, IGFBP2 has an Arg-Gly-Asp (RGD) domain, which is known to interact with integrin receptor. We confirmed that IGFBP2 interacts directly with integrin α5 through the RGD domain, we created an RGD→RGD mutant (D306E) IGFBP2 and stably over-expressed the mutant IGFBP2 in the same cell line. Co-immunoprecipitation then showed that D306E-IGFBP2 had no detectable binding with integrin α5. We further observed that IGFBP2-overexpressing cells had extensive cell surface lamellipodia, whereas D306E-IGFBP2-overexpressing cells showed abundant cell surface focal adhesions. Consistent with this, phenotype analysis then showed that IGFBP2-overexpressing cells have elevated migration rates compared with vector control; in contrast, the migration rates of the D306E-IGFBP2-overexpressing cells were not elevated and were comparable with that of vector control. Decreased expression of integrin α5 by small interference RNA in IGFBP2-overexpressing cells also reduced cell mobility. Therefore, we have concluded that one mechanism by which IGFBP2 activates IGFBP2-induced cell mobility is through its interaction with integrin α5 and this interaction is specifically mediated through the RGD domain on IGFBP2.

Cell migration is a highly regulated biological process and is associated with normal tissue modeling during early development. In most adult tissues, cell mobility is normally decreased but becomes highly active in cases of pathologic neoplasms, especially in advanced stages. This plus the increased production of basement membrane digestive enzymes are two key aspects of cancer invasion. Both are necessary for a cancer cell to successfully invade outside its original confines. With respect to cell mobility, cell surface receptors, such as integrins, must interact with the extracellular matrix in order for a cell to move. Participation of integrins in cell invasion has been shown to be critical in a wide variety of cancers (1–5). Once a signal is transmitted to the integrin receptor, a host of intracellular proteins are then activated and begin transmission of cascades of signals inside the cell. Aside from ligand binding, proteins are also able to interact with the integrin receptor inside the cell to modulate signaling and/or receptor affinity status, a process previously referred to as “inside-out” signaling (6). Eventually, certain intracellular protein signals are turned on (e.g. small molecule GTPases), enabling cells to increase their mobility. These proteins produce cytoskeletal rearrangements, such as stress fiber and membrane ruffle formation, that are conducive to cell movement. Here, we are specifically interested in IGFBP2-mediated mechanisms of cell mobility that contribute to invasiveness.

Gliomas are the most common primary tumors of the central nervous system, accounting for 80% of adult primary brain tumors (7). Patients with the most advanced stage of glioma, glioblastoma multiforme (GBM),2 face a very poor clinical outlook. By the time patients show clinical symptoms of GBM, they face a median survival of 8–10 months at the time of diagnosis. The characteristic that makes GBM so devastating and therapeutically intractable is its highly micro-invasive nature. Single-cell invasiveness typically renders GBM surgically incurable and results in significant neurological morbidity, with death being the outcome for most patients.

Previous studies using cDNA microarray profiling, followed by tissue microarray validation, have identified insulin-like growth factor-binding protein 2 (IGFBP2) overexpression as one of the most frequent molecular events in GBM (8–12). Subsequent functional studies showed that IGFBP2 promotes GBM invasion by activating the expression of a series of cell adhesion and cell mobility genes (13). The specific signal transduction events through which IGFBP2 produces enhanced cell invasion remain unresolved. Although it is known that IGFBP2 activates the expression of matrix metalloprotease 2, which contributes to cell invasiveness by breaking down matrix, the mechanism by which IGFBP2 activates cell mobility remains unknown. Under normal physiologic conditions, IGFBP2 is predominantly expressed in highly proliferative fetal tissues that exhibit extensive cell movement and tissue remodeling (14). Within the central nervous system, IGFBP2 is expressed in fetal glial cells, but after birth its expression significantly decreases (15). IGFBP2 is thus an important protein involved in cell movement during development. As is the case with many critical molecules in cancer biology, IGFBP2 has deviated from its normal role in the development of certain human cancers. In the present study, we seek to further elucidate the role of IGFBP2 in the context of cancer and also

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2 The abbreviations used are: GBM, glioblastoma multiforme; IGF, insulin-like growth factor; IGFBP2, IGF-binding protein 2; RGD, Arg-Gly-Asp domain; PBS, phosphate-buffered saline; siRNA, small interference RNA; TRITC, tetramethylrhodamine isothiocyanate.
attempt to delineate a mechanism of IGFBP2 signal transduction that leads to glioma cell migration, a critical step in IGFBP2-induced cell invasion.

