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Malignant Astrocytoma Cell Attachment and Migration to Various Matrix Proteins Is Differentially Sensitive to Phosphoinositide 3-OH Kinase Inhibitors

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Abstract Phosphoinositide 3-OH kinase (PI3-K) has been shown to play an important role in the signaling pathway necessary for cytoskeletal reorganization in non-astrocytic cells. We investigated the role of PI3-K in U-251MG human malignant astrocytoma cell adhesion and migration. Attachment of U-251MG cells to vitronectin, fibronectin, laminin, and collagen was inhibited in a concentration-dependent manner by two specific inhibitors of PI3-K (Wortmannin and LY294002). Attachment to vitronectin, fibronectin, and laminin was more sensitive to inhibition of PI3-K (45% inhibition at 10 nM Wortmannin) than attachment to collagen (25% inhibition at 100 nM Wortmannin). Similarly, migration toward these substrates showed differential sensitivity to inhibition. Attachment of the cells to these matrix proteins resulted in an increase in PI3-K activity, as compared to that of cells in suspension, with attachment to vitronectin resulting in the greatest increase in PI3-K activity. p125 focal adhesion kinase (p125FAK) was found to co-immunoprecipitate with PI3-K from the NP40-soluble cell fraction of a 1% NP40 detergent lysate of cells in the early stages of adhesion to vitronectin and fibronectin, but not during adhesion to collagen. The expression of p125FAK protein and level of phosphorylation were similar on adherence to all three substrates. These data indicate that the sensitivity of U-251MG cell attachment and migration to PI3-K inhibitors is substrate-dependent, and that complex formation of PI3-K and p125FAK correlates with this sensitivity to PI3-K inhibitors. Our data suggest a role for PI3-K and p125FAK complex formation in PI3-K activation. J. Cell. Biochem. 73:533–544, 1999.

Key words: PI3-K, p125FAK, adhesion; vitronectin; collagen; migration; astrocytoma; glioma; glioblastoma

Phosphoinositide 3-OH kinase (PI3-K) plays a central role in numerous signaling pathways, including those associated with cytoskeletal reorganization, cell motility, membrane ruffling, invasion, and cell proliferation [Shears, 1992; Toker and Cantley, 1997]. Activation of PI3-K results in the generation of D3 phosphoinositide metabolites, that interact with certain proteins containing pleckstrin homology domains; the latter domain module recruits proteins to cellular membranes [Shears, 1992; Toker and Cantley, 1997]. D3 phosphoinositides bind to a number of proteins that regulate actin assembly, e.g., phospholipase C, thereby contributing to filopodial actin assembly [Toker and Cantley, 1997]. In attached and spread platelets and osteoclasts, PI3-K is associated with the insoluble cell fraction (cytoskeleton) [Toker and Cantley, 1997; Guinebault et al., 1995; Lakkakorpi et al., 1997]. This localization of PI3-K to the cytoskeleton is supported by reports that PI3-K can be co-immunoprecipitated with the cytoskeletal protein α-actinin in NIH3T3 cells, and with p125 focal adhesion kinase (p125FAK) in spread platelets [Guinebault et al., 1995; Shibasaki et al., 1994]. Recent evidence suggests that the activity of PI3-K may increase when it is associated with the platelet cytoskeleton [Guinebault et al., 1995]. Furthermore, PI3-K modulates the reorganization of the actin cytoskeleton through interactions with the small GTPase protein, Rac, in mammary epithe-

Abbreviations used: PI3-K, phosphoinositide 3-OH kinase; PI3P, phosphatidylinositol 3-phosphate; p125FAK, p125 focal adhesion kinase; TLC, thin layer chromatography.

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lial and COS cells [Keely et al., 1997; Ma et al., 1998].

Cell adhesion is mediated by a family of receptors known as integrins [Clark and Brugge, 1995; Gladson and Cheresh, 1994; Schwartz et al., 1995]. Integrin ligation typically results in integrin clustering and the generation of a signalling complex at the submembranous portion of the cell adjacent to the clustered integrin. One of the signalling molecules that is activated (phosphorylated) upon integrin ligation and clustering and which localizes to this signalling complex is p125FAK, a cytoplasmic tyrosine kinase [Kornberg et al., 1992; Lipfert et al., 1992; Schaller et al., 1992]. p125FAK is thought to play a role in cell spreading, as overexpression of the p125FAK carboxy-terminus in fibroblasts inhibits cell spreading [Richardson et al., 1997]. p125FAK also plays a role in cell migration, as downregulation of p125FAK results in reduced cell motility [Ilic et al., 1995; Gilmore and Romer, 1996], and overexpression of p125FAK in CHO cells results in increased cell migration [Cary et al., 1996]. In cells transfected with p125FAK, as well as in Jurkat and H460 carcinoma cells, p125FAK is regulated through proteolytic cleavage by caspase 3 and probably caspase 6 of the carboxy-terminal-half of the molecule [Wen et al., 1997; Gervais et al., 1998]. Caspase 3 cleavage generates a fragment that acts as a competitive inhibitor of phosphorylation of the full-length p125FAK protein [Wen et al., 1997; Gervais et al., 1998; Schaller et al., 1993; Richardson and Parsons, 1996].

Activated p125FAK has been shown to interact with PI3-K in NIH3T3 fibroblasts [Chen and Guan, 1994]. The phosphorylated Tyr397 residue of p125FAK and the SH2 domain in the p85 subunit of PI3-K are necessary for the binding of PI3-K to p125FAK [Chen and Guan, 1994; Bachelot et al., 1996]. There is in vitro evidence that the recombinant p85 subunit of PI3-K is phosphorylated by p125FAK, suggesting that PI3-K may be a substrate of p125FAK, and that p125FAK may activate PI3-K [Chen and Guan, 1994]. As PI3-K has been shown to partially localize to focal adhesions in some cell types, the interaction of p125FAK with PI3-K may also recruit PI3-K to focal adhesions [Toker and Cantley, 1997].

In this study, we investigated the role of PI3-K in malignant astrocytoma cell adhesion and migration using PI3-K inhibitors. We found that PI3-K activity was necessary for attachment to and migration toward several substrates; however, the effect of specific inhibition of PI3-K varied with the substrate. Furthermore, we show that PI3-K forms complexes with p125FAK early in adhesion when the cells are adherent to substrates in which attachment is sensitive to PI3-K inhibitors; whereas no complex formation was detected when cells were adherent to a substrate in which attachment was relatively insensitive to PI3-K inhibitors. Our data implicate substrate-specific PI3-K signalling in the modulation of cell attachment and migration of malignant astrocytoma cells.

