The phosphatase and tensin homolog regulates epidermal growth factor receptor (EGFR) inhibitor response by targeting EGFR for degradation

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Edited by Webster K. Cavenee, Ludwig Institute, University of California, La Jolla, CA, and approved February 25, 2010 (received for review September 29, 2009)

The phosphatase and tensin homolog (PTEN) is a tumor suppressor that is inactivated in many human cancers. PTEN loss has been associated with resistance to inhibitors of the epidermal growth factor receptor (EGFR), but the molecular basis of this resistance is unclear. It is believed that unopposed phosphatidylinositol-3-kinase (PI3K) activation through multiple receptor tyrosine kinases (RTKs) can relieve PTEN-deficient cancers from their “dependence” on EGFR or any other single RTK for survival. Here we report a distinct resistance mechanism whereby PTEN inactivation specifically raises EGFR activity by impairing the ligand-induced ubiquitylation and degradation of the activated receptor through destabilization of newly formed ubiquitin ligase Cbl complexes. PTEN-associated resistance to EGFR kinase inhibitors is phenocopied by expression of dominant negative Cbl and can be overcome by more complete EGFR kinase inhibition. PTEN inactivation does not confer resistance to inhibitors of the MET or PDGFRA kinase. Our study identifies a critical role for PTEN in EGFR signal termination and suggests that more potent EGFR inhibition should overcome resistance caused by PI3K pathway activation.

Cbl | ubiquitylation | PTEN | glioma | drug resistance

The phosphatidylinositol-3-kinase (PI3K) pathway has emerged as the most frequently deregulated signaling pathway in human cancer. Activation of this pathway in cancer cells can occur through a variety of mechanisms, including mutations in genes encoding the catalytic (PIK3CA) or regulatory (PIK3R1) subunit of class IA PI3Ks, the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), Akt family members, Ras family members, neurofibromin 1, and/or various growth factor receptors. Inactivation of PTEN through missense mutations, deletions, and epigenetic mechanisms represents the most common cause of PI3K pathway activation in human cancer (1).

The PTEN protein exhibits dual protein and lipid phosphatase activity. Most of PTEN’s tumor suppressor functions have been attributed to its lipid phosphatase activity which hydrolyzes phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)_P3] at the D3 position of the inositol ring and directly antagonizes the function of phosphatidylinositol-3-kinase. PTEN loss leads to accumulation of PtdIns(3,4,5)_P3 which recruits proteins containing pleckstrin homology domains to cellular membranes, including the serine/threonine kinase Akt. In addition to its role in tumor suppression, PTEN has emerged as a determinant of tumor cell response to ATP-site competitive inhibitors of the epidermal growth factor receptor (EGFR) in EGFR amplified cancer cell lines (2) and in glioblastoma (GBM) patients whose tumors expressed the oncogenic EGFR variant III (EGFRvIII) mutant receptor (3).

How PTEN’s functions as tumor suppressor and drug response modifier relate to each other is currently unclear. One possibility is that PTEN inactivation relieves EGFR mutant cancer cells from their dependence on EGFR for survival by allowing sufficient PtdIns(3,4,5)_P3 accumulation and Akt activation through other growth factor receptors. However, although there is evidence for receptor tyrosine kinase coactivation in cancer (4) and certain growth factor receptors have been shown to mediate resistance to EGFR kinase inhibitors (5–7), it is unknown which kinase(s), if any, might substitute for EGFR in the setting of PTEN inactivation. Furthermore, PTEN inactivation has only been associated with clinical resistance to inhibitors of EGFR and its coreceptor HER2, but not other growth factor receptors. In this study, we sought to determine the molecular mechanism whereby PTEN inactivation confers resistance to EGFR kinase inhibitors.

Results

PTEN Knockdown Confers Resistance to EGFR, but Not MET or PDGFR Kinase Inhibitors. To test whether PTEN inactivation relieves cancer cells from their “dependence” on any single growth factor signal for survival, we selected a panel of cancer cell lines with distinct activated growth factor receptors, infected them with a retroviral PTEN shRNA, derived sublines with stable PTEN knockdown, and then determined how PTEN knockdown influenced the response of these cells to inhibitors of the respective oncogenic kinase. We focused on EGFR, platelet-derived growth factor receptor A (PDGFRA) and MET because mutations in these growth factors are found in multiple human cancer types and because small molecule inhibitors of these kinases are either already in use or in advanced stages of clinical development for the treatment of human cancer.