We hypothesize that one mechanism by which IGFBP2 promotes cell mobility is through its interaction with integrins. We arrived at this hypothesis as a result of several observations. First, our previous microarray analyses of genes affected by IGFBP2 overexpression showed that IGFBP2 activates the expression of integrin α5. Second, a sequence analysis of the IGFBP2 protein revealed an Arg-Gly-Asp (RGD) domain (at codons 304—306), which is a well characterized integrin-binding domain (16, 17). Insulin-like growth factor-binding protein 1 (IGFBP1) also contains an RGD domain in its protein sequence and has been shown to bind integrin (18). The IGFBP1-integrin interaction was further found to activate human trophoblast cell migration (19). Two groups have independently reported that IGFBP2 interacts with integrins in two different cell lines, breast (20) and sarcoma (21); however, the effects of this interaction identified by the two groups were different. In breast cancer, it was found that IGFBP2 interacts with integrin αv and that this interaction reduced tumor cell migration (20). In sarcoma, IGFBP2 was found to interact with integrin α5, and although no direct cell migration analysis was performed, the interaction promoted tumor cell de-adhesion (21), which is a characteristic consistent with increased cell mobility. Thus, IGFBP2 interacts with different integrin subunits in different cells and/or under different physiological conditions, affecting cell adhesion and mobility. In this study, we examined GBM cells, which are well recognized for overexpressing both IGFBP2 (2–6) and integrin interaction was further found to activate human trophoblast cell migration (19). Two groups have independently reported that IGFBP2 interacts with integrins in two different cell lines, breast (20) and sarcoma (21); however, the effects of this interaction identified by the two groups were different. In breast cancer, it was found that IGFBP2 interacts with integrin αv and that this interaction reduced tumor cell migration (20). In sarcoma, IGFBP2 was found to interact with integrin α5, and although no direct cell migration analysis was performed, the interaction promoted tumor cell de-adhesion (21), which is a characteristic consistent with increased cell mobility. Thus, IGFBP2 interacts with different integrin subunits in different cells and/or under different physiological conditions, affecting cell adhesion and mobility. In this study, we examined GBM cells, which are well recognized for overexpressing both IGFBP2 (2–6) and integrin (22, 23) compared with normal tissue. We show here that IGFBP2 interacts with integrin α5 through a specific binding domain on IGFBP2 and that this interaction is necessary for promoting IGFBP2-mediated cell motility.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Stable Transfection, and Mutagenesis—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). A vector control cell line was also created by transfection of an empty pcDNA3 expression vector (13). These cells have been maintained in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 10% fetal bovine serum in a humidified incubator containing 5% CO₂ at 37 °C. 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 A1. Transfected cells were subsequently selected in the presence of G418 (1,750 μg/ml) to establish the IGFBP2-overexpressing stable clones. The expression levels of IGFBP2 in the SNB19 clones were determined by Western blot analyses of cell extracts using anti-IGFBP2 antibody (C-18; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The mutation was introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), with specifically designed mutagenic oligonucleotide primers used to generate the respective mutants. The primer sequence (5’-3’) used for the sense strand is as follows: Asp³⁰⁶ → Glu³⁰⁶ (RGE mutant) TGCACCTCGGCTCCGGCG. In addition to the protocol specified by Stratagene, we added dimethyl sulfoxide (Me₂SO) (3.5 μl of Me₂SO/50-μl reaction) to the reaction mixture because of the high GC content of the IGFBP2 gene. Mutations were confirmed by DNA sequencing.

**Co-immunoprecipitation and Western Blotting—**For the co-immunoprecipitation done to check protein-protein interaction, briefly, 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) 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 blot. Briefly, cells were washed with PBS, detached by manual scraping, collected by centrifugation, and lysed. Equal amounts of proteins were loaded, boiled for 5 min, resolved by 10% SDS-PAGE, electroblotted onto Hybond ECL nitrocellulose membranes (Amer sham Biosciences), blocked in 5% skim milk in PBS, and probed with the following primary antibodies: anti–IGFBP2 polyclonal antibody C-18 (Santa Cruz Biotechnology), anti-actin polyclonal antibody I-19 (Santa Cruz Biotechnology), and anti-integrin α5 monoclonal antibody (BD Biosciences). Proteins were detected with an enhanced chemiluminescence (ECL) kit (Amer sham Biosciences).

