Tumor Suppressor PTEN Inhibition of Cell Invasion, Migration, and Growth: Differential Involvement of Focal Adhesion Kinase and p130Cas

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ABSTRACT

PTEN/MMAC1, a major new tumor suppressor gene that encodes a dual-specificity phosphatase with sequence similarity to the cytoskeletal protein tensin. Recently, we reported that PTEN dephosphorylates focal adhesion kinase (FAK) and inhibits cell migration, spreading, and focal adhesion formation. Here, the effects of PTEN on cell invasion, migration, and growth were investigated in U87MG glioblastoma cells missing PTEN. Cell invasion, migration, and growth were down-regulated by expression of phosphatase-active forms of PTEN but not by PTEN with an inactive phosphatase domain; these effects were correlated with decreased tyrosine phosphorylation levels of FAK and p130Cas. Overexpression of FAK concomitant with PTEN resulted in increased total tyrosine phosphorylation levels of FAK and p130Cas and effectively antagonized the effects of PTEN on cell invasion, migration and partially on cell growth. Overexpression of p130Cas increased total tyrosine phosphorylation levels of p130Cas without affecting those of FAK; however, although p130Cas could reverse PTEN inhibition of cell invasion and migration, it did not rescue cell growth in U87MG cells. In contrast to FAK, p130Cas could not be shown to interact with PTEN in cells, and it was not dephosphorylated directly by PTEN in vitro. These results suggest important roles of PTEN in the phenotype of tumor progression, and that the effects of PTEN on cell invasion, migration, and growth are mediated by distinct downstream pathways that diverge at the level of FAK.

INTRODUCTION

PTEN (also called MMAC1, mutated in multiple advanced cancers) is a major new tumor suppressor gene recently identified on human chromosome 10q23. It was identified as a candidate tumor suppressor gene based on the presence of inactivating mutations in human brain, breast, and prostate cancers (1, 2). PTEN is also frequently deleted or mutated in a wide range of human tumors and tumor cell lines such as glioblastoma, melanoma, and lymphoid, lung, and endometrial cancers (3–11). Furthermore, germ-line PTEN mutations have been found in patients with juvenile polyposis coli (12), Cowden disease, a multiple hamartoma syndrome with a high risk of breast and thyroid cancer (13), and the related hamartomatous polypsis syndrome, Bannayan-Zonana syndrome (14), suggesting that inactivation of PTEN plays important roles in tumorigenesis.

Recently, suppression of tumorigenicity and cell growth by expression of PTEN cDNA in glioma cells with mutated PTEN alleles was reported (15, 16). PTEN encodes the catalytic signature motif of protein tyrosine phosphatases and functions as a dual-specificity phosphatase in vitro (3, 17, 18). Although mutations of PTEN are seen throughout the gene in various cancers, the phosphatase domain seems to be critical for tumor suppression, because most missense mutations are observed to cluster around the phosphatase domain, and most remaining mutations are predicted to truncate the protein because of nonsense or frameshift mutations (1, 2, 10). In fact, growth suppression in glioma cells with mutated PTEN alleles by PTEN cDNA expression was shown to require a functional phosphatase catalytic domain (15). Furthermore, mutation or a reported decreased expression of PTEN is associated with advanced glioma, melanoma, and prostate cancer, implicating losses of PTEN mutation in tumor progression (6, 19, 20).

PTEN also has sequence similarity to the cytoskeletal protein tensin (1–3), which binds to actin filaments at focal adhesions. We have reported recently that PTEN inhibits cell migration, spreading, and focal adhesions (21). PTEN interacts with FAK and reduces its tyrosine phosphorylation, as well as that of a potential downstream effector, p130Cas. FAK is thought to be a key molecule implicated in integrin signaling pathways. The activation of integrins by cell binding to extracellular matrix leads to increases in FAK tyrosine phosphorylation and enhances kinase activity (22–27). Activation of FAK also leads to its association with several kinases and signal transduction molecules including Grb2, Src, and Shc, followed by activation of extracellular signal-regulated kinase 2/ MAP kinase (28–31). In addition to its potential involvement in integrin stimulation of MAP kinase, FAK promotes cell migration through the activation of p130Cas (32). FAK activation may lead to increases in tyrosine phosphorylation of p130Cas directly (33, 34) or via c-Src binding to FAK (35). In fact, this pathway may be essential for cell migration in several cell lines, and the FAK/p130Cas complex appears to target downstream pathways other than MAP kinases in mediating FAK-promoted cell migration (32, 36).

In the present study, we investigated the ability of PTEN to suppress glioblastoma cell invasion and migration and whether mutations in its phosphatase domain could ablate these abilities. To explore PTEN signaling pathways, we also tested whether overexpression of FAK and its downstream effector p130Cas could antagonize the effects of PTEN. We have found that FAK overexpression can rescue cell invasion, migration, and even to some extent growth, whereas p130Cas can rescue only cell invasion and migration. These findings suggest that PTEN suppresses cell invasion, migration, and growth, possibly through FAK dephosphorylation, and that p130Cas is a downstream mediator of FAK involved in mediating cell invasion and migration, but not in cell growth, showing distinct downstream pathways that diverge at the level of FAK.

