The tumor suppressor PTEN is a phosphatase with sequence homology to tensin. PTEN dephosphorylates phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$) and focal adhesion kinase (FAK), and it can inhibit cell growth, invasion, migration, and focal adhesions. We investigated molecular interactions of PTEN and FAK in glioblastoma and breast cancer cells lacking PTEN. The PTEN trapping mutant D92A bound wild-type FAK, requiring FAK autophosphorylation site Tyr$^{397}$. In PTEN-mutated cancer cells, FAK phosphorylation was retained even in suspension after detachment from extracellular matrix, accompanied by enhanced PI 3-K association with FAK and sustained PI 3-K activity, PIP$_3$ levels, and Akt phosphorylation; expression of exogenous PTEN suppressed all five properties. PTEN-mutated cells were resistant to apoptosis in suspension, but most of the cells entered apoptosis after expression of exogenous PTEN or wortmannin treatment. Moreover, overexpression of FAK in PTEN-transfected cells reversed the decreased FAK phosphorylation and PI 3-K activity, and it partially rescued PIP$_3$ levels, Akt phosphorylation, and PTEN-induced apoptosis. Our results show that FAK Tyr$^{397}$ is important in PTEN interactions with FAK, that PTEN regulates FAK phosphorylation and molecular associations after detachment from matrix, and that PTEN negatively regulates the extracellular matrix-dependent PI 3-K/Akt cell survival pathway in a process that can include FAK.

PTEN (phosphatase and tensin homologue deleted on chromosome 10, also called MMAC1 or TEP1) is a tumor suppressor gene identified on human chromosome 10q23 (1–3). PTEN is frequently deleted or mutated in a wide range of human cancers, including glioblastoma (4), melanoma (5), and prostate (6), breast (7), and endometrial cancers (8). Germ line PTEN mutations are present in patients with Cowden disease and Bannayan-Zonana syndrome (9, 10). Besides functioning as a tumor suppressor, PTEN is also essential for embryonic development (11–13).

Domains of PTEN share a high degree of homology with the family of protein-tyrosine phosphatases and the cytoskeletal protein tensin (1, 2). PTEN functions as a dual specificity phosphatase and lipid phosphatase in vitro (14, 15). Specific substrates include phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$) and focal adhesion kinase (FAK) (16–18). Many tumor-associated missense mutations cluster around the phosphatase domain, and most remaining mutations are predicted to truncate the protein due to nonsense or frameshift mutations (1, 2, 19), suggesting that the phosphatase activity of PTEN plays important roles in PTEN function. In fact, suppression of cell growth (20), focal adhesion formation (18), and cell migration and invasion (21) in PTEN-deficient glioblastoma cells by PTEN cDNA expression requires a functional phosphatase catalytic domain.

The cellular mechanisms of PTEN function are still not completely understood. Recent evidence demonstrates the ability of PTEN to directly dephosphorylate position D3 of PIP$_3$, a product of PI 3-K (16). In PTEN-mutated glioblastoma cells and mouse embryonic fibroblasts, the activity of Akt (also called protein kinase B) is constitutively elevated (17, 22). Akt is a survival-promoting serine-threonine protein kinase regulated by PIP$_3$ that is implicated in survival signaling in a wide variety of cells, including fibroblastic, epithelial, and neuronal cells (23). PTEN increases sensitivity to cell death in response to several apoptotic stimuli, including UV irradiation and treatment with tumor necrosis factor $\alpha$, by negatively regulating the PI 3-K/Akt pathway (17). In addition to its role in regulating the PI 3-K/Akt cell survival pathway, PTEN also inhibits growth factor-induced Shc phosphorylation and suppresses the mitogen-activated protein (MAP) kinase signaling pathway (24), suggesting that PTEN has roles in independent signaling pathways.

PTEN also interacts with FAK, a key molecule implicated in integrin signaling pathways, and it directly dephosphorylates tyrosine-phosphorylated FAK (18). The activation of integrins by cell binding to extracellular matrix leads to increases in FAK tyrosine phosphorylation levels and enhances kinase activity (25–29). Activation of FAK leads to its association with several kinases, signal transduction molecules, and cytoskel-
et al proteins including PI 3-K, Src, Grb2, and paxillin. Binding is mediated by specific tyrosine-phosphorylated residues within FAK and is followed by activation of downstream signaling pathways including extracellular signal-regulated kinase/MAP kinase and PI 3-K/Akt survival pathways (30–35). In fact, the integrin-mediated MAP kinase signaling pathway is also suppressed by PTEN (24).

Many mammalian cell types are dependent on adhesion to the extracellular matrix for their continued survival. When the signals from matrix are interrupted, normal cells may undergo apoptosis in a process termed anoikis (36). In contrast, the ability of malignant cells to proliferate in the absence of adhesion, termed anchorage independence of growth, correlates closely with tumorigenicity. In a study published while this paper was under review, Davies et al. (37) reported that PTEN expression in a cell line lacking PTEN increases the rate of apoptosis approximately 2-fold both before and especially after detachment from extracellular matrix. Another recent study reported that PTEN overexpression in human breast cancer cells induces apoptosis, even while the cells were substrate-attached and regardless of the presence of endogenous PTEN, and Akt was identified as a key molecule in this effect (38).

