PTEN blocks insulin-mediated ETS-2 phosphorylation through MAP kinase, independently of the phosphoinositide 3-kinase pathway

Liang-Ping Weng¹²⁴⁶, Jessica L. Brown¹²⁶, Kim M. Baker⁵⁶, Michael C. Ostrowski⁵⁶ and Charis Eng¹⁻⁷,*

¹Clinical Cancer Genetics Program, ²Human Cancer Genetics Program, ³Division of Human Genetics, Department of Internal Medicine, ⁴Division of Human Cancer Genetics, Department of Molecular Virology, Immunology and Medical Genetics, ⁵Department of Molecular Genetics and ⁶Comprehensive Cancer Center, The Ohio State University, Columbus, OH, USA and ⁷CRC Human Cancer Genetics Research Group, University of Cambridge, UK

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The tumor suppressor PTEN possesses lipid and protein phosphatase activities. It has been well established that the lipid phosphatase activity is essential for its tumor-suppressive function via the phosphoinositide 3-kinase (PI3K) and Akt pathways. The precise role of the protein phosphatase activity is still unclear. In the current study, we demonstrate that overexpression of wild-type PTEN in the MCF-7 breast cancer line results in phosphatase activity-dependent decreases in the phosphorylation of ETS-2, which is a transcription factor whose DNA-binding ability is controlled by phosphorylation. Exposure of MCF-7 cells to insulin, insulin-like growth factor 1 (IGF-1) or epidermal growth factor (EGF) can lead to the phosphorylation of ETS-2, Akt and ERK1/2. The MEK inhibitor PD590089 abrogates insulin-stimulated phosphorylation of ETS-2. In contrast, the PI3K inhibitor LY492002 has no effect on insulin-stimulated phosphorylation of ETS-2, despite the fact that it diminishes insulin-stimulated phosphorylation of Akt. Interestingly, overexpression of PTEN in MCF-7 leads to blockade of insulin-stimulated, but not EGF-stimulated, phosphorylation of ERK, accompanied by dramatic decreases in ETS-2 phosphorylation. We further show that the relationship of PTEN and ETS-2 has functional significance by demonstrating that PTEN abrogates activation of the uPA Ras-responsive enhancer, a target of ETS-2 action, in a phosphatase-dependent manner, irrespective of the presence or absence of insulin. Our observations, therefore, suggest that PTEN blocks insulin-stimulated ETS-2 phosphorylation through inhibition of the ERK members of the MAP kinase family independently of PI3K, and that the PTEN effect on the phosphorylation status of ETS-2 may be mediated through PTEN’s protein phosphatase activity.

INTRODUCTION

The tumor suppressor gene PTEN/MMAC1/TEP1 (1–3) encodes a dual-specificity phosphatase with N-terminal homology to the cytoskeletal proteins tensin and auxilin, and is almost ubiquitously expressed, to a greater or lesser extent, during human embryonic and fetal development as well as in many adult tissues (1,3,4). Germline mutations of PTEN cause the inherited tumor syndrome Cowden syndrome, which is characterized by multiple hamartomas and a high risk of breast, thyroid and endometrial cancers (5–8). Recent observations suggest that germline PTEN mutations can also cause a broad variety of developmental abnormalities that can lead to a subset of Bannayan–Riley–Ruvalcaba and Proteus syndromes (9–11). Germline PTEN mutation-positive families appear to be at higher risk of developing breast tumors compared with mutation-negative families (12,13). Interestingly, somatic PTEN mutations and deletions appear to be associated with either later stages or higher grades of sporadic neoplasia, for example those of the breast, prostate and brain, and melanoma (14–17). Thus, in addition to tumor initiation (illustrated by the consequences of germline mutation causing an inherited cancer syndrome), PTEN almost certainly can be expected to play an important role in tumor progression and metastases as well.