MATERIALS AND METHODS

Expression Plasmids. GFP expression plasmids based on pGZ21αZ containing no insert, full-length wild-type PTEN, or HA-tagged FAK were constructed as described (21). The point mutants D92A (Asp92→Ala), C124A (Cys124→Ala), or G129E (Glu129→Glu) were introduced into PTEN by site-directed mutagenesis as described (21). pSsrα-Cas-FLAG was constructed by inserting the epitope tag FLAG (Eastman Kodak Co., New Haven, CT) at the 3’ end of the p130Cas coding sequence in the expression vector pSSRα-Cas (37) that was kindly provided by Hisamatsu Hirai (University of Tokyo, Tokyo, Japan). The puromycin resistance plasmid pHA262pur (38)
was kindly provided by Hein te Riele (Netherlands Cancer Institute, Amsterdam, the Netherlands).

Antibodies. The monoclonal antibody 2A7 (Upstate Biotechnology, Lake Placid, NY) directed against FAK was used for immunoprecipitation, and a second monoclonal antibody against FAK (Transduction Laboratories, Lexington, KY) was used for immunoblotting. Monoclonal antibodies for p130Cas and phosphotyrosine (RC20) were obtained from Transduction Laboratories. Monoclonal antibody against FLAG (M2) was from Eastman Kodak, against HA (12CA5) was from Boehringer Mannheim (Indianapolis, IN), and against GFP was from Clontech. Rabbit polyclonal antibody MT478 against PTEN was raised by immunization with His6-tagged recombinant PTEN expressed in Escherichia coli as described (21). IgG was purified from antisera using Gammabind G Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ).

Cell Culture and Transfections. The PTEN-mutated glioblastoma cell line U87MG was obtained from American Type Culture Collection (Manassas, VA). Human foreskin fibroblasts were kindly provided by Susan Yamada (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD) and used as a positive control of cells expressing endogenous PTEN. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and cultured in 10% CO2 at 37°C. Calf serum (10%) was substituted for culturing NIH 3T3 cells. Transfections were performed by electroporation (39). Briefly, pGZ210×s (20 μg; alternatively, 10 μg in the case of cotransfection with either 10 μg HA-FAK or 10 μg FLAG-Cas) containing either no insert or PTEN was transfected into 1.5 × 10^6 U87MG cells by electroporation together with 10 μg of PHA262pur. Cells were subcultured at a 1:3 dilution 24 h after transfection and were maintained for 3 days in 1 μg/ml puromycin-containing medium. For cell invasion and migration assays, cells were serum starved by incubating in DMEM containing 0.5% serum during the last 24 h of puromycin selection to exclude any effects of growth suppression by PTEN on cell invasion and migration assays. For coimmunoprecipitation experiments, NIH 3T3 cells were transfected with either HA-FAK or FLAG-Cas (3 μg each) plus GFP-tagged wild-type PTEN or trapping mutant D92A (30 μg each) and used for immunoprecipitations 24 h after transfection. Cells were analyzed for expression of various constructs by Western blotting for PTEN, FAK, and p130Cas as described below; GFP-expressing cells were also quantified by scoring at least 100 cells per transfection using a fluorescence microscope.

Immunoprecipitation and Western Blotting. After puromycin selection of cells expressing the various constructs, U87MG cells (5 × 10^5) were allowed to spread for 2 h on 10-cm Petri dishes coated with 10 μg/ml fibronectin. The cells were washed with ice-cold PBS and solubilized in RIPA lysis buffer for 20 min on ice (40). For communoprecipitation experiments, NIH 3T3 cells were solubilized in modified CSK buffer (100 mM NaCl, 0.5% Triton X-100, 300 mM sucrose, 3 mM MgCl2, and 10 mM 1,4-piperazinediethanesulfonic acid, pH 6.8) containing 2 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and protease inhibitor mixture (Boehringer Mannheim). The homogenates were clarified by centrifugation at 20,000 × g for 15 min at 4°C, protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL), and samples were adjusted to equal protein concentration and volume. Immunoprecipitation was performed as described (40), except using anti-FAK (5 μg/ml), anti-p130Cas (4 μg/ml), anti-HA (4 μg/ml), or anti-FLAG (5 μg/ml) antibodies. Immunoprecipitates (or total cell lysates for anti-PTEN blotting) were resolved in 4–12% SDS-polyacrylamide gels (Novex, San Diego, CA) and electrophoretically transferred to nitrocellulose membrane (Novex) for 1.5 h at 150 mA. The filters were incubated with blocking buffer (5% nonfat dry milk; alternatively, 5% BSA for anti-phosphotyrosine antibody, in T-TBS: 150 mM NaCl, 50 mM Tris-HCl, and 0.1% Tween 20, pH 7.4) for 1 h and then incubated for 1 h with either anti-PTEN (2 μg/ml) or horseradish peroxidase-conjugated RC20 (1:2500) antibodies. Blots were visualized by the enhanced chemiluminescence (ECL) reaction (Amersham Life Science, Arlington Heights, IL). Membranes were then stripped with 2% SDS, 100 mM β-mercaptoethanol in 62.5 mM Tris-HCl (pH 6.8) for 30 min at 70°C, reprobed for FAK (1:1000), p130Cas (1:1000), or GFP (1:1000), and visualized again by ECL.