Other studies have implicated FAK in the general process of anoikis, i.e., apoptosis after loss of matrix interactions. Inhibition of FAK activity in fibroblasts or attenuation of FAK expression in tumor cells leads to apoptosis (39, 40). Constitutively activated FAK protects Madin-Darby canine kidney cells from apoptosis caused by loss of matrix contact, and Tyr397 of FAK is required for this effect (41). Association of the p85 subunit of PI 3-K with Tyr397 in FAK is induced by the attachment of cells to matrix (42, 43). PI 3-K is required for integrin-stimulated Akt activation (44). These results provide evidence that FAK is an important mediator of integrin-mediated survival signals upstream of the PI 3-K/Akt cell survival pathway. Several studies have also established that levels of FAK expression are often increased in proliferating cells or advanced cancers (45–47).

In the present study, we have investigated further the interactions between PTEN and FAK in trying to determine whether PTEN dephosphorylation of FAK is involved in processes related to cancer progression. Our results suggest that the major autophosphorylation site of FAK (Y397) is responsible for the initial in vivo association of PTEN with FAK, a prerequisite for FAK dephosphorylation by PTEN. A trapping mutant of PTEN (D92A) competed for the binding of Src and PI 3-K, which also bind to Tyr397 of FAK, without effects on binding to other sites. In order to explore PTEN signaling pathways, we also tested whether FAK dephosphorylation by PTEN was associated with effects on PI 3-K and downstream Akt cell survival signaling. In PTEN-mutated cancer cells, FAK phosphorylation was retained even in the absence of extracellular matrix contact, accompanied by sustained PI 3-K binding to FAK, activity of PI 3-K, levels of PIP3, and phosphorylation of Akt. PTEN-mutated cells were markedly resistant to apoptosis triggered by detachment from extracellular matrix. Expression of exogenous PTEN in PTEN-mutated cells inhibited FAK phosphorylation, and it restored a normal pattern of FAK/PI 3-K association, PI 3-K activity, PIP3 levels, Akt phosphorylation, and apoptosis in response to detachment from matrix. Furthermore, overexpression of FAK could effectively inhibit these effects of PTEN on PI 3-K activity and partially inhibited its effects on PIP3 levels, Akt phosphorylation, and apoptosis. Our results suggest that PTEN interactions with FAK may lead to inhibition of the PI 3-K/Akt cell survival pathway in parallel with its direct effects on PIP3, thereby promoting apoptosis in response to detachment from matrix.
phoresis, and analyzed by Western blotting with RC20 (1:2500), HA, GFP (1:1000), c-Src (2 μg/ml), PI 3-K (1:5000), pacllin (1:10,000), Grb2 (1:1000), FAK (1:1000), Akt (1:1000), phospho-Akt (1:1000), JNK (1:1000), or phospho-JNK (1:1000) antibodies using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

PI 3-K and PIP2 Assays—Determination of PIP2 in vivo was carried out as described (50). Briefly, after selecting transfected cells using puromycin, 1 × 105 cells were labeled with [32P]PO4 (500 μCi/ml) for 1 h. Cells were suspended by trypsinization and then either plated on fibronectin-coated dishes for 1 h in 10% serum-containing medium or incubated for an additional 1 h in suspension in the same serum-containing medium. Phospholipids were extracted from these infected or suspended cells as described (50). For examining plated-cell-derived growth factor (PDGF) stimulation, cells were serum-starved for 24 h before labeling with [32P]PO4 and then stimulated with 10 ng/ml PDGF-BB (Sigma) for 10 min. PIP2 levels were determined by using TLC (silica gel 60, EM Science, Gibbstown, NJ) and autoradiography (50). PI 3-K assays were performed as described (51). PI 3-K proteins were immunoprecipitated using a polyclonal anti-PI 3-K antibody (Upstate Biotechnology) and incubated with 20 μg of phosphatidylinositol 4,5-bisphosphate (Sigma) and [γ-32P]ATP (30 μCi) in kinase buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1 mM sodium orthovanadate, 20 mM MgCl2) for 10 min at 37 °C. Immunoprecipitates were removed by centrifugation, the supernatant solutions were extracted with chloroform, and aliquots were applied to TLC plates (51). Tyrosine phosphorylation of the p85 subunit of PI 3-K was measured by immunoprecipitating total tyrosine-phosphorylated proteins using monoclonal antibody 4G10 and then immunoblotting with anti-p85 PI 3-K monoclonal antibody (Transduction Laboratories).

Akt Phosphorylation Assay—Phosphorylation of Akt was evaluated using anti-Phospho-Akt antibodies as described (24). Transfected cells were selected using puromycin as described above. After attachment to fibronectin or detachment, cells were lysed with cold PBS and homogenized in the 1% Nonidet P-40 lysis buffer as described above. The cell homogenates were subjected to SDS-PAGE and immunoblotted with anti-phospho-Akt or total Akt antibodies.

