Enhanced expression of both integrin $\alpha_\beta_3$ and platelet-derived growth factor receptor (PDGFr) has been described in glioblastoma tumors. We therefore explored the possibility that integrin $\alpha_\beta_3$ cooperates with PDGFr to promote cell migration in glioblastoma cells, and extended the study to identify the Src family members that are activated on PDGF stimulation. Glioblastoma cells utilize integrins $\alpha_\beta_3$ and $\alpha_\beta_5$ to mediate vitronectin attachment. We found that physiologic PDGF stimulation (83 pM, 10 min) of vitronectin-adherent cells promoted the specific recruitment of integrin $\alpha_\beta_3$-containing focal adhesions to the cell cortex and $\alpha_\beta_5$-mediated cell motility. Analysis of PDGFr immunoprecipitates indicated an association of the PDGFr $\beta$ with integrin $\alpha_\beta_3$, but not integrin $\alpha_\beta_5$. Cells plated onto collagen or laminin, which engage different integrins, exhibited significantly less migration on PDGF stimulation, indicating a cooperation of $\alpha_\beta_3$ and the PDGFr $\beta$ in glioblastoma cells that promotes migration.

Further analysis of the cells plated onto vitronectin indicated that PDGF stimulation caused an increase in Src kinase activity, which was associated with integrin $\alpha_\beta_3$. In the vitronectin-adherent cells, Lyn was associated preferentially with $\alpha_\beta_3$ both in the presence and absence of PDGF stimulation. In contrast, Fyn was associated with both $\alpha_\beta_3$ and $\alpha_\beta_5$. Moreover, PDGF stimulation increased the activity of Lyn, but not Fyn, in vitronectin-adherent cells, and the activity of Fyn, but not Lyn, in laminin-adherent cells. Using cells attached to mAb anti-$\alpha_\beta_3$ or mAb anti-integrin $\alpha_\beta_5$, we confirmed the activation of specific members of the Src kinase family with PDGF stimulation. Down-regulation of Lyn expression by siRNA significantly inhibited the cell migration mediated by integrin $\alpha_\beta_3$ in PDGF-stimulated cells, demonstrating that the PDGFr $\beta$ cooperates with integrin $\alpha_\beta_3$ in promoting the motility of vitronectin-adherent glioblastoma cells through a Lyn kinase-mediated pathway. Notably, the data indicate that engagement of different integrins alters the identity of the Src family members that are activated on stimulation with PDGF.

Glioblastoma tumors (Grade IV tumors), the most malignant of astrocytic tumors, are associated with a poor prognosis and limited survival due to their rapid invasion into the normal brain (1). The enhanced expression of both PDGF$^1$ and its receptor, PDGFr, in Grade IV tumors (glioblastoma/malignant astrocytoma) (2, 3) suggests that the PDGF signaling pathway may play a role in the etiology or progression of these tumors, a concept that is supported by the finding that transgene expression of the PDGF B gene downstream of the promoter of nestin, an early glial cell-specific protein, or the promoter of GFAP, an astrocyte-specific protein, results in the development of gliomas in the mouse brain (4).

In several different cell types, PDGF stimulation has been shown to promote cytoskeletal reorganization, as evidenced by membrane ruffling and lamellipodia formation, and cell motility (5). PDGF dimers have been shown to bind to and activate the PDGFr, resulting in the activation of multiple downstream signaling molecules (5). Two forms of the PDGFr have been identified, PDGFr$\alpha$ and $\beta$, and the PDGF BB ligand binds both (5). The outcome of PDGFr signaling in terms of cell function is influenced by several factors, including the cell type, the dosage of PDGF, the PDGFr repertoire expressed on the cells, and the experimental conditions (5). PDGFr activation is known to result in the activation of proteins encoded by the Src family of proto-oncogenes (5), which bind to the phosphorylated PDGFr and participate in many ligand-induced cellular responses. The ability of the Src family to regulate the function of the cell is mediated by their association with growth factor receptors, integrin receptors, the actin cytoskeleton and other cell surface receptors (6–10). At this time, ten Src family members have been identified, i.e. c-Src, Yes, Lyn, Fgr, Lck, Hck, Yrk, Blk, and Frk (6). The expression of several of these molecules seems to be restricted to only a few types of cells; for example, Hck and Fgr are expressed primarily by hematopoietic cells (6).

There is a growing body of evidence to suggest that individual Src family members may mediate specific functions. Indirect evidence for this comes from the reports of the preferential association of an individual Src family member with a specific integrin(s) or other membrane receptor, for example, in platelets, integrin $\alpha_\beta_3$ co-immunoprecipitates with c-Src, rather than Fyn and Lyn (10), whereas Fyn and Lyn associate with the Fc receptor $\gamma$-chain (11). More direct evidence comes from the reports of preferential activation of individual Src family members, for example, in monocytes, Fgr has been shown to regulate the signaling of the $\beta_3$ but not the $\beta_2$ integrin (12), and

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*This work was supported by Grants CA59958 and CA97110 from the NCI, National Institutes of Health (to C. L. G.) and by Grant HL58655 from the HLB, National Institutes of Health and the Veterans Administration Merit Review Board (to M. A. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: PDGF, platelet-derived growth factor; PDGFr, PDGF receptor; BSA, bovine serum albumin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; FAK, focal adhesion kinase.
Integrin-specific Increase in Lyn or Fyn Activity with PDGF

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in EGF-stimulated A431 epidermoid squamous carcinoma cells transfected with a dominant negative Fyn construct inhibition of hemidesmosome disassembly and invasion was found (13). In human NK cells, Fyn and Lyn are activated by the clustering of integrin αβ3, and the addition of a soluble 120-kDa fibronectin fragment that had been cross-linked to integrin αβ3 has been shown to promote the activation of Fyn (9). In oligodendrocytes Fyn kinase activity is elevated during development, and in neuroblastomas tumors its overexpression promotes differentiation (14, 15). Lastly, differential activation of c-Src and Yes was reported in primary colon tumors as compared with metastatic colon tumors (16).