MATERIALS AND METHODS

Reagents

DMEM, and fetal bovine serum were purchased (Gibco BRL Life Technologies, Inc., Gaithersburg, MD). The following antibodies were purchased: rabbit anti-rat PI3-Kinase antisera (#06-195, UpState Biotechnology, Lake Placid, NY), rabbit anti-p125FAK IgG and mAb anti-phosphotyrosine (mAb PY20) (Upstate Biotechnology), mouse mAb anti-p125FAK directed toward the recombinant kinase domain (Transduction Laboratories, Lexington, KY), goat anti-mouse and goat anti-rabbit HRP-conjugated IgG (BioRad, Hercules, CA), and mouse sera (Sigma Chemical Co., St. Louis, MO). The IgG fraction of all antibodies was purified by chromatography over Protein A Sepharose (Sigma Chemical Co.). Two PI3-K inhibitors which inhibit the activity of PI3-K, Wortmannin (IC_{50} = 5 nM) and LY294002 (IC_{50} = 1.4 μM), were purchased (Calbiochem, La Jolla, CA), solubilized in DMSO, aliquoted, and stored at −70°C for no longer than 6 weeks. PD98059, which inhibits the activity of MAP kinase kinase, was purchased (Calbiochem) and stored as recommended. The caspase 3 inhibitor (DEVD-CHO) and the caspase 1 (ICE) inhibitor (YVAD-CHO) were purchased (BACHEM, Inc., Torrance, CA). Phosphatidylinositol and phosphatidylserine were purchased (Avanti Polar-Lipids, Inc., Birmingham, AL). Phosphatidylinositol 4-phosphate was purchased (Sigma Chemical Co.). Vitronectin was purified by the method of Yatohgo et al. [1988], and migrated on disulfide-reduced 7.5% SDS PAGE as a doublet at 70 kDa. Fibronectin, collagen type I, and laminin were purchased (ICN, Aurora, OH).

Cells and Cell Attachment Assays

U-251MG cells were purchased from the ATCC, and maintained in complete media as described [Gladson and Cheresh, 1991; Gladson et al., 1995]. Cells were free of Mycoplasma contamination, based on routine testing. Attachment assays were performed as described previously [Pijuan-Thompson and Gladson, 1997]. Briefly, cells were harvested with buffered EDTA, resuspended in adhesion assay buffer, incubated with inhibitors (1 h, 22°C), aliquoted onto wells coated previously with 10 μg/ml vitronectin, fibronectin, collagen, laminin, or ovalbumin, and allowed to attach (45 min, 37°C, 5% CO_2). Subsequently, the wells were washed with PBS 3×, cells fixed with paraformaldehyde, stained with Crystal Violet, dried, solubilized with acetic acid, and the absorbance read using an ELISA reader (ICN Titertek Multiskan Biochromatic, Irvine, CA) at 570 nm. Control cells were treated with 0.1% DMSO. Attachment to ovalbumin was subtracted. Samples were assayed in replicates of six, and the data analyzed and presented as the mean ± S.E.

Haptotactic Migration Assays

These assays were performed as described previously [Gladson et al., 1995]. Briefly, cells were harvested with buffered EDTA, resuspended in serum-free DMEM with 1% BSA, incubated with inhibitors (1 h, 22°C), aliquoted onto 8 μm pore filters that had been coated previously on the undersurface with 10 μg/ml of vitronectin, fibronectin, collagen, or ovalbumin, and allowed to migrate (3 h, 37°C, 5% CO_2). Subsequently, the cells on the upper filter were removed, cells on the bottom filter surface were fixed with paraformaldehyde, stained with Crystal Violet, washed, and counted. Control cells were treated with 0.1% DMSO. Migration toward ovalbumin was subtracted, and was always $<1\%$ of the total number of cells that migrated [Gladson et al., 1995]. Samples were assayed in replicates of four, and the data analyzed and presented as the mean ± S.E.

Immunoprecipitation and Western Blot Analyses

Cells adherent to a purified matrix protein in serum-free DMEM with 1% BSA were lysed in 1% NP40 lysis buffer with inhibitors on ice for 1 h [Chen and Guan, 1994]. Subsequently, the lysate was centrifuged (45,000 rpm, 1 h, 4°C), the protein concentration in the supernatant lysate determined, and equivalent μg of protein from each lysate immunoprecipitated with rabbit anti-PI3-K IgG coupled to Protein A Sepharose overnight (4°C), as described [Kornberg et al., 1992; Schaller et al., 1992; Chen and Guan, 1994]. Subsequently, the immunoprecipitates were washed 3× with PBS with 1% Tween 20 and 0.1% ovalbumin, 6× with lysis buffer, and 3× with PBS with 1% Tween 20, electrophoresed on a 7.5% SDS PAGE, and transferred to Immobilon [Kornberg et al., 1992; Schaller et al., 1992]. For Western blot analysis, the membrane was blocked with 5% BSA (3 h, 22°C), washed in TBS containing 1% Tween 20 (37°C), reacted with 1–5 μg/ml of primary antibody in 5% BSA (1 h, 22°C), washed 3× in TBS containing 1% Tween 20 at 37°C, reacted with 0.025 μg/ml of HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (45 min, 22°C), washed 3× in TBS containing 1% Tween 20 at 37°C, and developed with the Amersham Life Sciences ECL chemiluminescent system (Arlington Heights, IL) [Kornberg et al., 1992; Schaller et al., 1992]. The NP40-insoluble cell pellet was solubilized with RIPA buffer [Gladson and Cheresh, 1991].

PI3-K Activity Assay

The PI3-K activity assay was performed as described [Fry et al., 1992]. Briefly, 30 μl of immunoprecipitated PI3-K was incubated with 50 μl of 50 mM Tris-HCl (pH 7.6), containing 5 mM $MgCl_2$, 0.5 mM EGTA, 50 mM NaCl, 1 mM phosphatidylinositol, 400 μM phosphatidylserine, 10 μM ATP, and 1 μCi of ^{32}P-ATP (20 min, 30°C). The reaction was stopped with 100 μl of 1 N HCl, and the lipids were extracted with 160 μl of 1:1 chloroform:methanol. The organic phase was subjected to thin layer chromatography (TLC) in 50% chloroform, 47% methanol, and 11.3% ammonium hydroxide. Radioactive spots were visualized by autoradiography and quantified with a phosphorimager. Typically one band was detected on the TLC plate, which co-migrated with a phosphatidylinositol 4-phosphate standard, consistent with phosphatidylinositol 3-phosphate (PI3P) [Fry et al., 1992].