Cell Infection and Migration. Cell infection assays were carried out using modified Boyden chambers consisting of Transwell (Corning Costar Corp., Cambridge, MA) membrane filter inserts in 24-well tissue culture plates. The Transwell filters were 6.5-mm diameter, 8-μm thickness polycarbonate membranes. The upper surfaces of the Transwell membranes were coated with 1 mg/ml Matrigel matrix (Collaborative Biomedical Products, Bedford, MA; Becton Dickinson Labware, Franklin Lakes, NJ) overnight at 4°C, rinsed once with invasion buffer (DMEM containing 1% BSA and 0.5% serum), and then placed into 24-well tissue culture plates containing 600 μl of invasion buffer/well. After selection and serum starvation of transfected cells as described above, U87MG cells were harvested using 0.53 mm EDTA and resuspended in invasion buffer. Cells (2 × 10^5) in 100 μl of invasion buffer were added to each Transwell chamber and allowed to invade toward the underside of the membrane for 16 h. Noninvading cells were removed by wiping the upper side of the membrane, and the invaded cells were fixed and stained using Diff-Quik (Baxter Healthcare Corp., McGraw Park, IL). The number of invaded cells per membrane was counted under a light microscope at ×200.

Cell migration was measured using an in vitro wound healing assay as described (21). Briefly, in vitro “scratch” wounds were created by scraping confluent cell monolayers in fibronectin-coated (10 μg/ml) Petri dishes with a sterile pipette tip. After 24 h incubation in DMEM with 0.5% serum, migration was quantified by counting cell numbers migrating from the wound edge. Each determination represents the average of four individual experiments, and error bars represent SE. Ps were calculated by Student’s t test.

In-Blot Phosphatase Assay. PTEN dephosphorylation of FAK and p130Cas was examined by an in-blot phosphate assay as described (21). Briefly, histidine-tagged PTEN (His6-PTEN) was generated by inserting full-length PTEN cDNA into the pQE30 vector (Qiagen, Valencia, CA). The protein was purified using Ni-NTA beads (Qiagen) under denaturing conditions and then renatured by sequential dilution and concentration in renaturation buffer [PBS (pH 7.0) containing 2 mM MgCl2, 0.5 mM phenylmethanesulfonyl fluoride, 0.005% Tween 20, 10 mM DTT, and protease inhibitor mixture]. Purity (>90%) was confirmed by SDS-PAGE and Coomasie blue staining, and preparations were used within 1 h. U87MG cells that had spread on fibronectin for 2 h were lysed in RIPA buffer, and FAK and p130Cas were immunoprecipitated using either anti-FAK or anti-p130Cas antibodies, respectively. Equal amounts of immunoprecipitated proteins were subjected to 4–12% SDS-PAGE and electro transferred to nitrocellulose. Blots were incubated with 20 μg/ml recombinant His6-PTEN in 50 mM HEPES buffer (pH 7.0) containing 10 mM MgCl2, 10 mM DTT at 30°C for 1 h. Phosphoproteins were detected with anti-phosphotyrosine antibody.

Cell Growth. After puromycin selection for transfected cells, cells were harvested by trypsin-EDTA. Transfected U87MG cells (5 × 10^5) were cultured in six-well plates with 10% serum in DMEM. Cell number was counted after 24 h incubation by harvesting the cells using trypsin-EDTA and counting in a hemocytometer. [3H]Thymidine incorporation was measured using 1 μCi/ml [3H]thymidine (Amersham Pharmacia Biotech) for 1 h after 24 h incubation. The cells were then washed three times with ice-cold PBS and detached from the plates with trypsin-EDTA. The radioactivity of samples was determined with a liquid scintillation counter. In situ detection of cells undergoing apoptosis was done by fluorescent-conjugated annexin V staining (Clontech) as described (41) or by TUNEL assay (Boehringer Mannheim), which was detected by anti-fluorescein antibody conjugated with horseradish peroxidase according to the manufacturer’s protocol.