Data from many investigators indicate that tyrosine kinase growth factor receptors, such as the PDGFR, can cooperate with integrin receptors leading to the amplification of signaling pathways that regulate several different cell functions, including cell migration (17). The PDGFR has been shown to cooperate with integrin αβ3 in NIH 3T3 fibroblasts, endothelial cells, and oligodendrocyte precursor cells (18–20). Both co-immunoprecipitation of PDGFR with integrin αβ3, but not β3 integrin, and enhancement of the biologic action of PDGFR when αβ3 is engaged was demonstrated in these cells. Experimental manipulation of the receptors through transfection of porcine aortic endothelial and CHO cells with the α and β3 integrin subunits and PDGFRβ results in constitutive association of αβ3 with PDGFRβ, which occurs independently of stimulation with PDGF and in the presence of the PDGFR antagonist, AG1296 (21). The IRS-1 and the VEGF receptor 2 have also been shown to associate with integrin αβ3 (21, 22).

In this study, we characterized the activation of individual Src family members on PDGF stimulation of cells plated on different substrates and examined the potential cooperation of integrin αβ3 and PDGFR in the regulation of migration of glioblastoma cells. To our knowledge, no prior study has determined whether Src family member activation on PDGF stimulation is substrate- or integrin-specific. This study was based on our prior observations that the expression of integrins αβ3 and αβ5 is up-regulated on glioblastoma cells in vivo, they mediate glioblastoma cell attachment and migration toward vitronectin, and are regulated differentially (23, 24, 25). We found that PDGF stimulation activated Lyn kinase preferentially in vitronectin-adherent glioblastoma overexpressing integrin αβ3-mediated motility in a Lyn kinase-dependent manner. Furthermore, we show that the Src family member that is activated on PDGF stimulation depends on the substrate the cells are adherent to, or the integrin receptor that is engaged.

EXPERIMENTAL PROCEDURES

Cells and Reagents—U-87MG human glioblastoma or Grade IV malignant astrocytoma cells were purchased from the ATCC and were propagated in complete media (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 2 mM l-glutamine). Cells were monitored routinely for contamination with mycoplasma by nested RT-PCR (kit purchased from the ATCC) and found to be negative. Prior to PDGF stimulation, cells were cultured without serum for 48 h, and then harvested using buffered-EDTA, resuspended in serum-free media with 1% BSA and plated onto wells coated with vitronectin, laminin, collagen, or anti-integrin mAbs. The following neutralizing (blocking) antibodies were purchased: mAb anti-αv (Upstate Biotechnology, Inc., Lake Placid, NY), mAb anti-αβ3, (LM609), mAb anti-αβ5 (P1F6), mAb anti-αv, mAb anti-αv (P410), mAb anti-α5, and mAb anti-β3 (Chemicon, Inc., Temecula, CA). Neutralizing (blocking) mAb anti-αv (L230) hybridoma was obtained from the ATCC. Rabbit antibodies directed toward the cytoplasmic tail of the β3 and β5 integrin subunits were purchased (Chemicon, Inc.), and the optimal concentration for Western blotting was determined. The purified rabbit antibodies directed toward c-Src, Lck, Yes, Fyn, Lyn, and Fgr that were used for Western blotting and immunoprecipitation analyses were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the optimal concentration for Western blotting was determined. The purified rabbit antibodies that could only be used for Western blotting was purchased from Upstate Biotechnology. The following mAbs were purchased: anti-Lyn and anti-integrin α5 (Upstate Biotechnology, Inc.), anti-Fyn (Santa Cruz Biotechnology, Inc.), anti-v-Src (clone 327 Calbiochem, La Jolla, CA), anti-vinuculin (Sigma Chemical Co.), anti-Lamin A/C (Santa Cruz Biotechnology, Inc.), and anti-actin (Sigma Chemical Co.). The Src kinase inhibitor (PP1) was purchased from Calbiochem. PDGF BB (referred to as PDGF) was purchased from R&D Systems (Minneapolis, MN), and was utilized as it binds to both the PDGFα and β. The following rabbit antibodies were purchased: anti-phosphospecific Src [pY416] IgG (Santa Cruz Biotechnology, Inc.) (detects only activated Src), anti-phosphospecific Src[pY418] IgG (BioSource International) (detects only activated Src), anti-phosphospecific Src(Thr416) IgG (Upstate Biotechnology Inc.), anti-phosphospecific FAK(pY397) IgG (BioSource International), anti-PDGFRβ IgG (Santa Cruz Biotechnology, Inc.), anti-PDGFRβ IgG (Santa Cruz Biotechnology, Inc.), and anti-PDGFR IgG that recognizes both receptor forms (Upstate Biotechnology Inc.).

Attachment Assays—These were performed as described previously (26). Metabolically-labeled (35S) methionine/cysteine cells were harvested with buffered EDTA, resuspended in adhesion assay buffer with 1 mM MgCl2 and 100 μM MnCl2 with 1% BSA, or serum-free Dulbecco’s modified Eagle’s medium with 1% BSA, and aliquoted onto 96-well plates previously coated with 5 μg/ml vitronectin, 20 μg/ml collagen type I, or 10 μg/ml laminin. Cells were allowed to attach to the wells indicated (37°C, 5% CO2), and then washed twice with PBS prior to harvesting using trypsin. The amount of radioisotope in the harvested cells was determined using a beta scintillation counter. The attachment of the cells to immobilized anti-integrin mAbs was performed as described previously (24). Briefly, 20 μg/ml mouse IgG in PBS was coated onto wells (1 h, 22°C), the wells washed with PBS, blocked with 1% heat-inactivated BSA in PBS (1 h, 37°C), washed 1× with PBS, the mouse mAb anti-integrin (10 μg/ml) in PBS aliquoted onto the wells (1 h, 22°C), the wells washed 3× with PBS, and the cells plated.