**GTPase Activity Assay—**To confirm the presence of lamellipodia in IGFBP2-overexpressing cells, we performed a GTP molecule affinity pulldown assay to detect Rac1-GTP (active form of Rac1). In this experiment, cell lysates were incubated with PAK-1-agarose (Upstate Biotechnology, Lake Placid, NY) for 1 h at 4 °C to pull down the GTP-bound molecules. Beads were spun down, collected, and washed three times with magnesium-containing lysis buffer provided by the manufacturer. Samples were boiled and resolved by SDS-PAGE. Rac1 was detected with an anti-Rac1 antibody (Upstate Biotechnology). To determine the total Rac1 input, a separate immunoblot was performed on identical lysate using the same anti-Rac antibody. Signals were quantified using image-analyzing software (Kodak 1D Image Analysis Software, Rochester, NY). The assay was performed three times.

**In Vitro Cell Migration and Wound-healing Assays—**Cell migration rates were determined using a Boyden migration chamber, in this case 24-well Biocoat Control Insert chambers (BD Biosciences) 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, 2 × 10⁴ 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% CO₂. 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. 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 (done to confirm the Boyden chamber findings), cells were grown in 6-well plates until they reached 100% confluence. A small linear scratch was introduced into the middle of the well using a small 10-μl pipette tip. Cells were allowed to continued growing at 37 °C in a humidified incubator with 5% CO₂. Cells were only removed from the incubator at designated time points (0, 6, 12, and 24 h) when pictures were taken under a microscope.

**Immunostaining—**Cells were seeded onto fibronectin-coated glass slide chambers and incubated in the standard medium (as mentioned above) for 24 h. Cells were then fixed in 4% paraformaldehyde at room temperature for 10 min, permeabilized in 100% methanol for 30 min, incubated at −20 °C for 30 min, and blocked with blocking solution (PBS with 10% serum from the respective secondary antibody animal).
IGFBP2 and Integrin α5 Regulate Mobility

Results

IGFBP2 Up-regulates Integrin Receptor Expression—Previously, our microarray data showed that integrin α5 mRNA was up-regulated in stable IGFBP2-overexpressing SNB19 cells (7). We therefore sought to confirm this finding at the protein level by performing Western blot analyses in several SNB19 cell lines stably transfected with IGFBP2. Because the integrin α5 subunit only heterodimerizes with the integrin β1 subunit, we surmised that integrin β1 should also be up-regulated. Thus, we concurrently checked the expression level of integrin β1 as well. We found that both the α5 and β1 subunits were up-regulated at the protein level in IGFBP2-overexpressing cell lines, although the α5 subunit was induced to a higher level than was the β1 subunit (Fig. 1).

Thus, the expression of the entire integrin receptor (integrin α5β1) is up-regulated when IGFBP2 is overexpressed, confirming our preliminary microarray data.

IGFBP2 Interacts with Integrin α5—Because the IGFBP2 protein possesses an RGD domain, as shown by primary structure analysis, it is

FIGURE 1. IGFBP2 overexpression up-regulates the expression of the integrin receptor, integrin α5β1. Western blot of cell lysate from vector control SNB19 and three IGFBP2-overexpressing clones. Overexpression of IGFBP2 seen here is correlated with an up-regulation of integrin α5 and β1 expression. The higher molecular weight integrin β1 band corresponds to the mature glycosylated form of integrin β1. Thus, IGFBP2 up-regulates the expression of the entire integrin α5β1.

for 1–4 h. Primary antibody (1:50) in blocking solution was added and the cells incubated overnight at 4 °C. After the cells were washed with PBS, secondary antibody (1:200) in blocking solution was added and the cells incubated at room temperature for 1 h. After the slides were washed with PBS, they were mounted with ProLong® Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Molecular Probes) and covered with coverslips. The F-actin staining pattern was visualized with the phalloidin-TRITC stain, which indicates whether focal adhesion complexes, a feature of non-migrating cells, are present on the edges of the cell membrane.

siRNA Knock Down of Integrin α5—Integrin α5 siRNA (Santa Cruz Biotechnology) was transiently transfected into SNB19 cells using the siPORT NeoFX transfection agent from the Silencer Biotechnology (Ambion Inc., Austin, TX). An siRNA concentration of 60 nM was determined to be optimal, and a seeding cell count of 2.3 × 10⁶ cells/transfection well was used. Controls used were glyceraldehydes-3-phosphate dehydrogenase siRNA to check for transfection success and a scrambled sequence siRNA as a negative control, both provided by Ambion. Cells were incubated in the transfection reagent at 37 °C in a humidified incubator with 5% CO₂ for 72 h. Cells were then harvested and the protein expression changes (assayed via Western blotting) and phenotype analysis (cell migration assay).