RESULTS

PTEN Inhibits Cell Invasion, and Its Activity Is Phosphatase Dependent. U87MG cells (which have a PTEN frameshift mutation at codon 54) were cotransfected with GFP-PTEN (wild-type), GFP-PTEN phosphatase-inactivating mutant (C124A), the control mutant GFP-PTEN (G129E), or GFP without insert, together with a plasmid encoding a puromycin resistance gene to select cells coexpressing the expression plasmids. After selection with puromycin for 3 days, cells were analyzed for PTEN expression by immunoblotting. As shown in Fig. 1, a M_r ~82,000 band corresponding to GFP-PTEN was recognized by anti-PTEN antibody MT478 in wild-type or mutant PTEN-transfected cells. This antibody also recognized both recombinant full-length PTEN and endogenous PTEN in human foreskin fibroblasts (Fig. 1). PTEN protein migrated at M_r ~55,000, in agreement
with previous reports (3, 16). In contrast, no endogenous PTEN protein could be detected in U87MG cells transfected with GFP; the PTEN gene is mutated in this cell. We also determined the percentage of cells expressing GFP-PTEN by scoring for GFP-positive cells using a fluorescence microscope. The percentage of cells that were GFP positive after cotransfection with GFP, GFP-PTEN (wild type), GFP-PTEN (C124A), or GFP-PTEN (G129E) were 95 ± 6%, 91 ± 4%, 89 ± 4%, and 95 ± 1%, respectively (mean ± SE of four independent experiments).

To examine the effects of PTEN on cell invasion, U87MG cells were transfected with GFP plasmids either without insert or with wild-type PTEN or PTEN mutants and were selected by puromycin as described above. Because PTEN suppresses growth in cell lines missing the PTEN gene (Ref. 15; Fig. 6), cells were serum starved to suppress growth, thereby ruling out contributions of PTEN growth suppression on cell invasion assays. As shown in Fig. 2A, cell invasion through membranes coated with Matrigel was decreased by 45% (55.6 ± 7.5% of control) in cells expressing wild-type PTEN and by 42% (58.0 ± 7.0% of control) in cells expressing the phosphatase-active mutant of PTEN (G129E), whereas no significant change was observed after transfection with the phosphatase-inactivated mutant of PTEN (C124A), indicating that the phosphatase activity of PTEN is required for inhibition of cell invasion. There were no significant differences in total cell numbers of each transfectant, which were determined by counting invading plus noninvasing cells on Matrigel after 16 h incubation (data not shown). These results indicate that the suppression of cell invasion by PTEN was not due to PTEN effects on cell growth. The marked inhibition of cell invasion after PTEN expression was not accompanied by any change in cell viability (~95% viability by trypsin blue assay with or without PTEN expression).

We also examined whether tyrosine phosphorylation levels of FAK correlated with the effects of PTEN on cell invasion. Immunoprecipitation of FAK showed decreases in tyrosine phosphorylation of FAK by phosphatase-active forms of PTEN (wild type and G129E) but not by a phosphatase-inactive form of PTEN (C124A; Fig. 2B), consistent with our previous experiments that FAK is dephosphorylated both in vivo and in vitro (21). The amount of FAK protein in each sample assessed by immunoblotting was similar (Fig. 2B). Thus, PTEN inhibition of cell invasion is associated with FAK dephosphorylation.

FAK Overexpression Rescues PTEN Inhibition of Cell Invasion. The correlation of FAK dephosphorylation with inhibition of cell invasion suggests that the inhibition of invasion may be a downstream event of FAK dephosphorylation by PTEN. Next, we tested whether FAK overexpression could attenuate the effects of PTEN on cell invasion. As shown in Fig. 3A, overexpression of FAK resulted in an increase in total FAK protein levels and abrogated PTEN-induced down-regulation of FAK phosphorylation. We then examined the phosphorylation levels of p130Cas. Tyrosine phosphorylation of p130Cas was decreased in PTEN-expressing cells; however, it was increased after overexpression of FAK cotransfected with PTEN (Fig. 3A), suggesting that p130Cas is located downstream of FAK. Although FAK overexpression alone had minimal to only very slightly positive effects on cell invasion (115.6 ± 2.3% of control), coexpression of FAK with PTEN effectively rescued the ability of cells to invade (99.5 ± 4.0% of control) (Fig. 3B), suggesting that FAK is an important mediator of PTEN-induced inhibition of cell invasion.