PTEN is the major 3-phosphatase of phosphoinositide-3,4,5-trisphosphate (PIP3), a product of phosphoinositide 3-kinase

*To whom correspondence should be addressed at: The Ohio State University Human Cancer Genetics Program, 420 W. 12th Avenue, Suite 690 Tzagournis MRF, Columbus, OH 43210, USA. Tel: +1 6142922347; Fax: +1 6146883582; Email: eng-1@medctr.osu.edu
This lipid phosphatase activity is important in growth suppression through inhibition of the PI3K/Akt pathway (18–20). In addition to its lipid phosphatase activity, PTEN has been shown to dephosphorylate phosphopeptides at tyrosine and serine/threonine, at least in vitro (21,22). It was suggested that PTEN dephosphorylates focal adhesion kinase (FAK) directly to effect changes in cell motility and cell–cell interaction (23,24). Unfortunately, the phosphorylation levels of FAK in pten−/− embryonic stem cells were entirely normal. Therefore, the target and downstream pathways directly affected by PTEN’s protein phosphatase activity remain obscure. What is known, however, is that PTEN’s growth-suppressive consequences can be mediated via lipid phosphatase–PI3K/Akt-dependent and -independent pathways (25,26).

We have found that PTEN can exert its growth-suppressive effects by blocking insulin-stimulated phosphorylation of nitrogen-activated protein kinase (MAP kinase, MAPK) (27 and this report). The Ras/MAPK pathway is a central signal transduction pathway involved in the regulation of a broad range of cellular function. Well-defined nuclear targets of this pathway include members of the Ets family of transcription factors, including elk-1 and ETS-2 (reviewed in 28 and 29). For example, the factor ETS-2 is phosphorylated on threonine residue 72 in a Ras-dependent fashion, and phosphorylation of this site leads to an increased expression of ETS-2 target genes such as urokinase plasminogen activator (uPA), heparin-binding epidermal growth factor (HBE GF) and stromelysin (30–33). Phosphorylation of ETS-2 does not affect the DNA-binding properties of this factor, but increases its ability to transactivate target genes (30,31). Several studies have demonstrated that Ras can stimulate ETS-2 phosphorylation at position threonine 72 through the Raf/MAPK pathway (31,32,34,35). However, in macrophages, the PI3K/Akt pathway can also lead to phosphorylation of the same site through activation of p54 Jun N-terminal kinase (JNK) (36).

Overexpression of human ETS-2 transforms NIH 3T3 cells, allowing these cells to grow in soft agar and form tumors in nude mice (37). Similarly, an ETS-2 dominant-negative gene can inhibit Ras-dependent transformation of NIH 3T3 cells (38), and the same transdominant ETS-2 gene has been shown to abolish anchorage-independent growth and invasion of BT-20 breast carcinoma cells (39). Further, ETS-2 expression correlates with cell proliferation (37,40), and ETS-2 can activate the promoter of cyclin D1, a key positive regulator of the G1/S cell cycle progression and can upregulate cdc and cyclin A expression (41,42).

Because we have shown that PTEN can abrogate insulin-stimulated MAPK phosphorylation, and ETS-2 is immediately downstream of the MAPK and PI3K/Akt pathways, we explore the relationship of PTEN and ETS-2 phosphorylation in MCF7 cells in this study.

RESULTS

PTEN abrogates ETS-2 phosphorylation in a phosphatase activity-dependent manner

We began to investigate whether PTEN could be involved in the regulation of ETS-2 function by affecting its phosphorylation on the key threonine 72 residue, which is necessary for ETS-2 transactivation activity. We, therefore, chose to examine ETS-2 phosphorylation in the MCF-7 breast cancer line stably transfected either with wild-type PTEN (MCF-7/PTEN-wt) or phosphatase-dead C124S mutated PTEN (MCF-7/PTEN-cs), whose transcription is controlled by a Tet-off promoter system (see Materials and Methods). The phosphorylation status of ETS-2 was assessed by western blot analysis using a specific polyclonal antibody that recognizes ETS-2 only when phosphorylated at threonine 72. Because the endogenous, basal level of ETS-2 phosphorylation was extremely low in MCF-7 cells, both MCF-7/PTEN-wt and MCF-7/PTEN-cs were exposed to insulin for 30 minutes prior to evaluation of ETS-2 phosphorylation. Induction of wild-type PTEN in MCF-7 resulted in marked decreases in the phosphorylation levels of ETS-2, Akt and the ERK members of the MAPK family (ERK1/2) (Fig. 1: WT, Tet− versus Tet+). Decreased P-Akt in response to wild-type PTEN overexpression acted as a control. In contrast, induction of phosphatase-dead PTEN did not affect the phosphorylation status of ETS-2, Akt or MAPK (Fig. 1: CS, Tet+ and Tet−).