Immunofluorescence Analysis—The cellular distribution of specific integrin receptors under various conditions was assessed using immunofluorescence analysis, as described previously (30, 31). Briefly, cells were harvested with buffered EDTA, resuspended in the adhesion assay buffer with 1 mM MgCl2 and 100 μM MnCl2 with 1% BSA described above, and 40,000 cells were plated onto a coverslip coated previously with 5 μg/ml vitronectin for 5 h, followed by stimulation with PDGF or vehicle, washed, fixed in 4% buffered paraformaldehyde, dehydrized with 0.3% Triton X-100, blocked and reacted sequentially with both primary antibodies, followed by both secondary antibodies. Cells were viewed and photographed on a Nikon confocal microscope.

Scratch Motility Assay—Cells were harvested with buffered EDTA, resuspended in serum-free Dulbecco’s modified Eagle’s medium with 1% BSA, and plated onto 100-mm glass coverslips (60,000 cells/well) coated previously with a purified matrix protein, as described (29, 30). At 24 h, the confluent monolayer was scratched, photographed immediately after scratching, and again at the end of the assay. The number of cells in five representative fields (200×500 microns in the scratched area) was calculated for each condition. In experiments to inhibit cell proliferation Mitomycin C at 10 μg/ml was utilized, as at this concentration it inhibits the proliferation of these cells.

Immunoprecipitation and Western Blot Analyses—As described previously (31, 32), cell monolayers were washed twice with PBS then the cells were lysed for 60 min on wet ice with 1% Nonidet P-40 lysis buffer or for 10 min on wet ice with RIPA lysis buffer containing protease inhibitors (25,000 units/ml) for 30 min. The protein concentration in the supernatant determined. Except for the PDGFR and FAK immunoprecipitates, non-disulfide-reduced conditions were used for SDS-PAGE analysis of immunoprecipitates. Disulfide-reduced conditions were used in the electrophoresis of samples prior to Western blot analysis. The following antibody concentrations were utilized: Lyn IgG (1 μg/ml), anti-Phospho-Lyn IgG (0.5 μg/ml), anti-PDGFα or β IgG (0.2 μg/ml), rabbit anti-integrin β3 or β5, submit IgG (10 μg/ml), rabbit anti-phosphospecific Src[pY416] IgG (0.05 μg/ml), rabbit anti-Src family IgG (0.1 μg/ml), rabbit anti-FAK IgG (1 μg/ml), and rabbit anti-PDGF β1, anti-Lyn, anti-c-Src, anti-Yes, anti-Lck, and anti-Fgr IgG (0.2 μg/ml). In all cases, anti-αv mAb was utilized as an internal control. Anti-αv mAb was utilized as an internal control. Anti-αv mAb was utilized as an internal control. Anti-αv mAb was utilized as an internal control.
was measured based on phosphorylation of a specific Src kinase substrate peptide. 

Down-regulation of Lyn mRNA and Protein by siRNA—siRNA duplexes were synthesized by Dharmacon, Inc. (Lafayette, CO). The 21-nucleotide duplexes of the pattern AA(G/U)UU were selected to obtain symmetric 2-nt 3′ overhangs of identical sequence (33, 34). The Lyn sense sequence utilized, AAGUGGUGAACGAAUUGGCU, specifically targeted the kinase domain, and the Lyn sense sequence utilized, AAAGUGUCGAACGCCAU, specifically targeted the SH2 domain. Lamin A/C siRNA duplex and a nonspecific control siRNA duplex (VIII) were also purchased from Dharmacon, Inc. Transient transfection of siRNAs was carried out using 4 μl of LipofectAMINE 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s guidelines.

Qualitative RT-PCR Analysis—The software Primer 3 (Whitehead Institute) (35) was used to design PCR primers for the Src kinase family members. The following primers were used: c-Src, sense 5′-GGTGAACAAAGAAGAAGC-3′ and antisense 5′-GGCTTGCTG-3′; Fgr, sense 5′-GGACTGGACTGAGTCCAGG-3′ and antisense 5′-AATCTACTTACCCGCGACC-3′; Yes, sense 5′-TATGGCTGCTGATTGCTG-3′ and antisense 5′-TTCCAGGAGTCCTCAGGTA-3′; Lyn, sense 5′-TGTNGAGATCTACCCGTTCA-3′ and antisense 5′-TTTGTCTCCACCATCCTCC-3′; Fyn, sense 5′-TGAAGGCTGGAGGAGGAG-3′ and antisense 5′-GGTGGCATCTGGCGGATAA-3′; and, Lck, sense 5′-CTTCCACTGCTGACACAC-3′ and antisense 5′-GCAACCGTGTCCAGATTAC-3′. Total RNA was extracted from U-87MG cells using the RNasy Mini Kit (Qiagen, Inc.) according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed with 400 units of Maloney murine leukemia virus reverse transcriptase (Promega) in a 50-μl solution containing a standard reverse transcription buffer (Promega). Each PCR reaction was performed in a 50-μl solution containing 5 μl of the cDNA product from the same RT reaction. The PCR solution was subjected to 35 cycles of amplification. Each cycle consisted of denaturation at 95 °C for 30 s, primer annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, and an additional extension at 72 °C for 10 min. 