Jun Kinase Assay—Phosphorylation of JNK was evaluated using anti-phospho-JNK antibodies as described (24). A plasmid containing HA-JNK1 was co-transfected with the control GFP vector or wild type PTEN-GFP, and the cells were used for assays 24 h after transfection. Subconfluent cells were washed once with PBS and then exposed to UV light (200 mJ/cm2) using a Stratalinker UV cross-linker (Stratagene) followed by incubation for 1 h at 37 °C to induce JNK. Cells were lysed in Nonidet P-40 as described above.

Analysis of Cell Death—After puromycin selection for transfected cells, cells were harvested by trypsinization and plated on 10 μg/ml fibronectin-coated glass coverslips in 10% serum-containing medium for 2 h. Cells were then detached with trypsin and cultured in suspension in the presence of 10% serum-containing medium for the indicated times to ensure apoptosis. Air-dried, and fixed on glass coverslips in 4% paraformaldehyde/PBS for 30 min, incubated with blocking solution (0.3% horse serum in methanol) for 30 min, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. In situ detection of cells undergoing apoptosis was performed using a TdT-mediated dUTP nick end labeling (TUNEL) assay (Roche Molecular Biochemicals) according to the manufacturer’s protocol and visualized by DAB solution (Roche Molecular Biochemicals). As a control for nonspecific staining, the reaction mixture was incubated in parallel without enzyme.

Anti-IL-2R Bead Clustering Experiments—M-450 sheep anti-mouse IgG Dynabeads (Dynal, Great Neck, NY) were coated with anti-IL-2R monoclonal antibody according to the manufacturer’s protocol. NIH 3T3 cells were cotransfected with IL-2R-FAK (3 μg) and GFP-tagged PTEN D92A (10 μg) and used for assays 24 h after transfection. Cells (1 × 105 cells) were detached by trypsinization, recovered in 10% serum-containing medium for 2 h, and then HA-FAK was immunoprecipitated (IP) and immunoblotted for phosphotyrosine (pY), HA, and GFP. The whole cell lysates were immunoblotted for GFP to confirm the expression of similar amounts of GFP-containing proteins. C, visualization of in vivo interaction of IL-2R-FAK and GFP-PTEN in NIH 3T3 cells. The spatial association of GFP-PTEN with IL-2R-FAK clustered using anti-IL-2R antibody-coated beads was as described under “Experimental Procedures.” Each inset shows a higher magnification view. The arrowhead and arrows indicate corresponding locations of several arbitrarily chosen beads as visualized by GFP fluorescence or phase contrast microscopy.

FIG. 1. Interaction of PTEN with FAK in cells expressing mutant FAK molecules. A, HA-FAK mutants and IL-2R-FAK construct. Wild-type (WT) or mutant FAKs were fused to HA or to IL-2R from the N terminus through Trp359 as indicated at the end of the transmembrane (TM) domain. The tyrosine (Y) residues indicated were each mutated to phenylalanine (F), proline-rich domain; FAT, focal adhesion targeting domain. B, co-immunoprecipitation of GFP-PTEN (D92A) and HA-FAK. C, visualization of in vivo interaction of IL-2R-FAK and GFP-PTEN in NIH 3T3 cells. The spatial association of GFP-PTEN with IL-2R-FAK clustered using anti-IL-2R antibody-coated beads was as described under “Experimental Procedures.” Each inset shows a higher magnification view. The arrowheads and arrows indicate corresponding locations of several arbitrarily chosen beads as visualized by GFP fluorescence or phase contrast microscopy.

subsequently measured by FACS® (Becton Dickinson, San Jose, CA).

RESULTS

Tyrosine in FAK Is Required for Association with PTEN—We have recently reported that PTEN directly interacts with FAK and reduces its tyrosine phosphorylation, resulting in inhibition of cell migration, spreading, and focal adhesions (18). In order to investigate tyrosine residue is involved in the association of PTEN and FAK, six FAK mutants were constructed in which a tyrosine residue was replaced by phenylalanine (Fig. 1A). Integrin-stimulated FAK tyrosine phosphorylation occurs at six or more sites in vivo (25). Tyr397 is tyrosine-phosphorylated upon integrin clustering, and it is a critical residue for FAK activation by recruiting Src family protein kinases and PI 3-K to Tyr397 in FAK, resulting in the phosphorylation of other tyrosine residues in FAK and full catalytic activation. As shown in Fig. 1B, substitution of phenylalanine at Tyr397 caused a marked decrease in FAK phos-
phorylation, while other mutations showed little effects on FAK phosphorylation as described (32). We tested for physical interactions of PTEN with FAK in living cells using a “trapping” mutant of PTEN (18). Although PTEN D92A co-immunoprecipitated with wild-type and five mutant FAK molecules, only the Y397F mutant of FAK showed little or no association with PTEN (Fig. 1B). Expression levels of the HA-FAK mutants and of GFP-PTEN were similar (Fig. 1B). Wild-type PTEN could only weakly bind to FAK compared with PTEN D92A (18), and no association of GFP with HA-FAK could be seen in cells transfected with the control GFP plasmid (data not shown).