The MAPK, but not the PI3K, pathway is essential for growth factor-stimulated, receptor tyrosine kinase-mediated phosphorylation of ETS-2

Activated receptor tyrosine kinases can initiate signal transduction down several separate and overlapping pathways, the most important of which include the MAPK and PI3K pathways. To determine which of the major signaling pathways are involved...
in the stimulation of ETS-2 phosphorylation in the context of PTEN and the MCF-7 system, we analyzed the status of ETS-2 phosphorylation after exposure to several growth factors and related stimuli, in the first instance. When native MCF-7 cells, which have endogenous wild-type PTEN, were exposed to the growth factors insulin, epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1), marked increases in ETS-2 phosphorylation, accompanied by Akt and MAPK phosphorylation, were observed (Fig. 2A). Exposure to 2% serum also resulted in some increase in the phosphorylation of ETS-2, Akt and MAPKs. No differences in the phosphorylation levels of stress-activated protein kinase (SAPK) and p38 were observed in MCF-7 cells exposed to the three growth factors or to serum. Osmotic shock, achieved by exposure to 0.7M sodium chloride resulted in increases in phosphorylation of all three members of the MAPK family [ERK1/2 and MAP/ERK kinase (MEK)] and
p38, but not ETS-2 or Akt (Fig. 2A). Exposure of MCF-7 to ultraviolet irradiation resulted in marked increases in the phosphorylation levels of Jun/SAPK and p38, but not Akt or ERK1/2 (Fig. 2A). These observations suggest that the ERKs and/or PI3K could be important in regulating ETS-2 phosphorylation.

To further delineate whether the MAPK or PI3K pathway, or both, is important in growth factor-stimulated ETS-2 phosphorylation, we analyzed insulin-stimulated ETS-2 phosphorylation in the presence or absence of specific inhibitors of either the MAPK pathway or the PI3K pathway. Pretreatment of MCF-7 cells with PD98059, a MEK inhibitor, was found to abrogate insulin-stimulated phosphorylation of ETS-2 and ERK1/2, without affecting Akt phosphorylation (Fig. 2B: lane 3 versus lane 2). In contrast, pretreatment of MCF-7 cells with LY294002, a PI3K-specific inhibitor, had no effect on the phosphorylation of ETS-2 or ERK1/2, while inhibiting Akt phosphorylation (Fig. 2B: lane 4). These observations, therefore, suggest that the ERK members of the MAPK family, but not the PI3K pathway, must play an essential role in growth factor-stimulated ETS-2 phosphorylation.

PTEN does not affect the phosphorylation of FAK or Shc

Evidence in the literature suggests that PTEN inhibits integrin- and EGF-stimulated ERK1/2 activation by dephosphorylation of either FAK or Shc in the U87M glioma cell line (43). Thus, to explore the possible involvement of FAK and/or Shc in PTEN-mediated growth factor-stimulated ETS-2 phosphorylation, we examined the phosphorylation status of FAK and Shc in the MCF-7 context using immunoprecipitation–western blot analysis. Anti-FAK immunoprecipitates subjected to western blot with the anti-phosphotyrosine antibody 4G10 revealed only a weak signal at 120 kDa and two dominant bands/clusters at and below 45 kDa (Fig. 3A, top), irrespective of PTEN expression levels (Fig. 3A, top, middle two lanes). The two bands around 120 and 70 kDa elucidated by 4G10 remained unchanged before and after immunoprecipitation with anti-FAK antibodies (Fig. 3A, top: first two versus last two lanes). As a control, the same filter was subjected to western blot with anti-FAK antibody (Fig. 3A, bottom). This revealed that FAK was greatly enriched in the anti-FAK immunoprecipitate (Fig. 3A, bottom: middle two lanes) and depleted after immunoprecipitation (Fig. 3A, bottom: last two versus first two lanes). Thus, our observations would suggest that FAK is not the dominant tyrosine-phosphorylated protein in MCF-7 cells after insulin stimulation. Furthermore, overexpression of PTEN did not affect overall tyrosine phosphorylation or tyrosine phosphorylation on FAK.

Similarly, after separation through SDS–PAGE, anti-Shc immunoprecipitates were subjected to western blot with 4G10 (Fig. 3B). We show that tyrosine phosphorylation on Shc and total Shc protein were unaltered in anti-Shc immunoprecipitates, whether or not PTEN was overexpressed (Fig. 3B, top and bottom panels). As a control, anti-Shc immunoprecipitate was shown to be enriched for Shc, where all three isoforms are clearly evident (Fig. 3C: lane 3), compared with the pre-immunoprecipitation lysate (Fig. 3C: lane 1). The lysate remaining after immunoprecipitation was depleted of Shc (Fig. 3C: lane 2). Therefore, these latter experiments suggest that the phosphorylation of Shc on tyrosine appears to be unaffected by PTEN expression levels and insulin stimulation.