p130Cas Overexpression Also Rescues PTEN Inhibition of Cell Invasion. The adapter protein p130Cas is a putative downstream effector of FAK, and it is characteristically tyrosine phosphorylated after increases in FAK and c-Src kinase activity in response to attachment to the extracellular matrix (33–35). We therefore investigated whether p130Cas is also implicated in PTEN-induced inhibition of cell invasion as a downstream event of FAK dephosphorylation. Again, tyrosine phosphorylation levels of p130Cas were decreased in PTEN-expressing cells (Fig. 4A). Both total protein and tyrosine phosphorylation levels of p130Cas were increased after p130Cas overexpression of FAK cotransfected with PTEN (Fig. 4B), consistent with the correlation of FAK dephosphorylation with inhibition of cell invasion. The adapter protein p130Cas is an important mediator of PTEN-induced inhibition of cell invasion.
expression. The phosphorylation levels of FAK in p130 Cas-overexpressing cells were not affected, suggesting that p130 Cas phosphorylation was downstream of FAK. Although p130 Cas overexpression alone had minimal effects on cell invasion (109.8 ± 4.9% of control), coexpression of p130 Cas with PTEN effectively rescued the ability of cells to invade (increasing from 53.5 ± 6.5% to 96.5 ± 3.1% of control; Fig. 4B). These results are consistent with a role for p130 Cas in PTEN-induced inhibition of cell invasion as a downstream effector of FAK.

Direct Effects of PTEN on FAK but not on p130 Cas. Because our data suggested that p130 Cas was located downstream of FAK, we next tested whether PTEN could directly dephosphorylate tyrosine-phosphorylated p130 Cas in vitro using an in-blot phosphatase assay, with FAK as a positive control (21). Tyrosine-phosphorylated FAK and p130 Cas were immunoprecipitated from U87MG cells, electrobotted, and incubated with or without His<sub>6</sub>-PTEN (Fig. 5A). Recombinant PTEN reduced tyrosine phosphorylation of FAK by 78% (22 ± 15% of control, three experiments). No dephosphorylation occurred when blots were incubated with control E. coli lysate (data not shown) or without PTEN (Fig. 5A). In contrast, PTEN could not dephosphorylate p130 Cas (Fig. 5A).

We also tested for physical interactions of PTEN with p130 Cas in living cells using a “trapping” mutant of PTEN (21, 42). Overexpression of a PTEN mutant with inactivated phosphatase activity (potentially able to bind and protect a substrate) resulted in elevated phosphorylation of HA-FAK in NIH 3T3 cells that had endogenous PTEN (Fig. 5B), consistent with our previous report (21). Moreover, immunoprecipitated HA-FAK retained bound GFP-PTEN, indicating formation of a FAK-PTEN complex (Fig. 5B). In control plasmid GFP (-) transfected cells, no association of GFP with HA-FAK could be

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Fig. 3. FAK reversal of PTEN-induced reduction of cell invasion. In A, lysates from each U87MG transfectant were immunoprecipitated with either FAK (IP:FAK) or p130 Cas (IP:p130 Cas) and immunoblotted with anti-phosphotyrosine (pTyr), anti-FAK, or anti-p130 Cas. In B, cell invasion through Matrigel was analyzed using a modified Boyden chamber as described in “Materials and Methods.” Cells that invaded through the basement membrane extract to the lower surface of the membrane were fixed, stained, and counted from at least 10 fields and four experiments. Bars, SE. †, P < 0.005 versus control.

Fig. 4. p130 Cas reversal of PTEN-induced reduction of cell invasion. In A, lysates from each U87MG transfectant were immunoprecipitated with either FAK (IP:FAK) or p130 Cas (IP:p130 Cas) and immunoblotted with anti-phosphotyrosine (pTyr), anti-FAK, or anti-p130 Cas. In B, cell invasion through Matrigel was analyzed using a modified Boyden chamber assay as described in “Materials and Methods.” Cells that migrated to the lower side of membrane were counted from at least 10 fields and four experiments. Bars, SE. †, P < 0.005 versus control.

Fig. 5. Interactions of PTEN with FAK or p130 Cas. A, direct tyrosine dephosphorylation of FAK but not p130 Cas. Because our data suggested that p130 Cas was located downstream of FAK, we next tested whether PTEN could directly dephosphorylate tyrosine-phosphorylated p130 Cas in vitro using an in-blot phosphatase assay, with FAK as a positive control (21). Tyrosine-phosphorylated FAK and p130 Cas were immunoprecipitated from U87MG cells, electrobotted, and incubated with or without His<sub>6</sub>-PTEN (Fig. 5A). Recombinant PTEN reduced tyrosine phosphorylation of FAK by 78% (22 ± 15% of control, three experiments). No dephosphorylation occurred when blots were incubated with control E. coli lysate (data not shown) or without PTEN (Fig. 5A). In contrast, PTEN could not dephosphorylate p130 Cas (Fig. 5A).