We also confirmed the interaction between FAK and PTEN by microscopy using a chimeric IL-2R system (48) (Fig. 1A and C). FAK was expressed as a fusion protein anchored to the plasma membrane by the transmembrane and extracellular portions of the IL-2R α subunit by transient transfection of NIH 3T3 cells. Beads coated with anti-IL-2R antibody could induce transmembrane aggregation of GFP-PTEN Y925F around the beads in IL-2R-FAK wild-type co-transfected cells (Fig. 1C). Although this recruitment of GFP-PTEN to the beads by IL-2R-FAK could be also observed in IL-2R-FAK Y925F-transfected cells, it could not be seen in either IL-2R-FAK Y397F- or IL-2R-FAK Y925F-transfected cells (Fig. 1C). In cells transfected with only GFP-PTEN D92A, beads could not bind to the cells (Fig. 1C). Aggregation around the beads detected using GFP was not observed in cells transfected with HA-FAK wild-type or GFP-PTEN wild-type (data not shown). These data indicate that Tyr397 in FAK is required for association of the PTEN trapping mutant with FAK.

**Effects of PTEN on FAK Dephosphorylation after Matrix Detachment**—The PTEN-mutated glioblastoma cell line U-87MG and PTEN−/− embryonic stem cells are able to grow in an anchorage-independent manner. PTEN restoration in these cells causes suppression of this property (11, 52). We investigated FAK phosphorylation in U-87MG cells in suspension, because FAK plays important roles in matrix-dependent cell survival. As shown in Fig. 2A, in PTEN-mutated U-87MG cells, FAK phosphorylation was retained even after detachment from the matrix substrate and incubation of cells in suspension in serum-containing medium. This FAK phosphorylation persisted for more than 8 h in suspension (Fig. 3A). In cells expressing wild-type PTEN, levels of FAK phosphorylation were decreased compared with control and PTEN D92A cells as described (18), and the pattern of FAK phosphorylation became concordant with the normal pattern in cells with intact PTEN. Specifically, FAK was rapidly dephosphorylated after the loss of attachment (Fig. 2A), and very little tyrosine phosphorylation could be seen after an 8-h incubation in suspension (Fig. 3A). On the other hand, cells transfected with mutated PTEN D92A showed little dephosphorylation of FAK (Fig. 2A). As shown in Fig. 1A, PTEN D92A could be co-immunoprecipitated with HA-FAK even after cell detachment from the substrate (Fig. 2B).

**Effects of PTEN Interaction with FAK on Other FAK-binding Molecules**—Next, we examined the association of signaling molecules or a cytokeskeletal protein bound to activated FAK. In control U-87MG cells, c-Src, the p85 subunit of PI 3-K, paxillin, and Grb2 were all co-immunoprecipitated with FAK, as expected for FAK molecules with sustained phosphorylation (Fig. 2C). In contrast, all of these molecules were dissociated from FAK, and FAK was dephosphorylated after detachment from matrix in wild-type PTEN-expressing cells. Interestingly, in cells expressing PTEN D92A, both paxillin and Grb2, which bind to the C terminus of FAK and Y925 FAK, respectively (26), bound to FAK to levels similar to those of control cells. In contrast, neither c-Src nor PI 3-K, which bind to Tyr397 in FAK (26), could bind to FAK in cells expressing PTEN D92A (Fig. 2C). These findings suggest that enhanced binding of the PTEN trapping mutant D92A to FAK competes with the binding of these molecules to Tyr397 in FAK. These results support the notion that Tyr397 in FAK is required for the initial association of PTEN with FAK at a site that is also important for binding of other proteins.

**PTEN Inhibition of PI 3-K Association with FAK Is Correlated with Downstream Akt Phosphorylation**—PTEN inhibition of FAK may result in suppression of downstream signaling events, e.g. integrin-mediated MAP kinase signaling (24) and cell invasion and migration (18, 21) induced by p130Cas, a downstream effector of FAK (53). Because PTEN could affect association of molecules bound to Tyr397 in FAK, we next examined the downstream PI 3-K/Akt signaling pathway. U-87MG cells were co-transfected with GFP-PTEN (wild type), a GFP-PTEN phosphatase-inactivating mutant (C124A), or GFP without insert together with a plasmid encoding a puromycin resistance gene in order to select cells co-expressing the expression plasmids. After selection with puromycin for 2 days, cells were analyzed for PTEN expression by immunoblotting for GFP (Fig. 3A). Expression was also confirmed by determining the percentage of cells expressing GFP by fluorescence microscopy. The percentage of cells that were GFP-positive after co-transfection with GFP, GFP-PTEN (wild type), or GFP-PTEN (C124A) was 95 ± 2, 91 ± 4, and 89 ± 4%, respectively (mean ± S.E. of four independent experiments). In PTEN-mutated U-87MG cells, FAK phosphorylation and the physical association of PI 3-K with FAK were sustained for at least 8 h.
in suspension culture in serum-containing medium. In contrast, both FAK phosphorylation and PI 3-K binding were suppressed when cells were transfected with wild-type PTEN (Fig. 3A). The phosphatase-inactivated mutant PTEN C124A had no effects on FAK and PI 3-K.