RESULTS

Integrins αβ3 and αβ5 Mediate U-87MG Cell Attachment to Vitronectin—As a first step in exploring the potential cooperation of the PDGFr and integrin αβ3 on glioblastoma cells, we characterized the integrin receptors that mediate the attachment of U-87MG cells to vitronectin. We found that U-87MG cell attachment to vitronectin is mediated by integrins αβ3 and αβ5, as mAb anti-αβ3 (LM609), and mAb anti-αβ5 (P1F6) both partially inhibited the cell attachment to vitronectin (30 and 25% inhibition, respectively) (Fig. 1A). A combination of mAbs anti-αβ3 and anti-αβ5 resulted in 70% inhibition of attachment, and a mAb anti-αv (L230) resulted in 65% inhibition of attachment (Fig. 1A). A mAb anti-β3 (P4C10) did not inhibit cell attachment to vitronectin significantly, indicating that a β3 integrin does not participate in this attachment under the conditions utilized (Fig. 1A). 

Activation of the PDGFr on PDGFr Stimulation—Other investigators have shown that the U-87MG cell line expresses both PDGFrα and β (36). To confirm the activation of the PDGFr with PDGFr stimulation, serum-starved U-87MG cells were stimulated with a physiologic dose of PDGFr (83 pm, 10 min), lysed, immunoprecipitated with anti-PDGFr IgG and then blotted with anti-phosphotyrosine IgG. The PDGFr stimulation increased the phosphorylation of the tyrosines residues on a 180-kDa protein by 4-fold, consistent with activation of the PDGFr (Fig. 1, B and C). 

PDGFr Stimulation of U-87MG Glioblastoma Cells Results in the Specific Recruitment of Integrin αβ3-containing Focal Adhesions to the Cell Cortex: Requirement For Src Kinase Activity and a Correlation with the Activation of Rac and Rho—To determine the effect of PDGFr stimulation on the cytoskeletal organization of vitronectin-adherent glioblastoma cells, serum-starved U-87MG cells plated onto vitronectin in serum-free media for 5 h were stimulated with PDGFr (83 pm, 10 min), and then analyzed by double-label immunofluorescence. PDGFr stimulation resulted in the recruitment of integrin αβ3 to focal adhesions in the cell cortex (Fig. 2C), whereas vinculin-labeled focal adhesions could be found throughout the basal cell surface (Fig. 2D). In the absence of PDGFr stimulation, integrin αβ3 was localized to focal adhesions throughout the basal cell surface, in a pattern identical to that of vinculin (Fig. 2, A and B, respectively). To determine whether activation of Src was necessary for the PDGFr-mediated reorganization of the cytoskeleton, the Src kinase inhibitor, PP1, was added to the cell monolayer (30 min) prior to stimulation with PDGFr. PP1 (5 nm) blocked the recruitment of integrin αβ3 to the cell cortex (Fig. 2E), indicating that activation of Src is a necessary step in these processes. In contrast to the effects of PDGFr on the localization of integrin αβ3, the distribution of integrin αβ5 remained unaltered on PDGFr stimulation remaining in focal adhesions throughout the basal cell surface (Fig. 3, A–D). 

To confirm the reorganization of the cytoskeleton in the U-87MG cells on stimulation with physiologic levels of PDGFr, we determined the effect of PDGFr stimulation on the activity of Rac and Rho, which are small GTPases that play a role in the cytoskeletal organization (37). We found that stimulation with PDGFr (83 pm, 10 min) increased the activity of both Rac and Rho by 4-fold, and that the increase in activity was blocked by addition of the Src kinase inhibitor, PP1 (5 nm) (data not shown). These data suggest that PDGFr stimulation specifically recruits integrin αβ3 to focal adhesions in the cell cortex and that this recruitment requires Src kinase activity and correlates with the activation of Rac and Rho.
PDGF Stimulation Promotes U-87MG Cell Migration That Is Mediated by Integrin \( \alpha_\beta_3 \) Requirement for Src Kinase—To determine whether the PDGF stimulation promoted the motility of the U-87MG cells, a scratch motility assay was performed in serum-free conditions. In the absence of PDGF stimulation, there was no significant migration into the scratched area of cells plated onto vitronectin (Fig. 4A), collagen or laminin (data not shown) at 24 h. Stimulation with PDGF (83 pM, 10 min) immediately after scratching significantly promoted U-87MG cell migration on vitronectin into the scratched area (Fig. 4B). Although mAb anti-\( \alpha_\beta_3 \) (PF16) also could inhibit the migration, it was much less effective (30% inhibition) (Fig. 4H). To determine whether the PDGF\( \alpha \) cooperated specifically with integrin \( \alpha_\beta_3 \) in promoting and mediating this migration, the cells were plated onto collagen or laminin, which engage different integrins, scratched and then treated with PDGF (83 pM, 10 min). The migration into the scratched area when the cells were plated onto collagen or laminin was 83 and 89% less, respectively, than that exhibited by cells plated on vitronectin (data not shown). This indicates that the PDGF\( \alpha \) cooperates with \( \alpha_\beta_3 \) to promote migration of the U-87MG cells.
To determine the role of Src kinase in this motility, the cell monolayer was pretreated with PP1 (5 nM) prior to stimulation with PDGF. This pretreatment with PP1 completely blocked the PDGF-induced migration into the scratched area (Fig. 4D). To rule out a possible effect of PDGF on cell proliferation during this assay, the cell monolayer was treated with mitomycin C (10 μg/ml). Cell migration into the scratched area after PDGF stimulation was not reduced by pretreatment with mitomycin C (data not shown), indicating that cell proliferation does not contribute to the PDGF-induced migration into the scratched area. These data demonstrate that PDGF stimulation of U-87MG cells promotes migration in a scratch motility assay, that this migration is mediated predominantly by integrin αvβ3, and that the migration requires Src kinase activity.