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PTEN IN INVASION, MIGRATION, AND GROWTH

PTEN Inhibition of Cell Migration Is Phosphatase Dependent, and Both FAK and p130Cas Overexpression Rescue PTEN Inhibition of Cell Migration. We reported recently that PTEN overexpression reduces cell migration, whereas the expression of antisense PTEN enhances cell migration in stable transfectant lines of NIH 3T3 cells (21). In this study, we examined the effects of phosphatase mutants of PTEN and of FAK or p130Cas overexpression on PTEN suppression of cell migration. Phosphatase-active forms of PTEN significantly inhibited cell migration as measured by in vitro wound healing assays (Fig. 6A). In contrast, the expression of the phosphatase-inactive form of PTEN had no significant effect on cell migration (Fig. 6A). Next, either FAK or p130Cas was coexpressed with PTEN to test the involvement of FAK and p130Cas in PTEN-induced inhibition of cell migration. Both FAK and p130Cas overexpression individually could enhance U87MG cell migration (Fig. 6B) as reported previously in other cell types (32, 36, 43, 44), in clear contrast to their minimal effects on cell invasion (Figs. 3 and 4). Furthermore, in PTEN-expressing cells, both FAK and p130Cas overexpression effectively reversed the reduction of cell migration by PTEN (Fig. 6B), suggesting that both FAK and p130Cas are intermediates in PTEN-induced inhibition of cell migration; their effects on PTEN action were similar to the results of the cell invasion assay. Similar effects of FAK and p130Cas on PTEN-induced reduction of cell migration were observed in DBTRG-05 MG glioblastoma cells (data not shown).

FAK, but not p130Cas, Reverses Cell Growth Inhibition by PTEN. Because both FAK and p130Cas could rescue the inhibition of cell migration and invasion by PTEN, we examined for effects of FAK and p130Cas overexpression on cell growth. Cells were cultured for 24 h after puromycin selection for transfected cells. Cell growth in cells transfected with phosphatase-active forms of PTEN was suppressed by >50% (wild type: reduced to 45 ± 6% of control; G129E: 48 ± 7% of control; Fig. 7A). In contrast, no reduction was seen when cells were transfected with C124A, a phosphatase-inactive form of PTEN (Fig. 7A), compatible with a previous report (15). Next, cells were cotransfected with either FAK or p130Cas. Neither FAK nor p130Cas alone had any significant effect on the number of cells after transfection (Fig. 7B). In PTEN and FAK coexpressing cells, the cell number was significantly, but partially, increased compared with cells expressing PTEN alone (increasing from 44 ± 6% to 83 ± 4% of control, P < 0.005; Fig. 7B). The changes in cell number were also confirmed by [1H]thymidine incorporation (wild-type PTEN transfection reduced incorporation to 65 ± 5% of control; increased to 84 ± 6% by coexpression of FAK). On the other hand, p130Cas overexpression did not result in significant changes in cell numbers of

Fig. 6. Migration of U87MG cells expressing wild-type or mutant PTEN and modulation by FAK or p130Cas co-expression. In A, in vitro “scratch” wounds were created by scraping confluent cell monolayers in fibronectin-coated dishes. After 24 h, cell numbers migrating from the wound edge were counted. Bars, SE from four independent experiments. †, P < 0.0005 versus control. In B, the effects of FAK or p130Cas co-expression with PTEN on cell migration were analyzed by in vitro wound healing assay as described above. †, P < 0.0005; ‡, P < 0.001 versus control.

seen (data not shown). In contrast, phosphorylation levels of p130Cas were not increased, and no association of GFP-PTEN with FLAG-Cas was observed when cells were transfected with the PTEN trapping mutant (Fig. 5C). Thus, PTEN could not interact with p130Cas both in vitro and in vivo under conditions where FAK was readily dephosphorylated, indicating that p130Cas is not a physiological substrate for PTEN. Our results suggest that PTEN indirectly inhibits phosphorylation of p130Cas, e.g., through its effects on FAK.

Fig. 7. Growth-suppressive effect of PTEN in U87MG cells and modulation by FAK or p130Cas coexpression. In A, empty vector (control) or vector containing either wild-type or mutant PTEN was transfected in U87MG cells. After selection with puromycin, an equal number of cells were plated and cultured in 10% serum-containing medium for 24 h. Cell numbers were determined in four independent experiments and pooled. Bars, SE. †, P < 0.0005 versus control. In B, the effects of FAK or p130Cas coexpression with PTEN on cell growth were analyzed as described above. Bars, SE. †, P < 0.0005; ‡, P < 0.001 versus Lane 1.
PTEN expressing cells (Fig. 7B), suggesting that p130Cas is not involved in PTEN-induced regulation of cell growth.

FAK is also known to protect cells from apoptosis (45, 46). We next examined whether the marked suppression of cell growth after PTEN expression was related to apoptosis. U87MG cells were cotransfected with pH2A62pur either without or with GFP or GFP-PTEN (wild type). After puromycin selection for transfectants, cells undergoing apoptosis were detected by annexin V staining or TUNEL assays. The percentages of cells that were annexin V positive were very low without any treatment: 2.1 ± 0.5% (nontransfected cells), 1.8 ± 0.7% (GFP), and 2.2 ± 0.7% (GFP-PTEN), respectively (mean ± SE of three independent experiments), consistent with the results of trypan blue assays. In contrast, positive cells were increased to 74 ± 3% (nontransfected cells), 80 ± 6% (GFP), and 78 ± 7% (GFP-PTEN), respectively, after 24-h UV irradiation (200 mJ/cm²). The TUNEL assay also showed similar percentages of positive cells; there were no significant differences between nontransfected, GFP, and GFP-PTEN-transfected cells. Thus, apoptosis was not involved in the PTEN-mediated growth suppression observed in U87MG cells under standard cell culture conditions.