We next examined phosphorylation of Akt. Akt is activated by phospholipids (PIP_3) and/or PIP_2, which are downstream products of PI 3-K. Mechanisms include binding and activation loop phosphorylation at Thr^308 by phosphatidylinositol-dependent protein kinase 1 (PDK1) and also within the C terminus at Ser^473 by PDK2 in response to growth factor and integrin stimulation (44, 54–56). In adherent PTEN-mutated cells, Akt was highly phosphorylated compared with PTEN-expressing cells, consistent with recent reports that Akt activity is constitutively elevated in PTEN-deficient mouse embryonic fibroblasts (17) and PTEN-mutated tumor cells (12). Akt remained phosphorylated even after cells were maintained in suspension for 8 h (Fig. 3B). After transfection of PTEN, interestingly, Akt phosphorylation levels decreased markedly in response to detachment from matrix, along with decreased FAK phosphorylation and PI 3-K association with FAK (Fig. 3B). As shown in Fig. 3C, the levels of FAK phosphorylation, association of PI 3-K with FAK, and Akt phosphorylation were closely correlated before or after cells lose attachment to matrix. These findings suggested that decreased FAK phosphorylation by PTEN in cells in suspension might contribute to Akt down-regulation through the dissociation of PI 3-K and FAK, in addition to the known effects of PTEN on PIP_3 and Akt.

Direct Control of PI 3-K Activity and PIP_3 Levels by PTEN—We next investigated whether the altered interactions of FAK with PI 3-K might regulate PI 3-K activity and lead to increased levels of PIP_3. PI 3-K was immunoprecipitated from cells attached to fibronectin or cells in suspension, and the ability of PI 3-K to phosphorylate phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate was assayed. As shown in Fig. 4A, PI 3-K activity was retained even after detachment of PTEN-mutated cells from the matrix substrate and incubation in suspension, consistent with the sustained association of FAK with PI 3-K (Figs. 2 and 3). In contrast, PI 3-K activity was decreased in PTEN-expressing cells on fibronectin; notably, it was further decreased in response to detachment from fibronectin (Fig. 4A). FAK overexpression could increase PI 3-K activities in control cells, and it rescued this PTEN-induced PI 3-K down-regulation (Fig. 4A). These results were confirmed by examining tyrosine phosphorylation of the p85 subunit of PI 3-K, which is generally associated with activation (e.g. see Ref. 57). Tyrosine phosphorylation of PI 3-K p85 in PTEN-expressing cells was decreased and rapidly down-regulated after detachment from fibronectin (Fig. 4B). FAK overexpression rescued PTEN inhibition of PI 3-K p85 phosphorylation. These results indicate that association of FAK and PI 3-K is associated with PI 3-K activities and that FAK activation can enhance PI 3-K activity.

We next measured levels of PIP_3, a downstream product of PI 3-K. PIP_3 levels in control cells were also retained after detachment from the substrate (Fig. 4C), consistent with the retention of PI 3-K activity. In PTEN-expressing cells, PIP_3 levels were down-regulated when cells were plated on fibronectin and showed rapid further decreases after detachment. FAK overexpression in control cells could enhance PIP_3 levels as predicted by the PI 3-K assay. Interestingly, however, although PIP_3 levels were also increased in cells co-transfected with PTEN and FAK, PIP_3 levels were only partially rescued (~50% of control) by FAK overexpression although PI 3-K activity was high (Fig. 4C). This finding that complete rescue of PI 3-K by FAK overexpression is not sufficient for total recovery of PIP_3 levels suggests dual PTEN target sites in the FAK/PI 3-K/PIP_3 pathway: PTEN dephosphorylation of both FAK and PIP_3.

We also examined a different signaling pathway that leads to PI 3-K activation. There was no significant change in tyrosine phosphorylation of the p85 subunit of PI 3-K (Fig. 4D) and PIP_3 levels (Fig. 4E) after PDGF stimulation, which is consistent with the previous report that insulin-stimulated PI 3-K activity is not affected by PTEN expression (16) and that insulin or PDGF can similarly stimulate Akt regardless of PTEN (15, 17). These results suggest that the signal transduction pathway from growth factor receptors to PI 3-K is intact in PTEN-expressing cells, whereas integrin-induced activation of PI 3-K was down-regulated by PTEN. Furthermore, PIP_3 levels in unstimulated cells were decreased (Fig. 4E), consistent with previous reports that PTEN directly dephosphorylates PIP_3 (16).
PTEN Suppresses Phosphorylation of FAK and Akt in Other PTEN-mutated Tumor Cells—We also tested whether the PTEN effects on FAK and Akt could be observed in not only U-87MG glioblastoma cells but also in DBTRG-05MG glioblastoma and MDA-MB468 breast cancer cells in which PTEN is mutated (1). Cells were co-transfected with GFP-PTEN (wild type) or GFP without insert together with a puromycin resistance plasmid. After selection for transfectants with puromycin, cells were assayed for phosphorylation of FAK and Akt. As shown in Fig. 5, similar suppression of phosphorylation of FAK and Akt was observed when cells expressing PTEN were maintained in suspension for 1 h, compared with plating on fibronectin.