PTEN differentially regulates insulin- and EGF-stimulated ETS-2 phosphorylation

It is well established that Ras and Raf are the common central intermediates connecting peptide growth factors and their receptors (mainly receptor tyrosine kinases) to MAPK activation. We have shown that overexpression of PTEN leads to inhibition of insulin-stimulated phosphorylation of both ETS-2 and the ERKs (see above). Thus, to determine if PTEN can play a more generalized role in the negative regulation of ETS-2 phosphorylation in response to growth factor stimulation, we examined the effect of PTEN overexpression on ETS-2 phosphorylation in response to incremental time exposure to insulin and EGF (Fig. 4). Stimulation of MCF-7 cells with insulin resulted in dramatic increments in ETS-2 phosphorylation irrespective whether or not PTEN was overexpressed (Fig. 4, top panel: 10 min versus 0 min, Tet + and Tet−). Approximately 30 minutes after insulin exposure, ETS-2 phosphorylation was stable in the absence of PTEN induction, but began to decline rapidly when PTEN was overexpressed (Fig. 4, top panel: 30 min, Tet− versus Tet+). After 2 hours, phosphorylated ETS-2 levels returned to baseline. Thus, it would appear that PTEN did not affect the initiation and incremental phases of ETS-2 phosphorylation, but strongly inhibited sustained ETS-2 phosphorylation in response to insulin stimulation. In parallel, phosphorylation of ERK1/2 was dramatically increased 10 minutes after insulin stimulation, irrespective of PTEN expression status (Fig. 4 left, top panel: 10 min, Tet+ and Tet−), stabilized at 30 minutes and at 2 hours without PTEN induction, but rapidly declined in the presence of PTEN overexpression at 30 minutes and to baseline after 2 hours (Fig. 4, third panel: P-MAPK).

In direct contrast, while phosphorylation of ETS-2 also dramatically increased 10 minutes after EGF exposure, irrespective of PTEN expression status (Fig. 4 right, top panel: 10 min, Tet+ and Tet−), PTEN overexpression had little, if any, effect on ETS-2 phosphorylation after 30 minutes and after 2 hours (Fig. 4 right, top panel: 30 and 120 min, Tet+ and Tet−). PTEN had no obvious effect on ERK1/2 phosphorylation at all time points (Fig. 4 right, third panel). It should be noted, however, that EGF-stimulated increments in the phosphorylation of ETS-2 and ERK1/2 are much stronger than those induced by insulin exposure (Fig. 4, right versus left).

PTEN differentially regulates insulin- and EGF-stimulated activation of the uPA promoter by ETS-2

In order to determine if PTEN regulation of ETS-2 phosphorylation also had functional significance, the activation of the uPA Ras-responsive enhancer, a well-defined target of ETS-2 action (30,32) was studied in the MCF-7 cells that conditionally expressed PTEN (Fig. 5). For these experiments, the uPA firefly luciferase reporter activity was stimulated approximately 10-fold when co-transfected with an expression vector for human ETS-2 compared with empty expression vector. Insulin treatment stimulated uPA reporter activity approximately 2-fold. The combination of ETS-2 expression vector and
insulin resulted in a 30-fold induction of uPA reporter activity—an additional 3-fold stimulation over ETS-2 alone (Fig. 5A: first three bar graphs). That this cooperation between insulin and ETS-2 depended on phosphorylation of the threonine 72 residue was confirmed by experiments in which an expression vector for the mutated ETS2 T72A gene was co-transfected (Fig. 5A: two bar graphs in the middle). In this case, either in the presence or in the absence of insulin, about a 2-fold activation of the uPA promoter was observed, consistent with previously published results obtained with ETS2 T72A in response to Ras signaling events (30).

Conditional expression of PTEN-wt in MCF-7 cells following co-transfection of the uPA luciferase reporter and ETS-2 abolished activation of the uPA reporter by ETS-2 in either the absence or the presence of insulin (Fig. 5A: bars to right). Conditional expression of the PTEN-cs mutation had no significant effect on the ability of ETS-2 to activate the uPA promoter either in the absence or the presence of insulin (Fig. 5A), confirming that the phosphatase activity of PTEN was essential for regulation of ETS-2 phosphorylation and activity.