Specific Association of Integrin αvβ3 and the PDGF receptor in U-87MG Cells—To determine whether integrin αvβ3 associates specifically with the PDGF receptor in U-87MG cells, serum-starved U-87MG cells were plated onto vitronectin-coated flasks in serum-free media for 5 h, stimulated with PDGF (83 pM, 10 min, 37 °C) or vehicle. Lysed, 300 μg of lysate immunoprecipitated with mAb anti-αvβ3 or mAb anti-αvβ5, coupled to Sepharose, subjected to SDS-PAGE and then blotted with anti-PDGFrα- or β-specific IgG, followed by stripping and reprobing with rabbit anti-β3 or β5 IgG. E, lysate (300 μg) was immunoprecipitated with rabbit anti-PDGFrα IgG, subjected to SDS-PAGE, and then blotted with anti-PDGFrα IgG. The experiment was repeated two times.

PDGF Stimulation Results in Increased Src Kinase Activity Associated Specifically with Integrin αvβ3 in U-87MG Cells Adherent to Vitronectin—Based on the limited data from other investigators suggesting individual Src family members may associate preferentially with a specific integrin (10, 13, 9), we formed the hypothesis that PDGF stimulation could be affecting integrin αvβ3 signaling in the U-87MG cells by promoting the association of integrin αvβ3 with an active Src family member. To test this hypothesis, integrin αvβ3 was immunoprecipitated with mAb anti-αvβ3 (LM609) from a lysate of U-87MG cells plated on vitronectin, and then subjected to SDS-PAGE, and blotted with anti-phosphospecific Src[pY416] IgG, which recognizes only active Src family members. A 56–59-kDa band in intensity (3-fold) on PDGF stimulation (Fig. 6, lanes 1 and 2). Immunoprecipitation using a mAb anti-αvβ5 (P1F6), or anti-FAK IgG coupled to Sepharose, and the immunoprecipitates were then subjected to SDS-PAGE, transferred to Immobilon, and blotted with the indicated antibodies. The experiment was repeated two times.

As other investigators have shown that PDGF stimulation activates FAK and we have shown that FAK promotes the migration of malignant astrocytoma or glioblastoma cells (38), we examined the level of FAK activation in response to PDGF stimulation of the U-87MG cells. We found an increase in the intensity (4-fold) of a tyrosine-phosphorylated 125-kDa band, consistent with increased phosphorylation of FAK (Fig. 6, E and F). To determine whether this reflected an increase in the phosphorylation of FAK at Tyr397 (the autoactivation site), the cell lysate from PDGF stimulated or unstimulated cells was blotted with anti-phosphospecific FAK[pY397] IgG and a 3-fold increase in FAK phosphorylation on PDGF stimulation was detected (data not shown).

To confirm the above findings, Src kinase activity assays were performed on integrin αvβ3, integrin αvβ5, and Src immunoprecipitates from lysates of U-87MG plated on vitronectin. PDGF stimulation (83 pM, 10 min) resulted in an approximate
2-fold increase in the Src kinase activity associated with integrin αβ2 and a 2-fold increase in the Src kinase activity in the Src immunoprecipitate (Table I). In contrast, PDGF stimulation did not induce a significant increase in Src kinase activity in the αβ2 immunoprecipitate (Table I). These data confirm that PDGF stimulation results in increased Src kinase activity associated specifically with integrin αβ2.

**Characterization of the Src Family Members in U-87MG Cells**—Characterization of the repertoire of Src family members expressed in U-87MG cells by qualitative RT-PCR analysis with Src family member-specific primers (Fig. 7A) indicated that the levels of Fyn mRNA were highest, followed by Lyn mRNA (Fig. 7B). Lesser amounts of c-Src, Yes and Fgr mRNAs were detectable, but Lck mRNA appeared to be absent (Fig. 7B). As a control, we performed the same analysis on primary lung fibroblasts (19LU cells) and found that, in these cells, the levels of c-Src and Fyn mRNA were highest, with lower levels of Lyn and Yes mRNA (Fig. 7D). Amplification of Lck mRNA from Jurkat cells with Lck-specific primers confirmed our ability to detect a PCR product of the expected size (398 bp size) (data not shown). Amplification of the Src family member mRNAs from a second glioblastoma cell line (U-251MG) indicated that, similar to the findings in the U-87MG cells, the levels of Fyn mRNA were highest with relatively lower levels of Lyn mRNA (Fig. 7C). c-Src and Yes mRNA was barely detectable in the U-251MG cells (Fig. 7C), and Lck and Fgr mRNA appeared to be absent.

Lysates of the U-87MG and U-251MG cells were then Western blotted with Src family member-specific antibodies to evaluate the levels of expression of the proteins. Consistent with the RT-PCR analysis, in both cell lines the levels of Fyn protein were highest, with lower amounts of Lyn and very low levels of c-Src and Yes proteins (Fig. 7, E and G). Lck and Fgr proteins were not detected in either cell line.