**Inhibition of FAK and Akt Is Specific for PTEN**—Protein-tyrosine phosphatase 1B has been reported to regulate integrin- and cadherin-mediated signaling. PTP1B binds to N-cadherin and regulates the cadherin-actin linkage (58). Integrin-mediated adhesion and signaling is impaired in fibroblasts expressing a dominant-negative mutant of PTP1B, although they are not affected in cells expressing wild-type PTP1B (59), suggesting that PTP1B positively regulates integrin signaling. We examined whether PTP1B had similar effects as PTEN on the phosphorylation of FAK and Akt in cells attached to fibronectin in suspension. As shown in Fig. 6D, expression levels of PTEN and PTP1B were similar. PTP1B had no effects on FAK and Akt phosphorylation levels in cells that were either attached or in suspension (Fig. 6, A and B). On the other hand, both PTEN and PTP1B dephosphorylated p130Cas (Fig. 6C) as described (18, 60), suggesting that the effects on FAK and Akt are specific for PTEN.

**PTEN Induces Apoptosis by Loss of Matrix Attachment**—Since PTEN expression in PTEN-mutated cells caused matrix attachment-dependent suppression of Akt, we next examined whether PTEN reconstitution restored sensitivity to apoptosis by loss of matrix attachment. Akt is downstream of PI 3-K and is implicated in the matrix adhesion-dependent cell survival pathway (61–63). After selection with puromycin, cells were assayed for apoptosis. Morphological changes in PTEN-expressing cells spread on fibronectin were observed as described previously (18) (Fig. 7A). We used TUNEL assays to examine whether DNA condensation and fragmentation, a hallmark of apoptosis, occurred in the cells expressing PTEN (Fig. 7A). The percentages of cells undergoing apoptosis were similar in both control and PTEN-expressing cells on fibronectin. In contrast,
selected by puromycin. FAK or p130Cas were immunoprecipitated (IP) and immunoblotted for phosphotyrosine (pTyr) and each protein after cells were plated on fibronectin-coated dishes for 1 h in 10% serum-containing medium (FN) or after additional incubation in suspension for 30 min in the same serum-containing medium (A and C). Total cell lysates were immunoblotted for phospho-Ser473 Akt (p-Akt), total Akt (B), or GFP (D).

The percentage of PTEN-expressing cells that were TUNEL-positive was increased at 6 h, and most of these cells showed evidence of apoptosis after 18-h suspension in serum-containing medium (Fig. 7B). The induction of apoptosis was dependent on PTEN phosphatase activity (Fig. 7C), suggesting that PTEN triggers apoptosis through dephosphorylation of its substrate(s). We also tested whether detachment from matrix affected the cell cycle. No significant differences in cell cycle distribution between adherent and suspended cells were observed; the percentages of cells in S phase changed very little from 10.0% (adherent cells) to 8.9% (suspended cells) in control cells, while they were 6.2% (adherent cells) and 5.0% (suspended cells) in PTEN-expressed cells, consistent with the report that PTEN has no effects on the cell cycle in embryonic stem cells under normal culture conditions (11) or on U-87MG cells in 10% serum (64).

The Resistance to Apoptosis in PTEN-Mutated Cells Is Inhibited by a PI 3-K Inhibitor—PI 3-K is required for integrin-stimulated Akt activation (44). To confirm PI 3-K dependence of the sustained Akt activation in suspended PTEN-mutated cells, cells were incubated with the PI 3-K inhibitor wortmannin. In PTEN-mutated cells, although FAK phosphorylation levels did not change after incubation with wortmannin, Akt phosphorylation levels were markedly suppressed to the same levels as in PTEN-expressing cells (Fig. 8A). The suppression of Akt phosphorylation by wortmannin in suspended cells was accompanied by an increased incidence of apoptosis similar to that in PTEN-expressing cells (Fig. 8B). PD98059, a specific inhibitor of MEK1, had no effects on Akt phosphorylation, suggesting that the MAP kinase signal pathway, which is also down-regulated by PTEN (24), was not involved in the Akt activation. Detachment from matrix may activate JNK and promote apoptosis (65). In order to examine for possible involvement of JNK in PTEN-induced apoptosis, we next evaluated JNK phosphorylation levels. Although JNK phosphorylation levels in both control and PTEN-transfected cells were increased by UV irradiation, detachment from matrix did not cause any significant changes in JNK phosphorylation levels (Fig. 8C), suggesting that JNK is not involved in PTEN-induced apoptosis. Although extracellular matrix signals transduced by FAK inhibit p53 and suppress apoptosis in serum-deprived conditions (66), we could not observe any differences in p53 phosphorylation detected by anti-phospho-specific p53 (Ser392) antibody (data not shown).