Treatment of ETS-2 co-transfected cells with EGF resulted in an over 50-fold activation of uPA promoter activity—an effect that was significantly greater than the effect observed with insulin alone, and correlated with the increased stimulation of ETS-2 phosphorylation by EGF versus insulin (Fig. 4). This cooperative effect between ETS-2 and EGF also depended on the presence of ETS-2 threonine 72, since EGF did not further stimulate uPA reporter activity in the presence of the mutant expression vector for ETS-2 T72A (Fig. 5B: bar graphs in the middle). In contrast to the results obtained with insulin and ETS-2, the combination of EGF and ETS-2 was not inhibited by expression of PTEN-wt, (Fig. 5B: bar graphs to the right).
DISCUSSION

While the role of the lipid phosphatase activity in the action of the tumor suppressor PTEN is well accepted, recent work by our group and others indicates that the protein phosphatase activity is also critical for the growth-suppressive effects of this gene—at least in some types of cells (27,44,45). In particular, the protein phosphatase activity of PTEN appears to be responsible for the negative regulation of growth-stimulatory genes such as cyclin D1 and for the suppression of insulin-stimulated Raf/MEK-1/ERK activation in the breast cancer cell line MCF-7. One nuclear target of Ras signaling pathways is the transcription factor ETS-2 (reviewed in 29,46). This protein is rapidly phosphorylated in response to Ras signaling pathways at a specific residue, threonine 72 (30)—an event that leads to activation of a diverse set of target genes, including genes regulating the cell cycle (e.g. cyclin D1) and extracellular proteases involved in tumor invasiveness and metastasis (e.g. uPA).

ETS-2 was rapidly phosphorylated in response to insulin stimulation in MCF-7 cells, and conditional expression of PTEN in these cells decreased ETS-2 phosphorylation. Additionally, PTEN inhibited activation of the uPA promoter by insulin and ETS-2. In fibroblasts and ovarian tumor cells, the Raf/MEK/ERK pathway is the chief Ras-effector pathway that activates ETS-2 phosphorylation (32,34); however, in macrophages, both the Raf/MEK/ERK pathway and the PI3K/Akt pathway can mediate ETS-2 phosphorylation (36). PTEN modulation of ETS-2 phosphorylation could potentially deregulate both of these pathways. The use of pharmacological inhibitors specific for the two pathways demonstrated that ETS-2 phosphorylation correlated with activation of the ERK pathway. Thus, ETS-2 phosphorylation and its subsequent activation is negatively regulated through the phosphatase activity of PTEN. In the context of these data, therefore, one must consider what potential target genes are regulated by ETS-2 and which of these are subject to negative regulation by PTEN. In transient assays, PTEN negatively regulated ETS-2 activity when either the uPA reporter containing an ETS-AP1 Ras-responsive enhancer element or a palindromic ETS-site element based on the stromelysin Ras-responsive enhancer (M.C. Ostrowski et al., unpublished data) were used. However, these two pieces of data do not directly address which of the approximately 50 genes known to be regulated by ETS-2 are also negatively regulated by PTEN in breast cancer cells. Hypothetically, PTEN activity could downregulate expression of all ETS-2 target genes, including extracellular proteases such as uPA and stromelysin/MMP3, growth factors such as HBEGF (31), and cell cycle regulators such as cyclin D1 (41). Alternatively, PTEN may negatively regulate only discrete subsets of these genes, depending on the signal that initiates gene expression, the context of the ETS-enhancer element within the target gene, and the presence and activation of other transcription factors that interact with ETS-2 (e.g. AP1). The set of ETS-target genes that PTEN negatively regulates in breast cancer cells may have important biological significance, and a rigorous examination of this problem is now warranted based on the results presented here.

Where in the Ras-ERK-ETS-2 pathway does PTEN exert its effects? FAK activation or tyrosine phosphorylation of the Ras pathway adapter She do not seem to be involved in this regulation, despite a previous report indicating that FAK was a target for PTEN regulation of the ERK pathway (23,24). A kinetic analysis of ERK and ETS-2 activation in response to
insulin indicates that regulation must occur upstream of ERK.
In other words, PTEN most likely does not directly dephosphorylate ETS-2 but instead acts upstream of ETS-2 and ERK, and thus indirectly inhibits ETS-2 phosphorylation. Both ERK and ETS-2 phosphorylation occurred immediately after insulin stimulation, even in the presence of PTEN overexpression. However, inactivation of both ERKs and of ETS-2 occurred within 30 minutes in the presence of PTEN. These data are consistent with our recent observations indicating that the insulin receptor adapter molecule IRS-1 is likely the target for PTEN protein phosphatase activity in MCF-7 cells (27).