DISCUSSION

The PTEN gene is deleted or mutated in ~45% of endometrial cancers, ~30% of glioblastomas, and at lower frequencies in a wide range of other human tumors. Recent reports that reexpression of PTEN in human glioma cell lines with mutated PTEN alleles suppresses cell growth and tumorigenicity further established PTEN as a tumor suppressor (15, 16). However, studies characterizing the molecular mechanisms by which PTEN functions as a tumor suppressor have lagged considerably. In this study, we have: (a) established that PTEN can inhibit cell invasion in a phosphatase-dependent manner; (b) confirmed that PTEN can suppress cell growth and migration and established that the latter was also dependent on PTEN phosphatase; (c) demonstrated that overexpression of FAK can restore both FAK and p130Cas tyrosine phosphorylation, and that it can antagonize or reverse the inhibitory actions of PTEN on cell invasion, migration, and even partially on growth suppression; (d) established that p130Cas overexpression restores its own phosphorylation but not that of FAK and can antagonize effects of PTEN on cell invasion and migration, but not on cell growth; and (e) confirmed that PTEN can directly interact with FAK and reduce its tyrosine phosphorylation and established that, in contrast, PTEN does not interact with p130Cas and cannot dephosphorylate p130Cas directly. These effects are summarized in Fig. 8, and they suggest a working mechanistic scheme in which PTEN down-modulates FAK phosphorylation and suppresses all three biological activities. In contrast, p130Cas acts downstream of FAK, because its overexpression affects neither FAK phosphorylation nor growth, and it could not be shown to interact with PTEN either in cells or in vitro.

Our studies, provide, to our knowledge, the first evidence that PTEN can restrain the capacity of tumor cells to invade through a basement membrane matrix. Its inhibitory activity required an intact phosphatase domain. PTEN inhibition of tumor cell invasion could be reversed by intracellular overexpression of FAK at levels that restored its initially PTEN-suppressed tyrosine phosphorylation. 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. It was reported recently that PTEN dephosphorylates phosphatidylinositol 3,4,5-trisphosphate (47, 48) and decreases Shc tyrosine phosphorylation levels (49). It is therefore also possible that overexpression of FAK might play a dominant-negative role in PTEN growth suppression by substrate competition. In addition, we reported previously that cell migration was inhibited in NIH 3T3 cells overexpressing PTEN (21). In this report, we established that an intact PTEN phosphatase domain was needed for this activity, and moreover that FAK overexpression could also reverse PTEN inhibition of migration on a planar surface in two glioma cell lines. Although our similar findings in the invasion assay must be evaluated with caution because they are based on results from a single glioma cell line, rescue of tumor cells by FAK transfection and phosphorylation from PTEN-induced inhibition of tumor cell migration and invasion suggest that PTEN suppresses these activities through dephosphorylating FAK.

General proposed functions of FAK include roles in focal adhesion assembly, cell adhesion, and cell migration. Overexpression of dominant-negative FAK causes a transient reduction in cell spreading (50), consistent with our previous report that PTEN-induced FAK dephosphorylation leads to suppression of cell spreading and impaired focal adhesion and cytoskeletal formation (21). FAK is also an important regulator of integrin-mediated cell migration events. FAK expression is enhanced in rapidly migrating cells during wound healing (51, 52), and FAK overexpression stimulates cell migration (44). Cells in which FAK is inhibited by a tyrosine kinase inhibitor (52), cells from FAK-deficient (gene knockout) mice (43), and cells overexpressing the FAK COOH-terminal domain (53) all exhibit decreased cell migration. Our findings suggest a function for PTEN in down-modulating these FAK activities by dephosphorylation.