RESULTS

The Sensitivity of Malignant Astrocytoma Cell Attachment to Inhibition of PI3-K Activity is Substrate-Dependent

We have reported previously that attachment of U-251MG malignant astrocytoma cells

is mediated by different integrins in a substrate-specific manner [Gladson and Cheresh, 1991; Gladson et al., 1995; Pijuan-Thompson and Gladson, 1997]. Attachment of U-251MG cells to vitronectin is mediated by integrins αvβ3 and αvβ5, fibronectin attachment is mediated by α5β1, laminin attachment is mediated by α6β1, and collagen attachment is mediated by integrins α2β1 and α3β1 [Gladson and Cheresh, 1991; Gladson et al., 1995; Pijuan-Thompson and Gladson, 1997]. Activation of PI3-K upon cell adhesion or integrin ligation in non-astrocytic cell types has been reported [Toker and Cantley, 1997; Chen and Guan, 1994; Shaw et al., 1997; Hruska et al., 1995]. To determine whether PI3-K is necessary for adhesion of U-251MG human malignant astrocytoma cells, attachment assays were performed in the presence of specific inhibitors of PI3-K activity. We found that attachment of U-251MG cells to vitronectin, fibronectin, and laminin was inhibited (45%) by 10 nM Wortmannin (two-fold the IC_{50}) and 1.4 μM LY294002 (IC_{50} = 1.4 μM; Fig. 1A and B, respectively). In contrast, U-251MG cell attachment to collagen required significantly greater amounts of Wortmannin (20-fold the IC_{50}) to inhibit attachment by 25% (Fig. 1A). Preincubation of the cells with the MAP kinase kinase inhibitor (PD98059) failed to inhibit attachment to any substrate (Fig. 1C). Thus, the data indicate that U-251MG cell attachment to some, but not all, matrix proteins is sensitive to PI3-K inhibitors.

The Sensitivity of Haptotactic Migration of Malignant Astrocytoma Cells to Inhibition of PI3-K Activity is Substrate-Dependent

Integrin-mediated migration toward vitronectin and fibronectin has been reported to require PI3-K activation in other cell types [Klemke et al., 1998]. To determine whether PI3-K is necessary for malignant astrocytoma cell motility, U-251MG cell migration assays were performed in the presence of Wortmannin and LY294002. We found that Wortmannin and LY294002 at two- and one-fold the IC_{50} concentration, respectively, inhibited migration toward vitronectin and fibronectin by 45% (Fig. 2A,B). In contrast, migration toward collagen was unaffected by Wortmannin and LY294002 inhibitors at this concentration, and 100 nM Wortmannin only resulted in 10% inhibition of migration (Fig. 2A). These data indicate that haptotactic migra-

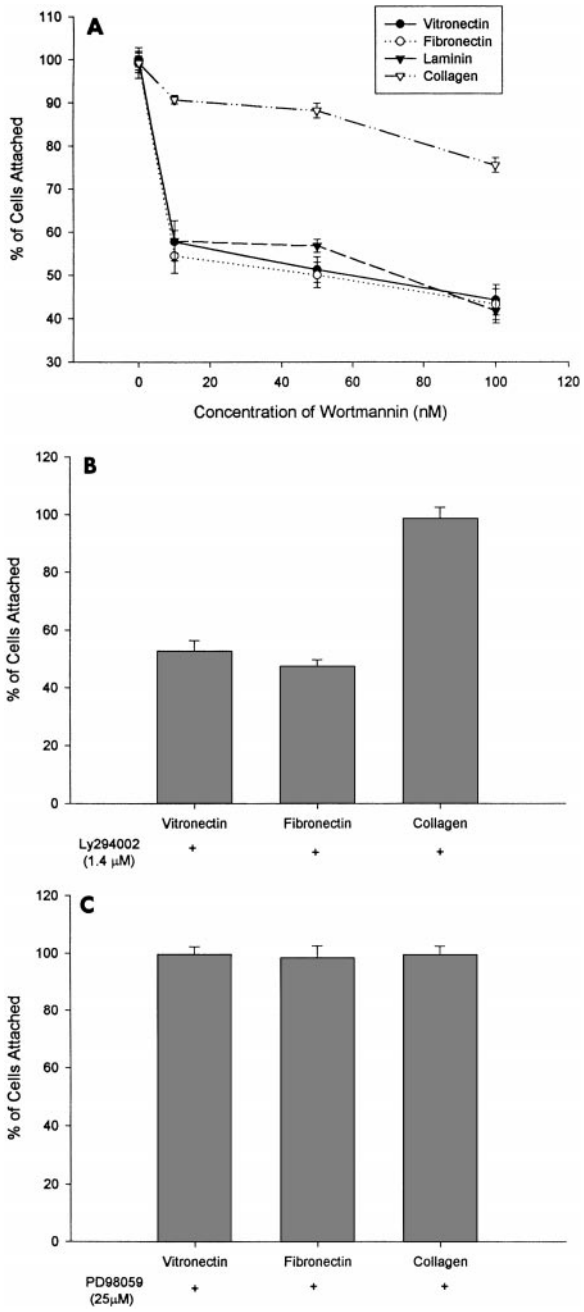

Fig. 1. U-251MG malignant astrocytoma cell attachment is differentially sensitive to PI3-K inhibitors, depending on the matrix protein. U-251MG cells were harvested with buffered EDTA, resuspended in adhesion assay buffer, incubated with inhibitors (1 h, 22°C), aliquoted onto 96-well plates that had been coated previously with vitronectin, fibronectin, laminin, or collagen, and allowed to attach (45 min, 37°C, 5% CO_2), as described in the Materials and Methods. **A:** Dose response of Wortmannin (10–100 nM; IC_{50} = 5 nM) treatment of U-251MG cells followed by attachment to vitronectin, fibronectin, laminin, or collagen. **B:** 1.4 μM LY294002 (IC_{50} = 1.4 μM) treatment of U-251MG cells inhibited attachment to vitronectin and fibronectin, but not to collagen. **C:** Twenty-five μM PD98059 (inhibits the activity of MAP Kinase Kinase) treatment of U-251MG cells was included as a control and failed to inhibit attachment.