While ERK1/2 activation, ETS-2 phosphorylation, and ETS-2 activation of the uPA Ras-responsive enhancer were stimulated by both insulin and EGF, EGF activation of these downstream effectors was refractory to PTEN protein phosphatase activity in MCF-7 cells. This is in contrast to the lipid phosphatase activity of PTEN, which prevents full activation of Akt whether insulin or EGF signaling is studied, even at early time points. This finding supports the hypothesis that the PTEN target in the insulin pathway is upstream of Ras and is receptor-specific. The idea that the protein phosphatase activity of PTEN is selective for certain tyrosine kinases may have implications for our understanding of the role of PTEN in tumor formation within different organ systems. In sporadic breast cancer, amplification of EGF family receptors is frequently encountered—an event that could render a tumor cell resistant to PTEN repression of the Ras/Raf/ERK pathway. Thus, inhibition of PI3K/Akt signaling alone may not alone provide an effective strategy for breast cancer treatment.

MATERIALS AND METHODS

Cell culture

The MCF-7 breast cancer cells expressing wild-type and the phosphatase-dead mutant C124S PTEN were generated as described previously (47). Cells were maintained in DMEM/10% fetal bovine serum (FBS) (Gibco BRL Life Technologies, Grand Island, NY) with 100 units/ml penicillin G (Sigma, St Louis, MO), 100 μg/ml streptomycin sulfate (Sigma) and in similar media plus 100 μg/ml Geneticin and 1 μg/ml tetracycline (Sigma).

Induction of PTEN expression

Induction of PTEN expression with the Tet-off system was performed as previously described (47). Briefly, subconfluent stock cells were washed twice with PBS, trypsinized by phenol red-free trypsin–EDTA (Gibco BRL Life Technologies) and

Figure 5. PTEN inhibits insulin, but not EGF, stimulation of uPA promoter activity by ETS-2. Transient transfection assays were performed in MCF-7 cells that expressed tetracycline-regulated forms of PTEN. Cells were transfected with 2 µg of uPA-luciferase reporter plasmid and 0.2 µg of ETS-2 or ETS-2 T72A expression vector as indicated. Renilla luciferase (0.2 µg) was included as an internal control. Cells were treated with 10 µg/ml of insulin (A) or 50 ng/ml of EGF (B), as indicated, for 4 h prior to cell harvest. PTEN or PTEN-cs were induced by removal of tetracycline from the cell culture media (see Materials and Methods). Firefly luciferase activity was adjusted for both total protein content of cell extracts and for Renilla luciferase activity, and is expressed in relative light units. The average of three independent experiments performed in duplicate is presented and error bars indicate standard deviation.
diluted at a 1:3 ratio into either estrogen-free culture media, which contain MEBM (phenol red-free, Clonetics, Palo Alto, CA), 5% charcoal/dextran-treated FBS (HyClone, UT) or regular DMEM (Gibco BRL Life Technologies) with 5% regular FBS for 3 days. Tetacycline (1 μg/ml) was included. After 3 days, equal numbers of cells were plated into the same medium in the absence of tetracycline (Tet+) to induce PTEN expression and into medium containing 1 μg/ml tetracycline (Tet−) as a control, and cultured for 48 h, unless otherwise noted.

Protein extraction and immunoblotting

After PTEN induction, cells were washed twice with ice-cold PBS and lysed in cold lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 5 μM PMSF, 5 μg/ml Leupeptin, pepstatin A and Aprotinin, 1 mM Na3VO4, 2 mM NaF, 2 mM Na4PO7, and 10 mM β-glycerophosphate) for 10 min on ice. Insoluble material was removed from cell lysates by centrifugation at 4°C. Protein concentration was calculated using the Bradford reagent. The Bradford reagent and other chemicals were resolved by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 5 μM PMSF, 5 μg/ml Leupeptin, pepstatin A and Aprotinin, 1 mM Na3VO4, 2 mM NaF, 2 mM Na4PO7, and 10 mM β-glycerophosphate) for 10 min on ice. Insoluble material was removed from cell lysates by centrifugation at 4°C. Protein concentration was calculated using the Bradford reagent. The Bradford reagent and other chemicals were purchased from Sigma. Cell lysates were mixed with equal volumes of 2× Laemmli sample buffer, boiled for 10 min, resolved by 10% SDS–PAGE, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in TBST (10 mM Tris–HCl, pH 8.0, 100 mM NaCl and 0.05% Tween-20) or TBST with 5% BSA for 1 h at room temperature, and were then incubated with appropriate primary antibody for 2 h at room temperature or overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody (Promega, Madison, WI) at 1:5000 dilution for 1 h at room temperature. Protein signals were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ).