Results

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FIGURE 2. IGFBP2 binds to integrin α5. Co-immunoprecipitation was performed in which IGFBP2 was pulled down using goat anti-IGFBP2 antibody. Following SDS-PAGE electrophoresis, the separated proteins were probed with anti-integrin α5 antibody. Lysate from cells overexpressing IGFBP2 demonstrate the interaction of IGFBP2 with integrin α5. Normal goat IgG was used as a negative control. IP, immunoprecipitation; WB, Western blotting.

FIGURE 3. Binding of IGFBP2 to integrin α5 occurs through the RGD (integrin-binding) domain on IGFBP2. A, generation of a mutant IGFBP2 expressing an RGD→RGE mutation. A single nucleotide substitution at codon 306 changed aspartic acid (D) to glutamic acid (E), resulting in disruption of the RGD domain on IGFBP2. We therefore named the mutant protein D306E-IGFBP2. B, we then transfected the D306E-IGFBP2 into SNB19 cells and created stable clones. We selected clones overexpressing levels of the mutant IGFBP2 comparable with levels of IGFBP2 expressed by the IGFBP2-overexpressing V, vector control cells. C, repeated co-immunoprecipitations on the D306E-IGFBP2 clones showed an abolishment of binding to integrin α5, signifying the specific involvement of the RGD domain in the binding of IGFBP2 to integrin α5.
IGFBP2 and Integrin α5 Regulate Mobility

Possible that IGFBP2 not only induces the expression of integrin α5 but also physically interacts with integrin α5 via the RGD domain. We tested this potential protein-protein interaction via co-immunoprecipitation studies. In IGFBP2-overexpressing cells, we co-immunoprecipitated IGFBP2 and then detected integrin α5 by immunoblot. In contrast, co-immunoprecipitation with control antibody did not bring down integrin α5 (Fig. 2).

IGFBP2 Binds to Integrin α5 through the RGD Domain—We next studied whether the binding of IGFBP2 to integrin α5 occurs through the RGD sequence. Using polymerase chain reaction (PCR)-based mutagenesis, we generated an IGFBP2 expression vector with a point mutation in which Asp306 was switched to Glu306 (D306E), thus mutating the RGD domain to RGE (Fig. 3A). We then transfected the D306E-IGFBP2 mutant vector into SNB19 cells and created stable expressing clones. We chose several high expressing D306E-IGFBP2 clones for investigation. D306E-IGFBP2 clones were selected that expressed mutant IGFBP2 at levels comparable with the control clone expressing IGFBP2 (Fig. 3B) for use in further assays in this study. Co-immunoprecipitation was repeated with the D306E-IGFBP2 clones to measure the IGFBP2-integrin α5 binding changes. Cells expressing D306E-IGFBP2 showed no detectable amounts of co-immunoprecipitated integrin α5 (Fig. 3C) compared with cells expressing IGFBP2. Interestingly, D306E-IGFBP2-overexpressing clones and IGFBP2-overexpressing clones up-regulated integrin α5 protein expression to comparable levels (data not shown). Thus, the changes in the amount of D306E-IGFBP2/integrin α5 binding were due to the mutation and not due to a difference in the integrin α5 expression levels. Therefore, not only does IGFBP2 bind to integrin α5, but the interaction also appears to be mediated specifically through the integrin binding RGD motif on IGFBP2.

Interaction of IGFBP2 with Integrin α5 Induces Changes in Cell Morphology—We observed marked changes in the cell shape that depended on whether the cells overexpressed IGFBP2 or D306E-IGFBP2. Specifically, on staining for F-actin, we clearly visualized numerous focal adhesion complexes on the edges of the cell membrane in parental, vector, and D306E-IGFBP2-overexpressing cells. In contrast, the IGFBP2-overexpressing cells were much larger, showing marked lamellipodia (Fig. 4).