Recently, p130Cas has been reported to be a mediator of FAK-promoted cell migration (32, 36). We have shown that p130Cas is also dephosphorylated as well as FAK by PTEN expression (21). We established in this report that either FAK or p130Cas overexpression could induce rephosphorylation of p130Cas and could effectively rescue PTEN-induced inhibition of both cell migration and invasion. p130Cas is a signal transduction protein that binds directly to FAK and is phosphorylated upon cell adhesion to extracellular matrix proteins in a FAK- and Src-dependent manner (33–35, 54). The prolinc-rich region of FAK spanning amino acids 711–717 has been demonstrated.
to be a binding site for the SH3 domain of p130Cas (55). We have been unable to detect direct dephosphorylation of p130Cas by PTEN using an in-blot phosphatase assay under conditions where FAK is readily dephosphorylated, nor could we detect physical association of PTEN with p130Cas. These findings collectively suggest that p130Cas is a downstream effector in a signal transduction pathway leading to cell migration and invasion after PTEN dephosphorylation of FAK (Fig. 8). The findings that both p130Cas and FAK were rephosphorylated after overexpression of FAK, but that p130Cas overexpression resulted in only its own phosphorylation without affecting the phosphorylation level of FAK also indicates that rescue by p130Cas is a downstream event of FAK. Thus, the signaling pathways of PTEN modulating cell invasion, migration, and growth appear to be mediated by distinct downstream pathways that diverge at the level of FAK (Fig. 8). The evidence that both FAK and p130Cas play important roles not only in cell migration but also in cell invasion suggests that FAK/p130Cas activation may contribute to the invasiveness of tumors. Thus, the elevated FAK observed in transformed cells (56–58) may form complexes with p130Cas, leading to invasion events involved in the dissemination of tumors.

In this study, manipulating the amounts of phosphorylated FAK could also antagonize the biological effects of PTEN on cell growth. Although overexpression of FAK rescued the action of PTEN on cell invasion, migration, and even partially on growth suppression, p130Cas did not affect growth, suggesting that the cell growth-suppressive effect of PTEN is mediated by FAK and downstream effectors other than p130Cas. Activation and autophosphorylation of FAK in response to integrin binding to extracellular matrix leads to its binding to a number of intracellular signaling molecules besides p130Cas including Src, Grb2, and phosphatidylinositol 3-kinase (28, 29, 59). FAK/Src association can lead to activation of the MAP kinase pathway through Grb2 binding to FAK (28, 30), although other mechanisms of integrin-mediated MAP kinase activation also exist (60, 61). Cell adhesion also promotes the association of FAK with phosphatidylinositol 3-kinase (59), which has been implicated in integrin-mediated cell survival through the proto-oncogene Akt and MAP kinase activation (62, 63). The requirement for phosphatase activity in PTEN-mediated growth suppression (Ref. 15 and this study) strongly suggests that the dephosphorylation of a substrate(s) of PTEN such as FAK is critical for growth. Recently, however, phosphatidylinositol 3,4,5-triphosphate, which is produced by phosphatidylinositol 3-kinase and can activate Akt (64), has also been identified as a substrate of PTEN besides FAK (47, 48). PTEN also decreases phosphorylation levels of Shc in response to growth factor receptor activation and suppresses MAP kinase activity (49). Taken together with our results that PTEN can directly dephosphorylate FAK, but also that FAK rephosphorylation could rescue only about 60% of elevated FAK observed in transformed cells (56–58) may form complexes with p130Cas, leading to invasion events involved in the dissemination of tumors.

Most types of normal cells require attachment to extracellular matrix components to be able to grow. The loss of integrin-mediated cell-matrix contact induces apoptosis, termed anoxia, in many nontransformed cell types (65–68). In contrast, the ability of malignant cells to proliferate in the absence of adhesion, termed anchorage independence of growth, correlates closely with tumorigenicity. Recent studies have implicated FAK in this cell survival. Inhibition of FAK in fibroblasts and endothelial cells results in growth suppression (53) and apoptosis (46), although FAK may not mediate survival in all cases (69). Conversely, overexpression of activated FAK can rescue MDCK cells from anoxia (45). In one case, activated FAK has even been reported to induce anchorage-independent growth and tumor formation by MDCK cells (45). These results provide evidence 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 (56–58).

Interestingly, although PTEN inhibits FAK and suppresses cell growth, we could not detect PTEN-induced apoptosis in this study. PTEN might affect tumor progression by its role in down-regulating cell invasion through basement membranes by changes in focal adhesion or cytoskeletal formation, because mutation or decreased expression of PTEN is associated with tumor progression in certain cancers (6, 19, 20). Moreover, PTEN inactivation has been shown recently to enhance the ability of ES cells to grow in an anchorage-independent manner (70).

In summary, we have shown that PTEN restoration in glioblastoma cells with mutated PTEN alleles results in inhibition of cell invasion, migration, and growth in a process that involves phosphorylation of FAK. Alterations in p130Cas were also implicated in PTEN inhibition of cell invasion and migration but not in its effects on cell growth, suggesting that there are different downstream signaling pathways that diverge at the level of FAK. As documented in this study, such cell surface roles of PTEN appear to distinguish it from other tumor suppressors, such as p53 or Rb, which typically function in the nucleus (71). Recent studies indicate a role for PTEN inactivation in tumor progression, and our findings suggest that potential therapeutic targets of antimitostatic approaches may include FAK and p130Cas.

REFERENCES

PTEN IN INVASION, MIGRATION, AND GROWTH


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