**PDGF Stimulation Increases Lyn Activity in Vitronectin-Adherent U-87MG Cells:** Preferential Recruitment of Lyn to Integrin αβ2—To determine whether Fyn or Lyn are activated upon PDGF stimulation of vitronectin-adherent U-87MG cells, serum-starved cells were stimulated with PDGF (83 pm, 10 min), and the lysate immunoprecipitated with rabbit anti-Lyn or rabbit anti-Fyn IgG. The immunoprecipitate was resolved by SDS-PAGE and Western blotted with phosphospecific anti-Src[pY416] IgG. Lyn activity was increased ~3-fold on PDGF stimulation (Fig. 8A, lanes 1 and 2). In contrast, the intensity of the Fyn band was not altered by PDGF stimulation (Fig. 8A, lanes 3 and 4). Similar results were obtained in response to a higher concentration of PDGF (199 pm, 10 min), with an increase in the activity of Lyn (~3-fold) and unaltered Fyn activity (data not shown). Very high concentrations of PDGF (~1660 pm) resulted in cell rounding (data not shown).

To determine whether Lyn or Fyn associates specifically with integrin αβ2 in the U-87MG cells, αβ2 immunoprecipitates of vitronectin-adherent cells were subjected to SDS-PAGE and then Western blotted with Src family member-specific antibodies. A band at 56 kDα, when blotting with rabbit anti-Lyn, and a band at 59 kDα, when blotting with rabbit anti-Fyn IgG, were observed and these exhibited equivalent intensity in the presence and absence of PDGF stimulation (Fig. 8, D and E). Identical results were obtained when mAbs directed toward Lyn or Fyn were used (data not shown). As shown above in Fig. 6 there was a 3-fold increase in Src kinase activity after PDGF stimulation associated specifically with integrin αβ2 that was detected with anti-phosphospecific Src[pY416] IgG. Blotting of the αβ2 immunoprecipitate with rabbit anti-c-Src or rabbit anti-Yes IgG failed to reveal bands of the appropriate relative molecular mass (data not shown). As the Fyn protein is expressed at significantly higher levels than Lyn in the U-87MG cells, these data suggest that Lyn is preferentially recruited to integrin αβ2 in vitronectin-adherent U-87MG cells. Similar analyses of anti-integrin αβ2 immunoprecipitates indicated that Fyn is associated constitutively with integrin αβ2, independent of PDGF stimulation, and that Lyn fails to associate with integrin αβ2 irrespective of the presence or absence of PDGF stimulation (Fig. 8, F and G). These data indicate that PDGF stimulation of vitronectin-adherent U-87MG cells increases Lyn activity, and that Lyn is preferentially recruited to integrin αβ2.

**Src Family Member Activation on PDGF Stimulation Is Dependent on the Substrate the Cells are Adherent to** and the Integrin Receptor That is Engaged—To determine whether the activation of Lyn kinase on PDGF stimulation of the vitronectin-adherent U-87MG cells is dependent on the engagement of integrin αβ2 or can be mediated by the engagement of other integrin receptors, the U-87MG cells were plated onto collagen or laminin in serum-free media for 5 h and then stimulated with PDGF (83 pm, 10 min), lysed, and immunoprecipitated with rabbit anti-Lyn or rabbit anti-Fyn IgG. Blotting of the Lyn and Fyn immunoprecipitates with anti-phosphospecific Src[pY416] IgG demonstrated an increased activation of Fyn with PDGF stimulation only on adherence of the U-87MG cells to laminin, with no increase in either Lyn or Fyn activity with PDGF stimulation on adherence of the U-87MG cells to collagen (Fig. 9A). We determined by cell attachment assays that integrins αβ3 and αβ5 do not participate in U-87MG cell attachment to laminin or collagen (Fig. 9, C and D, respectively), indicating that the engagement of different integrin receptors did not result in increased Lyn activity with PDGF stimulation. In addition, U-87MG cell attachment to laminin and collagen required more time (60 min), as compared with cell attachment to vitronectin (20 min), see Fig. 1, suggesting differences in the affinity or avidity of the laminin and collagen receptor integrins on these cells, as compared with the vitronectin receptor integrins.

To confirm that engagement of a specific integrin receptor could affect the Src family member that is activated on PDGF stimulation, serum-starved U-87MG cells were plated onto immobilized anti-integrin mAbs. Plating of the cells onto mAb anti-αβ3 (4 h) followed by 83 pm PDGF stimulation (10 min) resulted in increased Lyn activity (3-fold), but not Fyn activity (Fig. 10). In contrast, plating the cells onto mAb anti-αβ5 IgG followed by 83 pm PDGF stimulation (10 min) resulted in increased Fyn activity (4-fold), but no change in Lyn activity (Fig. 10). These data suggest that PDGF stimulation increases the activity of individual Src family members in a manner that
is dependent on the substrate the cells are adherent to, or the integrin receptor that is engaged.

Down-regulation of Lyn mRNA and Protein by siRNA Inhibits PDGF-stimulated Integrin αᵥβ₃-mediated Migration—To determine whether Lyn is necessary for PDGF-stimulated migration mediated by integrin αᵥβ₃, Lyn mRNA and protein were downregulated with siRNA. Lamin A/C siRNA was used to optimize the transfection conditions and efficiency in the U-87MG cells (Fig. 11A). Lyn and as a control Fyn expression were downregulated with Lyn siRNA or Fyn siRNA, respectively, in U-87MG glioblastoma cells and the respective mRNA analyzed by RT-PCR using Src family member specific primers (Fig. 11B). The respective proteins were analyzed by Western blot with Src family member specific antibodies (Fig. 11C). Additional controls of nonspecific siRNA duplex or vehicle alone were evaluated, and found to not alter Lyn or Fyn mRNA and protein expression. Transfection of Lyn siRNA specifically inhibited Lyn mRNA and protein expression but not Fyn mRNA and protein expression (Fig. 11 B and C), while transfection of Fyn siRNA specifically inhibited Fyn mRNA and protein expression but not Lyn mRNA and protein expression (Fig. 11, B and C). In vitronectin-adherent U-87MG cells, transfection of Lyn siRNA, but not Fyn siRNA, significantly inhibited PDGF-stimulated migration into a scratched area (70% inhibition) (Fig. 11D). These data indicate that Lyn is required for PDGF-stimulated migration mediated by integrin αᵥβ₃.