Overexpression of FAK Can Increase Akt Phosphorylation and Rescue PTEN-expressing Cells from Apoptosis—We previously reported that overexpression of FAK antagonizes the effects of PTEN on cell spreading, migration, and invasion and partially on cell growth and shape (18, 21). Because dephosphorylation of FAK by PTEN was correlated with decreased association of PI 3-K with FAK; reduced PI 3-K activity, PIP2 levels, and Akt phosphorylation; and also apoptosis, we examined the effects of FAK overexpression on Akt phosphorylation and apoptosis. As shown in Fig. 9A, overexpression of FAK resulted in an increase in total FAK protein and tyrosine phosphorylation levels, and it abrogated PTEN-induced down-regulation of FAK phosphorylation dependent on the amount of
transfected DNA. FAK overexpression could also increase Akt phosphorylation levels along with the increase in FAK phosphorylation (Fig. 9, A and B). Although FAK phosphorylation was markedly increased by FAK overexpression in both control and PTEN-expressing cells, FAK overexpression could only partially increase Akt phosphorylation (Fig. 9, A and B), which was accompanied by only partial protection of PTEN-expressing cells from adhesion-dependent apoptosis (Fig. 9, C). These results suggest that FAK contributes to, but can only partially account for, PTEN-induced adhesion-dependent apoptosis.

**DISCUSSION**

Many recent lines of evidence have implicated functional inactivation of the PTEN gene in the pathogenesis of tumors of various tissues, indicating that *PTEN* acts as a tumor suppressor gene. In fact, recent reports that re-expression of *PTEN* in human glioma cell lines with mutated *PTEN* alleles suppresses cell growth and tumorigenicity further establish *PTEN* as a tumor suppressor (20, 52). Increased proliferation in PTEN mutant embryos also suggests that PTEN plays roles in regulating cell proliferation (17). It is important to elucidate the cellular functions of PTEN in order to understand how PTEN regulates normal cell behavior and acts as a tumor suppressor in vivo. Recently, both lipid and protein candidate substrates for PTEN have been identified, including PIP$_3$, FAK, and Shc (16, 18, 24). In this study, we have (a) established that Tyr$^{397}$ in FAK is required for the interaction between PTEN and FAK; (b) found that the interaction between FAK and the molecules bound to Tyr$^{397}$ in FAK including PI 3-K is inhibited by PTEN trapping mutant binding to FAK; (c) established that FAK tyrosine phosphorylation is maintained in *PTEN*-mutated cells
even after detachment from matrix substrates, which is accompanied by sustained FAK and PI 3-K association, PI 3-K activity, PIP3 levels, Akt phosphorylation, and resistance to apoptosis triggered by loss of matrix contact; (d) shown that expression of exogenous PTEN in mutant cells restores both their sensitivity to matrix-dependent apoptosis and normal patterns of FAK and Akt phosphorylation, association of FAK and PI 3-K, and levels of PI 3-K activity and PIP3, (e) found similar effects of PTEN on FAK and Akt phosphorylation in three glioblastoma and breast cancer cell lines, whereas another nonreceptor protein-tyrosine phosphatase, PTP1B, had no effects similar to PTEN; (f) established that the ability to maintain Akt phosphorylation and to protect cells from apoptosis was inhibited by a PI 3-K inhibitor but not by a MEK1 inhibitor; and (g) demonstrated that FAK overexpression could manipulate PI 3-K activity, PIP3 levels, and Akt phosphorylation and partially rescue suspended cells from PTEN-induced apoptosis. These results indicate that PTEN interacts with FAK through residue Tyr397 in the FAK molecule and suggest that it may down-regulate the downstream PI 3-K/Akt cell signaling and invasion, spreading, and focal adhesions (18, 21). PTEN inhibition of FAK leads to phosphorylation of other tyrosine residues in FAK in concert with activated Src.

Our data also provide interesting insights into FAK regulation upon detachment. It has been thought that FAK may be negatively regulated by putative tyrosine phosphatases that dephosphorylate Tyr397 of FAK in response to the detachment from matrix (75). In PTEN-mutated cells, we found that FAK remained abnormally phosphorylated even in suspension but could be almost completely dephosphorylated after the expression of PTEN. These findings are consistent with a role for PTEN in matrix-dependent regulation of FAK phosphorylation.