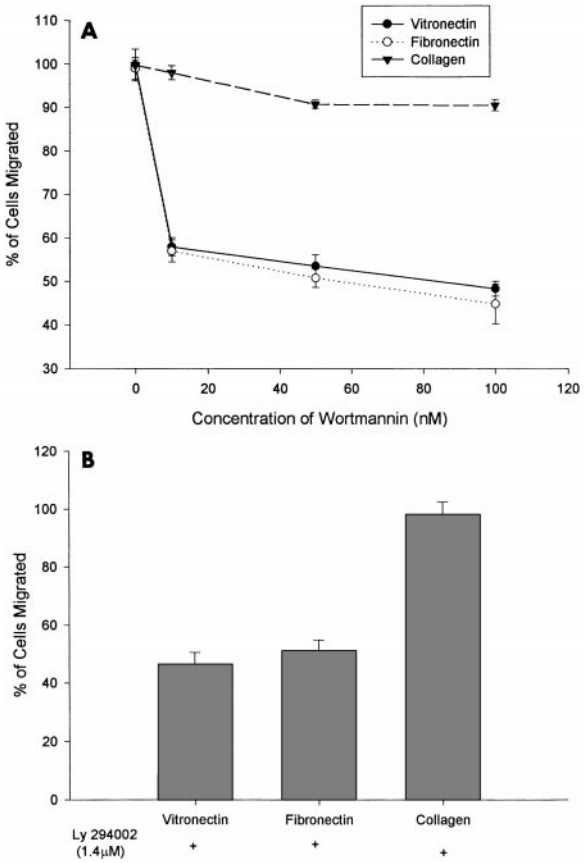

Fig. 2. Malignant astrocytoma cell migration is differentially sensitive to PI3-K inhibitors, depending on the matrix protein. U-251MG cells were harvested with buffered EDTA, resuspended in serum-free DMEM, incubated with inhibitors (1 h, 22°C), and aliquoted onto filters that had been coated previously on the under surface with vitronectin, fibronectin, or collagen, and allowed to migrate (3 h, 37°C, 5% CO_2), as described in the Materials and Methods. **A:** Dose response of Wortmannin (10–100 nM, IC_{50} = 5 nM) treatment of U-251MG cells, and its effect on cell migration toward vitronectin, fibronectin, and collagen. **B:** 1.4 μM LY294002 (IC_{50} = 1.4 μM) treatment of U-251MG cells inhibited migration toward vitronectin and fibronectin, but not toward collagen.

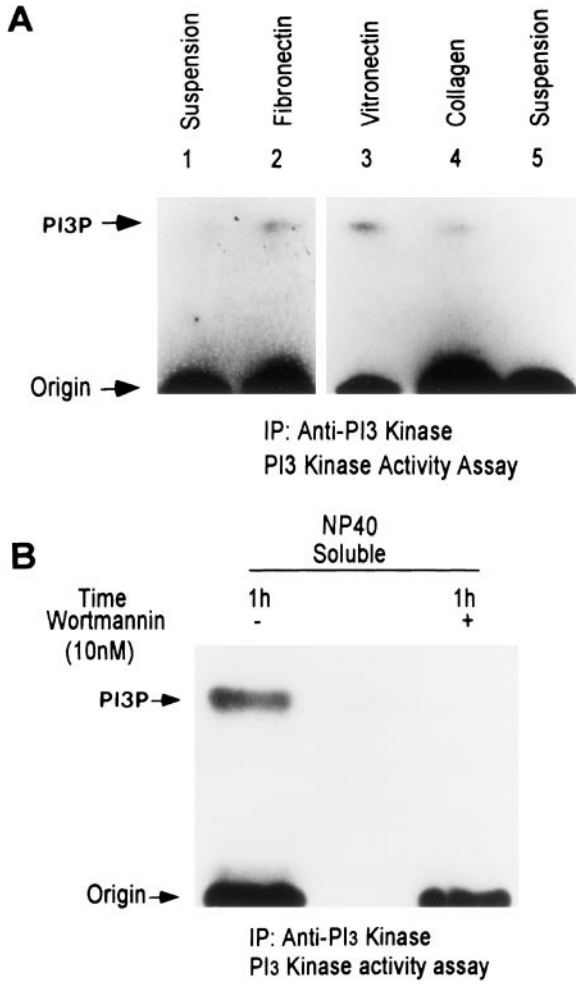

Fig. 3. Attachment of U-251MG cells increases PI3-K activity. U-251MG cells were harvested, washed, and lysed in 1% NP40 lysis buffer at 0 time (suspension), or after 1 h of adhesion to vitronectin, fibronectin, or collagen. Subsequently, equivalent μg of protein lysate were immunoprecipitated with anti-PI3-K IgG, and the immunoprecipitates subjected to a PI3-K activity assay, as described in the Materials and Methods. PI3-K activity, detected as PI3P production, was not detected in cells in suspension (**A**). PI3-K activity was maximal in cells adherent to vitronectin, and the least PI3-K activity was detected in cells adherent to collagen (A, arrow). To confirm that the product, PI3P, was due to PI3-K phosphorylation of the substrate, Wortmannin was preincubated with the cell monolayer prior to beginning the assay and no band was detected (**B**).

tion of U-251MG cells is differentially sensitive to PI3-K inhibitors depending on the matrix.

PI3-K Activity is Increased Upon U-251MG Cell Attachment

To determine whether PI3-K is activated upon adhesion to vitronectin, fibronectin, or collagen, PI3-K activity was assessed in cells adherent to these substrates for 1 h, and compared with the activity of cells in suspension. PI3-K activity, detected as PI3P production, was increased in adherent cells, as compared to that of cells in suspension in the absence of matrix protein (Fig. 3A). The intensity of the PI3P band on densitometric scanning was three-fold greater in cells adherent to vitronectin as compared to cells adherent to collagen (Fig. 3A). To confirm that this band represented PI3-K phosphorylation of the substrate, and thus PI3P formation, Wortmannin was preincubated with the cell monolayer prior to cell lysis and the PI3-K activity assay. Wortmannin inhibited PI3P production, indicating PI3-K phosphoryla-

tion of the substrate (Fig. 3B). These data indicate that PI3-K is activated in malignant astrocytoma cells upon cell attachment, and that vitronectin attachment results in a greater PI3-K activity as compared to collagen attachment.