**DISCUSSION**

In this study, we found that stimulation of vitronectin-adherent U-87MG glioblastoma/malignant astrocytoma cells with PDGF promotes the recruitment of integrin αᵥβ₃-containing focal adhesions to the cell cortex and cell migration in a Src kinase-dependent manner. Stimulation with PDGF of U-87MG cells adherent to laminin or collagen, when different integrin receptors are engaged, promoted cell migration to a much lesser extent. These results suggested a potential cooperation between αᵥβ₃ and PDGFr in these cells. PDGF stimulation of the vitronectin-adherent cells or of cells adherent to mAb anti-αᵥβ₃ resulted in a specific increase in Lyn kinase activity, and an increase in Src kinase activity that was associated specifically with integrin αᵥβ₃. These affects were apparent despite the fact that Fyn is the predominant Src family member expressed in these cells, and that both Lyn and Fyn associated constitutively with integrin αᵥβ₃ under the conditions utilized. Confirming an important or perhaps necessary role for Lyn in PDGF-stimulated integrin αᵥβ₃-mediated cell migration, we show that down-regulation of Lyn mRNA and protein with siRNA significantly inhibited this migration. Furthermore, we show that the specific integrin receptor that is engaged determines which Src family member is activated on PDGF stimulation.

We found that PDGF stimulation of U-87MG cells adherent to vitronectin recruited integrin αᵥβ₃ to adhesion structures in the cell cortex in a Src-kinase-dependent manner, in contrast to integrin α₅β₅. This re-localization of integrin αᵥβ₃ from focal adhesions found throughout the basal cell surface to the cell cortex after PDGF stimulation is probably part of the cytoskeletal reorganization preceding cell motility. It is consistent with the Src kinase-dependent increase in Rac and Rho activity that we detected on PDGF stimulation. Other investigators have shown that the small GTPases, Rho, Rac, and cdc42, can be activated by PDGF, and that they regulate cytoskeletal reorganization and cell migration (37, 39). Src kinase activity has been shown to be necessary for PDGF-induced focal adhesion remodeling in Swiss 3T3 cells (40). Other investigators have demonstrated that PDGF stimulation (996 pM, 10 min) of
vitronectin-adherent NIH3T3 fibroblasts resulted in loss of integrin H9251 localization to focal adhesions; whereas identical treatment of RFC cells adherent to vitronectin failed to alter the focal adhesion localization of integrin H9251 (19). This occurred despite the observation that integrin H9251/H9252 and PDGFr/H9252 cooperate in both cell types to promote migration (19). This suggests that the effect of PDGF stimulation on H9251/H9252 localization in vitronectin-adherent cells is dependent on multiple factors and maybe cell type-specific.

Our data also showed that PDGF stimulation increased the activation and overall tyrosine phosphorylation of FAK in the U-87MG cells, consistent with studies in non-glial cells indicating that PDGF can increase FAK activity and overall tyrosine phosphorylation (41, 42). FAK promotes cell migration (38, 43–45), and the importance of the Src/FAK interaction in migration has been demonstrated by the impaired haptotactic migration of FAK-null fibroblasts expressing a mutant FAK (Y397F) construct (Src binds to the phosphorylated Tyr397 residue in FAK) (46), and the observation that cells derived from mouse embryos deficient in c-Src, Fyn, and Yes show significantly reduced integrin-mediated activation of FAK and integrin-mediated cell migration (47). These data are consistent with a critical role for the Src/FAK complex in promoting cell migration.

PDGF stimulation was found to promote glioblastoma cell migration of vitronectin-adherent cells into a scratched area in a Src kinase-dependent manner and in a mechanism mediated primarily by integrin H9251/H9252. In multiple cell types, PDGF stimulation has been shown to promote chemotactic or haptotactic migration, as well as migration into a scratched area (5). The mechanism by which the PDGFr and integrin H9251/H9252 cooperate to promote cell migration is not understood fully. A direct physical association between these two cell surface receptors likely plays a role in this cooperation, as the PDGFr binds the extracellular domain of the integrin H9252 subunit in a manner that is independent of PDGFr activation (21). Consistent with the latter finding, we show here a specific association of the PDGFr and integrin H9251/H9252 in the U-87MG cells by co-immunoprecipitation analysis of the cell lysate.

We also show that PDGF stimulation specifically increased the activity of Lyn kinase in vitronectin-adherent U-87MG cells, and that Lyn was associated preferentially with integrin H9251/H9252. Lyn was also co-immunoprecipitated with integrin H9251/H9252, but its activity in the U-87MG cells adherent to vitronectin was independent of PDGF stimulation. In contrast, Fyn but not Lyn...
was constitutively associated with integrin $\alpha_6\beta_4$. There is a precedent for tyrosine kinase growth factor receptor signaling to a specific Src family member; Mariotti et al. (13) have shown in A431 epidermoid squamous carcinoma cells that transfection with a dominant negative Fyn construct inhibited EGF-stimulated hemidesmosome disassembly. They also showed that Fyn was preferentially associated with integrin $\alpha_6\beta_4$ (13).