To confirm the presence of lamellipodia in IGFBP2-overexpressing cells, we assayed for the activity of the small GTPase, Rac1, a molecule contributing to lamellipodia formation. This showed that IGFBP2-overexpressing cells contained more Rac1-GTP than did D306E-IGFBP2-overexpressing cells. In particular, IGFBP2-overexpressing cells showed a >2-fold increase (over vector control cells) in the Rac1-GTP level (Fig. 5A). We then immunostained for Rac1, which showed Rac1 in the lamellipodia of IGFBP2-overexpressing cells (Fig. 5B). We also determined the fractions of cells with lamellipodia (among 100 cells for each
We counted the cells showing observable lamellipodia and found that a significantly higher percentage of IGFBP2-overexpressing cells had lamellipodia than did vector control and D306E-IGFBP2-overexpressing cells (Fig. 5C). Thus, on the basis of our cell morphology data, it appears that D306E-IGFBP2-overexpressing cells should have a decreased migratory potential compared with IGFBP2-overexpressing cells, and this was assessed in the experiment described in the next section.

Interaction of IGFBP2 with Integrin α5 Is Necessary for IGFBP2-induced Cell Migration—Because the ability of IGFBP2 to interact with integrin α5 induced the formation of lamellipodia, we next performed a Boyden chamber cell migration assay to determine whether these cell surface features are associated with cell mobility. This showed that SNB19 cells overexpressing IGFBP2 migrated significantly faster than did vector control cells. However, SNB19 cells overexpressing D306E-IGFBP2 did not migrate faster and, in fact, migrated at roughly the same rate as the parental SNB19 and vector control cells (Fig. 6A).

Wound-healing assays confirmed the quantitative migration differences seen between cells overexpressing IGFBP2 and cells overexpressing D306E-IGFBP2. Specifically, SNB19 cells overexpressing IGFBP2 migrated into and covered the wound area in ~24 h, whereas vector control and D306E-IGFBP2-overexpressing cells took significantly longer to migrate into the wound area (Fig. 6, B–D). Thus, the Boyden chamber assay and wound-healing assay both showed that D306E-IGFBP2-overexpressing cells and vector control cells had a comparably attenuated migration rate.

Knock Down of Integrin α5 Attenuates IGFBP2-induced Cell Migration—To further confirm the necessity of the interaction between IGFBP2 and integrin α5 in IGFBP2-induced cell migration, we used siRNA to decrease the expression of integrin α5 and examined the effects of reducing integrin α5 in IGFBP2-overexpressing cells. Integrin α5 expression was dramatically reduced in the integrin α5 siRNA-transfected cells compared with the negative control siRNA-transfected cells, while IGFBP2 expression levels remained unchanged (Fig. 7A). The transfected cells were then analyzed for phenotypic changes. On cell migration assay we found that the knock down of integrin α5 significantly attenuated the cell migration rate of the IGFBP2-overexpressing cells (Fig. 7B). Hence, the reduction in cell migration rate here is correlated to the decreased levels of integrin α5.
IGFBP2 and Integrin α5 Regulate Mobility

**FIGURE 7.** Decreased integrin α5 expression results in attenuated cell migration in IGFBP2-overexpressing cells. A, IGFBP2-overexpressing cells transfected with integrin α5 siRNA showed a significant decrease in integrin α5 expression (72 h post-transfection). Cells transfected with negative control siRNA showed no changes in integrin α5 expression compared with untransfected cells. IGFBP2 expression levels remained unchanged throughout. B, the cell migration rate of IGFBP2-overexpressing cells transfected with integrin α5 siRNA was accordingly attenuated and migrated at a rate much slower than IGFBP2-overexpressing cells transfected with negative control siRNA but remained faster than parental controls (P). 2% fetal bovine serum was added to the bottom chamber and used as chemoattractant during cell migration.

**DISCUSSION**

The enhanced expression of IGFBP2 was recently found to be one of the most frequent alterations in GBM (8–12) and in some advanced stage cancers of the prostate (24, 25) and ovaries (26, 27). The fact that IGFBP2 is overexpressed only in advanced stages of these tumors suggests that IGFBP2 affects functional pathways critical in determining the phenotypes of these cancers, such as increased cell proliferation and invasion. In prostate cancer, IGFBP2 was shown to promote cell proliferation (28, 29). In both brain and ovarian cancers, IGFBP2 has been found to enhance tumor cell invasion but not cell proliferation (28, 29, 30). Although IGFBP2 is a member of a group of proteins (IGFBP 1–6) known to modulate the bioavailability of insulin-like growth factors (IGFs), the naming scheme of these proteins is largely misleading. This is because several reported functions of IGFBPs also exist that are independent of IGFs. These include direct effects on cellular growth, adhesion, migration, and apoptosis independent of IGFs.