Complex Formation of PI3-K and p125FAK in Cells Adherent to Vitronectin or Fibronectin, But Not in Cells Adherent to Collagen

PI3-K is thought to play a central role in signalling events, and there is evidence that PI3-K activation may require complex formation with p125FAK [Shears, 1992; Toker and Cantley, 1997; Chen and Guan, 1994]. To determine whether p125FAK complex formation with PI3-K occurs in cells adherent to vitronectin, fibronectin, or collagen, PI3-K was immunoprecipitated from the 1% NP40-soluble cell fraction of cells, and the immunoprecipitates western blotted with mAb anti-p125FAK. A complex of p125FAK and PI3-K was precipitable from cells adherent to vitronectin and fibronectin early in adhesion (1 h); however, by 19 h of adhesion the complex was undetectable in the NP40-soluble cell fraction of cells adherent to vitronectin and minimally detectable in the cells adherent to fibronectin (Fig. 4A,C, respectively). Stripping and reprobing of the membrane with mAb anti-phosphotyrosine (mAb PY20) resulted in detection of a 125-kDa band, indicating that the p125FAK co-immunoprecipitated with PI3-K is active (data not shown). In contrast, a complex of PI3-K and p125FAK was not detectable in the NP40-soluble cell fraction of cells adherent to collagen for 1 or 19 h (Fig. 4C). The difference in p125FAK associated with PI3-K when the cells are adherent to different substrates is not due to differing amounts of immunoprecipitated PI3-K, as stripping and reprobing of the membranes in Figure 4A and C

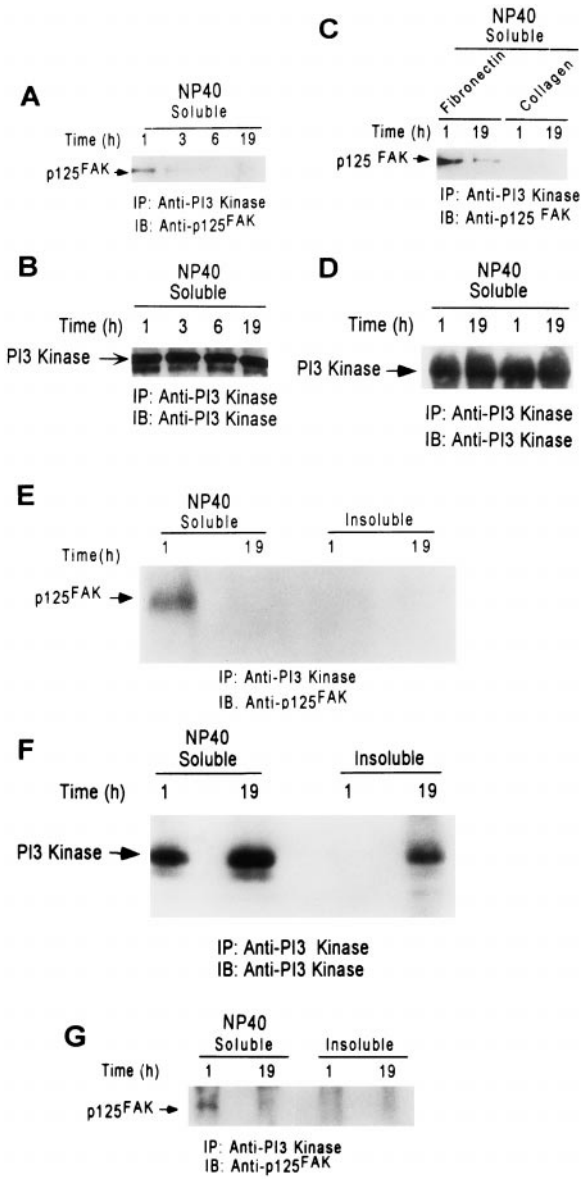

Fig. 4. PI3-K and p125FAK form a complex in U-251MG cells adherent to vitronectin and fibronectin, but not in cells adherent to collagen. U-251MG cells plated onto purified matrix proteins in serum-free media were lysed at various time points with 1% NP40 lysis buffer, equivalent µg of protein immunoprecipitated with anti-PI3-K IgG, the immunoprecipitates were subjected to SDS PAGE, and then Western blotted with mAb anti-p125FAK, or anti-PI3-K IgG, as described in the Materials and Methods. **A,B:** A complex of PI3-K and p125FAK was detected in the NP40-soluble cell fraction at 1 h of attachment to vitronectin, but not at 6 or 19 h (A; chemiluminescent exposure time 1 min). The membrane was stripped and reprobed with anti-PI3-K IgG and similar amounts of PI3-K protein were detected in each lane (B; chemiluminescent exposure time 1.5 min). **C,D:** A complex of PI3-K and p125FAK was detected in the NP40-soluble cell fraction of cells adherent to fibronectin for 1 h and much less complex was detected at 19 h; whereas no complex was detected in cells adherent to collagen for 1 or 19 h (C; chemiluminescent exposure time 1 min). The membrane was stripped and reprobed with anti-PI3-K IgG and similar amounts of PI3-K protein were detected in each lane (D; chemiluminescent exposure time 1.5 min). **E–G:** A complex of PI3-K and p125FAK was detected in the NP40-soluble cell fraction at 1 h of attachment to vitronectin (E; chemiluminescent exposure time 2 min) or fibronectin (G; chemiluminescent exposure time 1 min); after solubilization of the NP40-insoluble cell fraction (pellet) in RIPA buffer no complex was detected on either substrate. The membrane in E was stripped and reprobed with anti-PI3-K IgG and PI3-K protein was detected in the NP40-soluble cell fraction at 1 and 19 h, whereas, in the NP40-insoluble cell fractions PI3-K protein was only detected at 19 h (F; chemiluminescent exposure time 2 min).

with anti-PI3-K IgG demonstrated similar amounts of PI3-K protein in all lanes (Fig. 4B,D, respectively). After solubilization of the NP40-insoluble cell fraction (pellet) with RIPA buffer (1% desoxycholate detergent with 0.1% SDS), a complex of PI3-K and p125FAK could not be detected (Fig. 4E,G). This could be due to RIPA buffer disruption of the PI3-K and p125FAK interaction, as RIPA buffer is more chaotropic than 1% NP40. Stripping and reprobing of the membrane in Panel E with anti-PI3-K IgG demonstrated PI3-K protein in the NP40-soluble fractions at 1 and 19 h, whereas in the NP40-insoluble fractions PI3-K protein was only detected at 19 h (Fig. 4F). These data indicate that during adhesion of U-251MG cells to vitronectin or fibronectin, complex formation of p125FAK and PI3-K occurs in the NP40-soluble cell fraction during early adhesion.