FAK also binds to PI 3-K and increases its activity in response to integrin-mediated cell adhesion (42). In this study, we showed that the association of PI 3-K with FAK and Akt phosphorylation are closely correlated. Furthermore, Akt phosphorylation was down-regulated when the cells were detached from matrix and lost FAK phosphorylation by PTEN expression in PTEN-mutated cells, suggesting that Akt might be partially regulated by integrin-mediated FAK activation. The demonstration that inhibition of PI 3-K completely suppressed Akt phosphorylation suggests that PI 3-K activation is necessary for the FAK-mediated Akt activation. These results are consistent with previous reports that PI 3-K is required for integrin-stimulated Akt activation (44, 61). This sequence of signaling events was further confirmed by our finding that PTEN down-regulated both PI 3-K activity and PIP3 levels and that PTEN inhibition of Akt could be partially reversed by intracellular overexpression of FAK. We speculate that overexpressing FAK may enhance its phosphorylation by increasing the total amounts of FAK available for phosphorylation and out-competing the phosphatase activity of PTEN. PTEN also dephosphorylates PIP3 (16) and decreases Shc tyrosine phosphatase levels (24). It is therefore also possible that overexpression of FAK might play a dominant negative role by substrate competition. However, the finding that FAK overexpression could totally overcome the effects of PTEN on PI 3-K p85 phosphorylation and PI 3-K activity while it only partially rescued PIP3 levels suggests that PTEN can affect the PI 3-K/ Akt pathway at more than one stage of signaling, i.e. at both the level of PIP3 and at an upstream FAK-dependent point.

Activation of Akt has been implicated in protection from apoptosis in response to several signals including growth factors (23, 76), cytokines (77), c-myc overexpression (78) UV irradiation (23), and matrix detachment (61, 62). Activation of Akt leads to phosphorylation of the Bel-2 family member Bad, thereby suppressing apoptosis and promoting cell survival (63). Loss of PTEN in mouse embryonic fibroblasts results in decreased sensitivity to cell death in response to various apoptotic stimuli by increasing basal Akt activity (17). Constitutively increased Akt phosphorylation levels are also reported in PTEN-mutated tumor cells (15), consistent with our data indicating that Akt phosphorylation levels were decreased in PTEN-expressing cells plated on matrix. The acquisition of anchorage independence and apoptosis resistance are critical for tumor malignancy (36). PTEN-mutated U-87MG glioblastoma cells that we used in this study also have the ability to grow in suspension (52). Recent studies have implicated FAK in this type of cell survival (reviewed in Ref. 79). Inhibition of FAK in several cell types results in growth suppression (71) and apoptosis (39, 80), although FAK may not mediate survival in all cases (40). Conversely, overexpression of activated FAK can rescue Madin-Darby canine kidney cells from anoikis (41).
These results suggest that FAK is an important mediator of integrin-mediated survival signals. In fact, several studies have established that levels of FAK expression are often increased in proliferating cells or advanced cancers (45–47).

In this study, we demonstrated that loss of PTEN protected cells from apoptosis triggered by matrix detachment and that re-expression of PTEN in PTEN-mutated cells caused apoptosis in cells in suspension. Our studies combined with those of Davies et al. (37) establish that PTEN plays important roles in anchorage-dependent cell survival. Sustained association of FAK with PI 3-K and Akt phosphorylation levels were closely correlated with the ability to survive in suspension. Furthermore, inhibition of PI 3-K suppressed Akt phosphorylation and resulted in apoptosis, suggesting that the ability to survive in suspension in PTEN-mutated cells is dependent on a PI 3-K/Akt pathway. In addition, however, FAK overexpression could manipulate PI 3-K activity and PIP3 levels, suggesting a level of PTEN action beyond a simple effect directly on PIP3.

Death signals activated in the absence of integrin-mediated adhesion may also include the JNK pathway, although its function in induction and protection from anoikis remains controversial (65, 81, 82). JNK phosphorylation levels showed normal reactions in our cells in response to UV irradiation (enhanced dephosphorylation) after detachment from matrix and results in inhibition of FAK phosphorylation (66), but we could not detect the involvement of p35 in PTEN-mediated apoptosis under our culture conditions in regular serum-containing medium.

In summary, we have elucidated interactions and signaling processes involving PTEN and FAK. We demonstrated that Tyr397 in FAK is important for FAK-FAK interaction. PTEN restoration in tumor cells with mutated PTEN alleles results in inhibition of PTEN phosphorylation (enhanced dephosphorylation) after detachment from matrix and results in inhibition of PI 3-K association with FAK. PTEN reconstitution also restores matrix-dependent regulation of FAK and Akt phosphorylation and apoptosis in cells in suspension. Overexpression of FAK antagonized the effects of PTEN. Our data demonstrate that PTEN functions to suppress the ability of cells to survive in suspension. Our studies combined with those of Davies et al. (37) establish that PTEN plays important roles in anchorage-dependent cell survival. Sustained association of FAK with PI 3-K and Akt phosphorylation levels were closely correlated with the ability to survive in suspension. Furthermore, inhibition of PI 3-K suppressed Akt phosphorylation and resulted in apoptosis, suggesting that the ability to survive in suspension in PTEN-mutated cells is dependent on a PI 3-K/Akt pathway. In addition, however, FAK overexpression could manipulate PI 3-K activity and PIP3 levels, suggesting a level of PTEN action beyond a simple effect directly on PIP3.
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