For growth factor stimulation, cells were starved by exposure to serum-free medium for 24 h before adding growth factor(s). Insulin, IGF-1, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and EGF were purchased from Gibco BRL. Wortmannin and phorbol-13-myristate-13-acetate (PMA)–EGF were purchased from Sigma. PD590089 and 1-oleoyl-lysophosphatidic acid (LPS)–EGF were purchased from New England Biolabs (Boston, MA) and Cayman Chemical (Ann Arbor, MI), respectively. The anti-PTEN monoclonal antibody 6H2.1 was raised against the C terminus of PTEN, and has been shown to be specific (48,49). The polyclonal anti-phospho-Akt, anti-Akt, anti-phospho-MAPK, anti-MAPK, anti-phospho-MEK1/2, anti-phospho-SAPK and anti-phospho-p38 (New England Biolabs) were used at 1:1000 dilution. Polyclonal anti-IRS-1, anti-IRS-2, anti-IRβ, anti-Sos1/2, anti-MEK1 and anti-MEK2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were employed at 1:250 dilution, 4G10 and polyclonal anti-p85 PI3K (Upstate Biotechnology Inc., Lake Placid, NY) at 1 μg/ml, and monoclonal anti-α-tubulin (Sigma) at 1:5000 dilution. Monoclonal and polyclonal anti-Shc and monoclonal and polyclonal anti-Grb2 (Transduction Laboratory, San Diego, CA) were used at 1 μg/ml, 0.1 μg/ml, 0.5 μg/ml and 1 μg/ml, respectively. Anti ETS-2 antibodies (both anti-pT72 and non-phospho-discriminating antibodies) have been previously described (32).

All experiments were performed in triplicate.

Immunoprecipitation

After PTEN induction, cells were washed twice with ice-cold PBS and lysed in cold lysis buffer. After removal of insoluble material, the supernatant was precleared with protein A/agarose (Santa Cruz) or anti-mouse IgG/agarose (Sigma). 500 ml of the cell lysates (500 μg protein/ml) were incubated with 2.5 μg of the appropriate monoclonal or polyclonal antibodies for 2 h or overnight at 4°C, followed by 10 μl of packed protein A/agarose or anti-mouse IgG/agarose for 2 h. The immunoprecipitates were washed three times with lysis buffer and then resuspended in Laemmli sample buffer. The immunoprecipitates were boiled for 5 min, centrifuged, and the protein in the supernatant resolved by SDS–PAGE.

Transient transfection assays

MCF-7 cells expressing either PTEN-wt or PTEN-C124S were transfected by the calcium phosphate method as previously described (30), with the following modifications. One day before transfection, 1 x 106 cells were plated in 100 mm dishes in estrogen-free media containing 1 μg/ml tetracycline as described above. The following day, cells were transfected with 10 μg/ml total DNA. The urokinase plasminogen activator Ras-responsive enhancer/promoter–firefly luciferase reporter and expression vectors for ETS2 wild type and ETS2 T72A have been previously described (30). An expression vector for Renilla luciferase (pRL–CMV, Promega, Madison, WI) was included as an internal control for transfection efficiency (0.2 μg per DNA precipitate).

After 16 h, DNA precipitates were removed and cells were placed in media with 0.5% FCS for 24 h, either with or without tetracycline as indicated in the legend to Figure 5. Cells were treated with either 10 μg/ml insulin or 50 ng/ml EGF for 4 h. Cell extracts were prepared and assayed for both firefly and renilla luciferase activity, and the protein concentration of extracts was determined. Firefly luciferase activities were normalized to both Renilla luciferase activity and protein concentration. Transfection data were analyzed by ANOVA using pairwise comparisons to determine the statistical significance of the differences measured.

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