PI3-K Activity and Protein is Distributed Between the NP40-Soluble and NP40-Insoluble Cell Fractions During Late Adhesion

PI3-K is thought to locate to the cytoskeleton (NP40-insoluble cell fraction) upon cytoskeletal organization [Toker and Cantley, 1997; Guinebault et al., 1995; Lakkakorpi et al., 1997; Hartwig et al., 1996]. To determine whether PI3-K activity redistributed entirely to the NP40-insoluble cell fraction late in adhesion and was thus unavailable to complex with p125FAK, we examined PI3-K activity in the NP40-soluble and the NP40-insoluble cell fractions, early and late in adhesion. The NP40-insoluble cell fraction (pellet) was solubilized in RIPA buffer. We found that at 19 h of adhesion to vitronectin, PI3-K activity, based on PI3P production, was distributed between the NP40-soluble and NP40-insoluble cell fractions (Fig. 5A). At 1 h of adhesion, PI3P was found in the NP40-soluble cell fraction (Fig. 5A), but could not be detected in the NP40-insoluble cell fraction (data not shown). The total amount of PI3-K protein in the NP40-soluble and the NP40-insoluble cell fractions at 1 h was similar to the combined PI3-K protein at 19 h in cells adherent to vitronectin (Fig. 5B). Nearly identical findings were detected in cells adherent to fibronectin (data not shown). Total PI3-K protein determined from cells adherent to vitronectin, fibronectin, or collagen and completely solubilized in RIPA buffer was found to be nearly identical at 1 and 19 h, independent of the substrate (Fig. 6A,B,

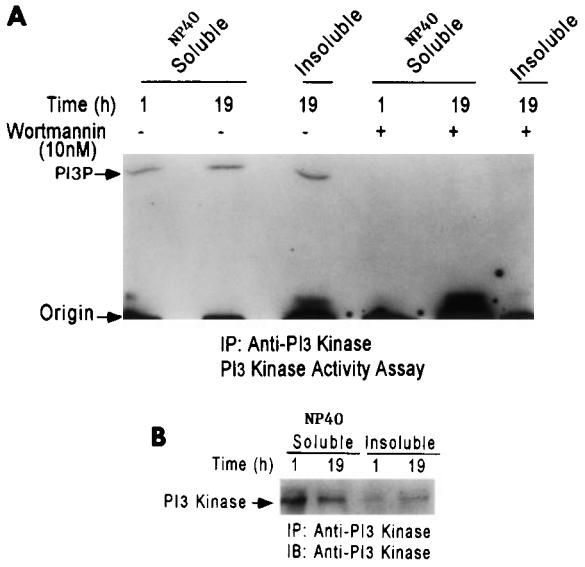

Fig. 5. PI3-K activity and protein are distributed between the NP40-soluble and the NP40-insoluble cell fractions during late adhesion of U-251MG cells to vitronectin. U-251MG cells plated onto purified vitronectin in serum-free media were lysed with 1% NP40 lysis buffer, equivalent µg of protein immunoprecipitated with anti-PI3-K IgG, and the immunoprecipitate subjected to a PI3-K activity assay (**A**) or to a Western blot analysis with anti-PI3-K IgG (**B**), as described in the Materials and Methods. The NP40-insoluble cell fraction (pellet) was solubilized in RIPA buffer, immunoprecipitated with anti-PI3-K IgG, and then subjected to the PI3-K activity assay (A) or subjected to Western blot analysis with anti-PI3-K IgG (B). PI3-K activity was detected as PI3P production in A (arrow), and found in both the NP40-soluble and insoluble cell fractions at 19 h of adhesion. The combined densitometric reading of PI3-K protein in the NP40-soluble and insoluble cell fractions at 1 h was similar to the combined densitometric reading of the NP40-soluble and insoluble cell fractions at 19 h (B).

and C, respectively). To determine whether p125FAK also redistributed to the NP40-insoluble cell fraction late in adhesion, p125FAK protein was Western blotted from the NP40-insoluble cell fraction at 1 and 19 h of adhesion to vitronectin or fibronectin after solubilization of the pellet in RIPA buffer (Fig. 7A and B, respectively). p125FAK was found in the NP40-insoluble cell fraction late in adhesion, indicating it also partially redistributes to the cytoskeleton late in adhesion (Fig. 7). These data indicate that during malignant astrocytoma cell adhesion, PI3-K and p125FAK partially redistribute to the cytoskeleton over time. Also, the data indicate that the lack of detectable p125FAK-PI3-K complex formation in the NP40-soluble cell fraction late in adhesion of cells to vitronectin cannot be attributed to an absence of PI3-K activity.

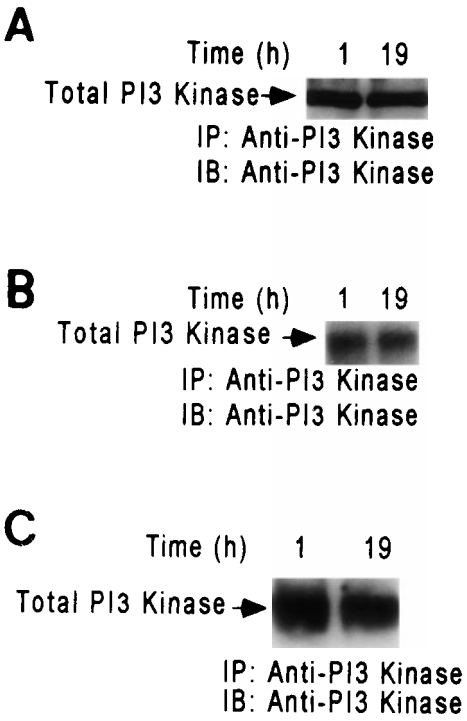

Fig. 6. Equivalent amounts of PI3-K protein in U-251MG cells adherent to vitronectin, fibronectin, or collagen at 1 and 19 h. U-251MG cells were plated onto purified vitronectin (**A**), fibronectin (**B**), or collagen (**C**) in serum-free media, and at 1 and 19 h the cells were completely lysed with RIPA buffer, equivalent µg of protein lysate immunoprecipitated with anti-PI3-K IgG, the immunoprecipitate subjected to SDS PAGE, and then western blotted with anti-PI3-K IgG, as described in the Materials and Methods. Similar amounts of PI3-K protein were detected at 1 and 19 h, independent of the substrate.