Our studies do not define whether the association of integrin $\alpha_3\beta_1$ or $\alpha_6\beta_4$ with a Src family member is direct or indirect; however, it is likely indirect. When the U-87MG cells were

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FIG. 10. Integrin-specific Src family member activation with PDGF stimulation. Serum-starved U-87MG cells in serum-free media were plated onto immobilized mAb anti-$\alpha_3\beta_1$ (LM609), mAb anti-$\alpha_5\beta_1$, or mAb anti-$\alpha_6\beta_4$ for 4 h (37 °C, 5% CO$_2$), stimulated with 83 pM PDGF or vehicle (10 min, 37 °C), detergent lysed, the lysate precleared with Gammabind Sepharose, and 30 $\mu$g of lysate immunoprecipitated with rabbit anti-Lyn or anti-Fyn IgG. The immunoprecipitates were subjected to SDS-PAGE and blotted with anti-Src[pY418] IgG (detects only activated Src) (A), stripped, and reprobed with rabbit anti-Lyn or anti-Fyn IgG (B). The experiment was repeated two times.

FIG. 11. Down-regulation of Lyn mRNA and protein by siRNA inhibits PDGF-stimulated integrin $\alpha_3\beta_1$-mediated cell migration. U-87MG cells were transfected with 160 nM Lamin A/C siRNA (A), 320 nM Lyn siRNA (B–D), 320 nM Fyn siRNA (B–D), or with 320 nM nonspecific control siRNA (A–C). U-87MG cells (3 x 10$^5$) 48 h post-transfection were detergent lysed, subjected to disulfide-reduced 10% SDS-PAGE, and blotted with mAb anti-Lamin A/C (A), stripped, and reprobed with mAb anti-actin (A), or blotted with rabbit anti-Lyn or anti-Fyn-specific IgG (C), stripped, and reprobed with mAb anti-actin (C). The experiment was repeated four times. B, total RNA from U-87MG cells 48 h post-transfection was extracted and subjected to RT-PCR with Lyn- or Fyn-specific primers, followed by electrophoresis of identical PCR product volumes on a 2% agarose gel, as described under “Experimental Procedures.” The experiment was repeated four times. D, as described in Fig. 4, a confluent monolayer of serum-starved U-87MG cells adherent to vitronectin in serum-free media was scratched 20 h after transfection with 320 nM Lyn or Fyn siRNA, stimulated with 83 pM PDGF (10 min, 37 °C) or vehicle, followed by washing and incubation (37 °C, 5% CO$_2$). At 24 h post-scratching, the scratched area was photographed. Conditions were assayed in replicas of six and the experiment was repeated two times.

was constitutively associated with integrin $\alpha_3\beta_1$. There is a precedent for tyrosine kinase growth factor receptor signaling to a specific Src family member; Mariotti et al. (13) have shown in A431 epidermoid squamous carcinoma cells that transfection with a dominant negative Fyn construct inhibited EGF-stimulated hemidesmosome disassembly. They also showed that Fyn was preferentially associated with integrin $\alpha_6\beta_4$ (13). Our studies do not define whether the association of integrin $\alpha_3\beta_1$ or $\alpha_6\beta_4$ with a Src family member is direct or indirect; however, it is likely indirect. When the U-87MG cells were
adherent to laminin through integrins $\alpha_\beta_1$, $\alpha_\beta_1$, and $\alpha_\beta_3$, or to immobile mAb anti-integrin $\alpha_\beta$, PDGF stimulation resulted in increased Fyn, but not Lyn, activity. These data suggest that the specific Src family member activated with PDGF stimulation is dependent on the substrate the cells are adherent to, or the integrin(s) that is engaged. We do not know the mechanism for this PDGF-stimulated specific increase in Lyn activity in the U-87MG cells adherent to vitronectin, and this is an area of future study. However, our data with siRNA directed toward Lyn indicate Lyn is necessary for the cooperative signaling of the PDGF and integrin $\alpha_\beta_3$ that promotes glioblastoma cell migration. These data may point to a potent specific pairing of individual Src family members with specific integrins in their cooperative signaling with growth factor receptors.

Other investigators have reported that PDGF stimulation activates Fyn in fibroblasts plated in 10% serum-containing media until confluent, and then serum-starved in 0.5% serum-containing media for 40 h, followed by PDGF stimulation at 1660 pm for 10 min (48). We show that the 19LU pulmonary fibroblasts express a different repertoire of Src family members, as compared with the U-87MG and U-251MG cells; predominantly Fyn and c-Src mRNAs were detected in the 19LU cells and barely detectable levels of Lyn and Yes mRNAs. This difference in Src family member activation upon PDGF stimulation of fibroblasts reported previously versus what we demonstrate here in the U-87MG glioblastoma cells could be due, at least in part, to the different repertoire of Src family members expressed and the different experimental conditions.

Previous studies in our laboratory have shown that integrins $\alpha_\beta_3$ and $\alpha_\beta_3$ are up-regulated on glioblastoma cells in vitro as compared with astrocytes in the normal brain, and that these tumors express at least two ligands for integrin $\alpha_\beta_3$, osteopontin, and vitronectin (23, 25, 49). Therefore, these data taken together with the previous reports of an up-regulation of PDGF and the PDGFr in these tumors (2, 3), suggest that the PDGFr and integrin $\alpha_\beta_3$ are both available and could cooperate in promoting glioblastoma cell migration and invasion in vivo. In summary, we demonstrate in glioblastoma cells that: 1) PDGF stimulation of vitronectin-adherent U-87MG cells or of cells adherent to mAb anti-$\alpha_\beta_3$ results in the increased activity of Lyn kinase specifically and that Lyn is recruited preferentially to integrin $\alpha_\beta_3$; 2) the PDGFr$\beta$ cooperates with integrin $\alpha_\beta_3$ to promote cell migration in a Lyn kinase-dependent manner; and 3) the individual Src family member activated upon PDGF stimulation depends on the substrate the cells are adherent to or the integrin receptor(s) that is engaged.

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