In this study, we demonstrated that IGFBP2 enhances the migration of GBM cells through its interaction with integrin α5; this interaction is dependent on the IGFBP2 RGD domain. We used a GBM cell line (SNB19) with a low endogenous level of IGFBP2. Because overexpression of IGFBP2 is a common molecular change found in GBM (8–12), we decided to overexpress IGFBP2 in SNB19 cells since this cell line provided an optimal GBM model system that was not already overexpressing this gene. Further, integrin α5 is also expressed in low levels in SNB19 cells. In our transfected SNB19 cells, by overexpressing IGFBP2 (and resulting overexpression of integrin α5) we were able to observe the interaction between these two proteins (IGFBP2 and integrin α5). In parental SNB19 cells, the low endogenous IGFBP2 and integrin α5 perhaps also interact to play a role in base line cell mobility.

We further found that, in gliomas, IGFBP2 participates in the decreased formation of focal adhesions, which are membrane-anchoring structures. Thus, the ability of IGFBP2 to engage integrin α5 contributes to the ability of the glioma cell to relinquish anchor points at the focal adhesions and adopt a more mobile morphology (i.e., lamellipodia). In SNB19 cells that overexpress IGFBP2, we observed increased formation of lamellipodia, little to no focal adhesions, and the ability of the cells to migrate significantly faster compared with vector control and D306E-IGFBP2-overexpressing cells. Consistently, Rac1, a GTPase molecule in the signal transduction pathway that leads to the formation of lamellipodia, is activated, and it localizes to the leading edge areas of IGFBP2-overexpressing cells. Conversely, in cells that overexpress D306E-IGFBP2, we saw focal adhesions but no appreciable lamellipodia and a considerably attenuated migration rate. Although activation of Rac1 (Rac1-GTP) was attenuated in cells overexpressing D306E-IGFBP2, we still observed more activated Rac1 in the D306E-IGFBP2 clones than in the vector control. This suggests that overexpression of IGFBP2 is able to activate Rac1 independent of whether IGFBP2 binds to integrin α5. However, the mechanism through which IGFBP2 activates Rac1 independent of integrin α5 remains to be investigated.

During the course of our study, Pereira et al. (20) also independently showed that IGFBP2 bound to integrins and regulated cell movement. The effect and the specific integrin involved, however, differed to some extent depending on the particular neoplasm under study. In breast cancer, the interaction of IGFBP2 with integrin αv decreased cell migration (20). In the glioma cells that we used for this study, integrin αv is detectable; however, IGFBP2 co-immunoprecipitation experiments did not co-precipitate integrin αv (data not shown). Thus, the interaction between IGFBP2 and integrin proteins is cell-and, perhaps, tissue-specific through a mechanism that is not presently known. In addition, the differing partnerships between IGFBP2 and the various integrin subunits may produce differing effects on cell migration.

In sarcoma cells, IGFBP2 has been shown to interact with integrin α5 and results in increased cell de-adhesion and reduced cell proliferation (21); both of these features are characteristic of a cancer cell that is adopting a mobile phenotype. The same authors reported their findings to be IGF independent. In our study, we did not directly test whether the interaction between IGFBP2 and integrin α5 was modulated by IGF. Our findings here in glioma are supportive of what was found in sarcoma, although our study, using a site-directed mutation approach, provided mechanistic data demonstrating that the interaction between IGFBP2 and integrin α5 is specific and mediated through the RGD domain.

In knocking down the expression of integrin α5, we noted that the migration rates of IGFBP2-overexpressing cells were decreased, supporting the notion that IGFBP2 needs to interact with integrin α5 to enhance cell migration. In the parental controls we also observed a slight decrease in parental cell migration rate when transfected with integrin α5 siRNA. Although the observed decrease here could be small, it does point to the possibility, as suggested earlier, that the small endogenous amount of IGFBP2 and integrin α5 expression in SNB19 cells are indeed interacting and contribute to base line cell mobility.

In summary, the present study further characterizes the role of IGFBP2 in glioma pathology. Not only does IGFBP2 enhance cell invasion by increasing the extracellular matrix digestive process, it also pro-
motes cell mobility through an integrin-mediated pathway, which is known to be activated in glioblastoma.

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