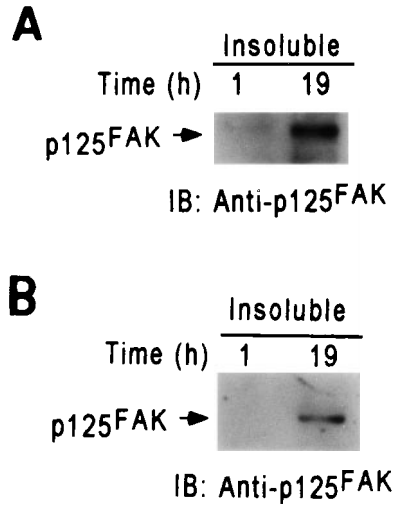

Fig. 7. p125FAK protein is detected in the NP40-insoluble cell fraction late in adhesion to vitronectin and fibronectin. U-251MG cells were plated onto purified vitronectin (**A**) or fibronectin (**B**) in serum-free media, the cells lysed with 1% NP40 lysis buffer, centrifuged, and the pellet (NP40-insoluble fraction) solubilized with RIPA buffer. Equivalent µg of protein lysate from the solubilized pellet was immunoprecipitated with mAb anti-p125FAK, the immunoprecipitates subjected to SDS PAGE, and then Western blotted with anti-p125FAK IgG, as described in the Materials and Methods. p125FAK protein was detected in the NP40-insoluble cell fraction at 19 h of adhesion to both vitronectin and fibronectin.

p125FAK Expression and Phosphorylation in Malignant Astrocytoma Cells Adherent to Vitronectin, Fibronectin, or Collagen

Other investigators have reported that p125FAK in Jurkat and H460 carcinoma cells, as well as in lymphoid cells transfected with p125FAK, is cleaved by caspase 3, and probably caspase 6, to generate 90- and 30-kDa protein fragments [Wen et al., 1997; Gervais et al., 1998]. To determine whether the absence of p125FAK and PI3-K complex formation in cells adherent to collagen could be due to caspase cleavage of p125FAK, the levels of p125FAK protein and phosphorylation were evaluated in the presence of caspase-specific inhibitors. U-251MG cells adherent to the various substrates for 1 h in the presence of the caspase 3 inhibitor DEVD-CHO, or the control caspase 1 (ICE) inhibitor YVAD-CHO, were lysed and Western blotted with mAb or rabbit anti-125FAK IgG (Fig. 8A, and B, respectively). The protein expression of p125FAK was similar in

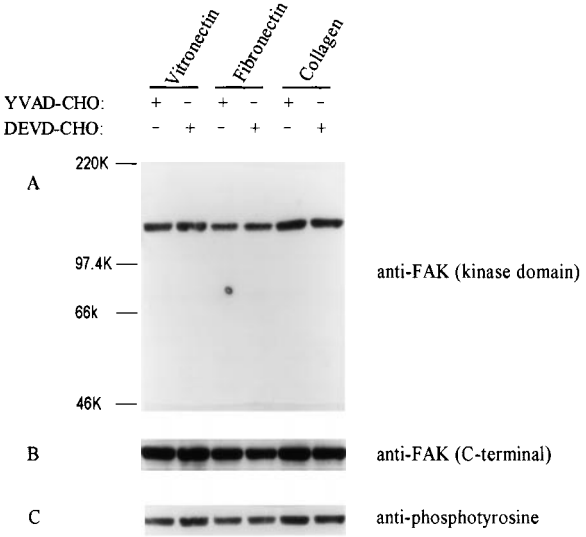

Fig. 8. p125FAK protein expression and phosphorylation in cells adherent to vitronectin, fibronectin, or collagen. U-251MG cells were treated with 200 µM of the caspase 1 (ICE; YVAD-CHO) or the caspase 3 (DEVD-CHO) inhibitor for 1 h, lysed, and subjected to Western blot analysis with mAb anti-p125FAK IgG (**A**), rabbit anti-p125FAK IgG (**B**), or mAb anti-phosphotyrosine IgG (**C**). A 125-kDa band of similar intensity was detected on each blot, independent of the caspase 3 inhibitor. No degradation products, consistent with caspase 3 cleavage, were detected (A).

cells adherent to all three substrates, independent of the caspase 3 inhibitor, indicating the absence of detectable caspase 3 cleavage of p125FAK in cells adherent to vitronectin, fibronectin, or collagen for 1 h. In addition, the level of p125FAK phosphorylation was similar when the cells were adherent to vitronectin, fibronectin, or collagen for 1 h (Fig. 8C). These experiments were repeated when the cells were adherent to vitronectin, fibronectin, or collagen for 19 h, and the results were identical (data not shown). These data indicate that p125FAK is not cleaved by caspase 3 in U-251MG cells adherent to collagen for 1 or 19 h. Thus, the lack of p125FAK and PI3-K complex formation in malignant astrocytoma cells adherent to collagen is not due to caspase 3 cleavage of p125FAK.

DISCUSSION

In previous work we have reported that integrins αvβ3 and αvβ5 mediate vitronectin-directed attachment and migration of the U-251MG human malignant astrocytoma cell line [Gladson and Cheresh, 1991; Gladson et al., 1995]. In addition, we have shown that in vivo, malignant astrocytoma cells synthesize vitronectin and that expression of integrins αvβ3 and αvβ5 marks the malignant astrocyte phenotype [Gladson and Cheresh, 1991; Gladson et al., 1995]. To understand the signals generated by vitronectin-attachment of these cells, we investigated the role of PI3-K, a centrally-located signaling molecule. In this report, we demonstrate that PI3-K activity is necessary for human malignant astrocytoma cell attachment to and migration toward multiple substrates; however, the sensitivity to PI3-K inhibitors varies considerably according to the substrate. Furthermore, we found that PI3-K activity was increased upon cell adhesion, as compared to cells in suspension, and complex formation of PI3-K and p125FAK was found in cells attached to those matrix proteins in which U-251MG cell attachment and migration exhibited sensitivity to PI3-K inhibitors.

Our findings regarding the requirement for PI3-K activity in malignant astrocytoma cell attachment utilized two specific inhibitors of PI3-K activity, Wortmannin and LY294002. Several other investigators have reported previously that β1 and β2 integrin-mediated adhesion of T lymphocytes, macrophages, and HL-60 myelomonocytic cells requires PI3-K activity [Zell et al., 1996; Nielsen et al., 1996; Shimizu et al., 1995; Meng et al., 1998; Nagel et al., 1998]. In contrast, other investigators who have examined the role of PI3-K in carcinoma cell attachment to fibronectin and laminin have reported that attachment is not inhibited by Wortmannin and LY294002 [Shaw et al., 1997; Klemke et al., 1998; Li et al., 1998]. Further evidence that there is cell-type specificity in the requirement for PI3-K in cell attachment, is provided by the report that osteoclasts require PI3-K for attachment to bone whereas osteoblasts do not [Lakkakorpi et al., 1997]. The inhibition found in this report is unlikely to be due to nonspecific effects of these inhibitors, as we found nearly 50% inhibition of attachment to vitronectin and fibronectin utilizing Wortmannin and LY294002 at two- and one-fold the IC_{50}, respectively. We have reported previously that collagen attachment of U-251MG human malignant astrocytoma cells is mediated by integrins α2β1 and α3β1, and that collagen attachment is independent of the integrins used for vitronectin, fibronectin, and laminin attachment [Pijuan-Thompson and Gladson, 1997]. Thus, the differential sensitivity to PI3-K inhibitors of the U-251MG cells when attached to various substrates suggests that there may be substrate-specific and probably integrin-specific signaling of PI3-K in these cells. There is a precedent for integrin-specific signalling of PI3-K, as Shaw et al. [1997] reported previously that β1 and β4 integrins differentially signal PI3-K in colon carcinoma cells. Thus, PI3-K plays a role in cell attachment of some, but not all, cell types, and this role is further limited by the specific matrix.

We also found that U-251MG human malignant astrocytoma cell migration toward vitronectin and fibronectin, as opposed to collagen-directed migration, was sensitive to PI3-K inhibitors. Other investigators have shown a requirement for PI3-K activity in carcinoma cell motility toward fibronectin and vitronectin [Klemke et al., 1998]. Collagen-directed migration maybe uniquely independent of PI3-K activity.

Our findings are not due to decreased numbers of cells attaching to or migrating toward collagen; although the numbers of cells attaching to or migrating toward collagen were lower than when vitronectin was used as the substrate, the numbers were higher than when fibronectin was used as the substrate. Also, these differences are not due to fewer collagen integrin receptors on the cell surface, as inte-

grin α3β1, a collagen receptor mediating in part collagen attachment of these cells, is expressed at greater numbers on these cells than integrins αvβ3, αvβ5, α5β1, or α6β1, based on mean fluorescent intensity on FACS analysis [Pijuan-Thompson and Gladson, 1997].

In parallel with the PI3-K inhibitor studies of cell attachment and migration, we examined PI3-K activity in cells adherent to vitronectin, fibronectin, or collagen for 1 h, and found that PI3-K activity was increased upon attachment to all three substrates; however, less PI3-K was activated in cells adherent to collagen, as compared to cells adherent to vitronectin. In addition, when cells were lysed in suspension, PI3-K activity was not detectable. Other investigators have shown that the PI3-K activity of NIH3T3 cells increases with cell attachment, as compared to cells in suspension [Chen and Guan, 1994], and that attachment of colon carcinoma cells that over-expressed integrin α6β4, as compared to those with low levels of integrin α6β4 expression, also was associated with higher levels of PI3-K activity [Shaw et al., 1997]. Thus, our data indicate that, in human malignant astrocytoma cells, integrin-mediated cell adhesion results in activation of PI3-K.

PI3-K may be activated by more than one mechanism. We found that complex formation of PI3-K and p125FAK correlated with increased sensitivity of cell attachment and migration to PI3-K inhibitors. The binding of PI3-K to p125FAK may be necessary for PI3-K activation in certain instances, as another investigator has shown that in NIH3T3 cells p125FAK complex formation with PI3-K is necessary for phosphorylation and activation of PI3-K by p125FAK [Chen and Guan, 1994]. Certain growth factors, such as platelet-derived growth factor (PDGF), bind and activate tyrosine kinase growth factor receptors, resulting in the phosphorylation and activation of PI3-K [Domin et al., 1996]. The low levels of PI3-K activity generated during collagen attachment of malignant astrocytoma cells, could be due to PI3-K activation by another mechanism that is independent of p125FAK. For example, autocrine PDGF has been reported to be synthesized by these cells [Harsh et al., 1990], and this may provide an alternative mechanism for PI3-K activation. Since other investigators have shown that PI3-K is activated upon cell adhesion but that it is not required for cell adhesion of colon carcinoma cells, this suggests that PI3-K serves different functions in different cells.

The distribution of PI3-K over time in the U-251MG cells adherent to vitronectin or fibronectin indicates that PI3-K partially redistributes to the NP40-insoluble cell fraction with cytoskeletal organization. This should not be surprising, as other investigators have shown that PI3-K becomes associated with the cytoskeleton after platelet activation and osteoclast attachment [Toker and Cantley, 1997; Guinebault et al., 1995; Lakkakorpi et al., 1997; Hartwig et al., 1996]. The fact that a complex of p125FAK and PI3-K can no longer be detected in the NP40-soluble cell fraction late in adhesion, despite the presence of p125FAK protein and PI3-K activity, suggests that the subset(s) of p125FAK and PI3-K remaining in the NP40-soluble cell fraction serve a different function, associate with a different affinity, or that the kinetics no longer favor association. In support of the hypothesis that p125FAK may serve multiple functions in the cell, Polte and Hanks [1997] have shown that different subsets (pools) of p125FAK exist in the cell. Thus, our data indicate that p125FAK and PI3-K form a complex detectable in the NP40-soluble cell fraction in cells attaching to vitronectin or fibronectin, and that with cytoskeletal organization a subset of PI3-K and p125FAK redistributes to the NP40-insoluble cell fraction.

The role of vitronectin and collagen in these tumors in vivo is quite different. We have reported previously [Gladson and Cheresh, 1991], along with other investigators [McComb and Bigner, 1985; Chintala et al., 1996], that collagen expression in malignant astrocytomas is largely limited to the perivascular mesenchymal tissue and to the pial-glial membrane. In contrast, malignant astrocytoma cells synthesize vitronectin in vivo, and it is tumor cell-associated [Gladson et al., 1995]. Tumor cell synthesis of vitronectin and the resultant tumor cell attachment to vitronectin, probably results in increased PI3-K activation and signalling, pointing to extracellular matrix control of cell signalling.

In summary, we have shown that U-251MG human malignant astrocytoma cell attachment and haptotactic migration toward various substrates is PI3-K-dependent; however, the sensitivity of these cells to PI3-K inhibitors is dependent on the substrate. As the sensitivity to PI3-K inhibitors correlates with complex forma-

tion of PI3-K and p125FAK, our data suggest a role for PI3-K and p125FAK complex formation in PI3-K activation early in adhesion. These studies also suggest that the lack of PI3-K involvement in collagen-directed migration may provide a useful tool for dissection of the role of PI3-K in migration toward other substrates.

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