Treatment of EGFR-amplified A431 cells with the irreversible EGFR kinase inhibitor EKB-569 resulted in dose-dependent induction of cell death as determined by the trypan-blue method and cleavage of the Caspase3 substrate PARP. In contrast to parental A431 cells, A431 cells with stable PTEN knockdown (Fig. L4) were markedly resistant to cell death induction by EKB-569.


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0911188107/DCSupplemental.
PTEN inactivation did not affect the response of transduced with a PTEN shRNA. (Fig. S1sensitivity of A431 PTENshRNA cells to EGFR kinase inhibitors lacks lipid phosphatase activity [mPTEN(G129E)], restored the wild-type PTEN, but not a naturally occurring PTEN mutant that lacks the targeting sequence of our hairpin. Reintroduction of PTENshRNA cells using a murine PTEN cDNA (mPTEN) that of the PTENshRNA construct, we reconstituted PTEN in A431 due to PTEN knockdown, rather than potential off-target effects 6460

Increased EGFR Activity in PTEN De

(Fig. 1B and Fig. S1A). To confirm that this resistance was indeed due to PTEN knockdown, rather than potential off-target effects of the PTENshRNA construct, we reconstituted PTEN in A431 PTENshRNA cells using a murine PTEN cDNA (mPTEN) that lacks the targeting sequence of our hairpin. Reintroduction of wild-type PTEN, but not a naturally occurring PTEN mutant that lacks lipid phosphatase activity [mPTEN(G129E)], restored the sensitivity of A431 PTENshRNA cells to EGFR kinase inhibitors (Fig. S1B). This result documents the specificity of our PTEN shRNA construct and indicates that the lipid phosphatase activity of PTEN is the critical mediator of PTEN-associated EGFR kinase inhibitor resistance.

The EGFR mutant lung cancer cell lines HCC827 (EGFRdel746-750) and HCC4006 (EGFRdel746-749) underwent cell death at lower EKB-569 concentrations than A431 cells, consistent with the reported enhanced sensitivity of these EGFR mutants to EGFR kinase inhibitors. This effect was again markedly blunted by PTEN knockdown (Fig. 1A and C). In contrast to our findings with EGFR mutant cancer cell lines, PTEN knockdown did not protect cancer cell lines harboring amplification of the MET growth factor receptor kinase (MKN45 and EBC1 cells) from cell death in response to the MET kinase inhibitor SU11274. PTEN knockdown also did not protect cell lines with amplification of the PDGFRα gene (H-1703 and TS-543 cells) from cell death induction by the PDGFR inhibitor imatinib (Fig. 1D). These results were confirmed with other inhibitors of the MET and PDGFR kinase.

**Increased EGFR Activity in PTEN Deficient Cells.** Our finding that PTEN inactivation did not affect the response of MET or PDGFR amplified cancer cells to inhibitors of the MET and PDGFR kinase, respectively, pointed toward a more intricate relationship between PTEN and EGFR. We therefore examined the biochemical effects of PTEN on the EGFR protein. Immunoblotting of A431 whole cell lysates with phosphosite-specific antibodies against EGFR demonstrated dose-dependent EGFR inhibition by EKB-569 in both parental and PTEN knockdown cells. However, PTEN knockdown cells consistently showed greater EGFR phosphorylation than their matched controls in the absence of drug and at equimolar concentrations of EKB-569 (Fig. 2A Upper). PTEN knockdown did not increase phosphorylation of the MET receptors under steady-state conditions and also did not right-shift the biochemical response of these receptors to SU11274 (Fig. 2A Lower), respectively.

We also examined the relationship between PTEN, EGFR phosphorylation, and EGFR kinase inhibitor response in pretreatment tumor samples from GBM patients who either responded to (n = 5) or failed (n = 5) EGFR kinase inhibitor therapy in the context of our previously reported clinical trial (3). The group of PTEN-deficient tumors that failed treatment showed a statistically significant increase in EGFR phosphorylation compared to PTEN-expressing tumors from clinical responders (P = 0.021, Fisher’s exact test) (Fig. 2B). Loss of PTEN protein expression was similarly associated with increased EGFR phosphorylation in glioblastomas, which did not express the EGFRvIII mutant (Fig. S2).

Because EGFR phosphorylation provides only a limited read-out for EGFR kinase activity, we next examined the effects of PTEN on a broader panel of EGFR substrates. These experiments took advantage of a microfluidic-based phosphopeptide array, which measures phosphorylation rates of 144 matrix-spotted
peptides following the addition of whole cell lysates, adenosine-5′-triphosphate, and a fluorescently labeled antiphosphotyrosine antibody (Fig. S3A). We previously identified 10 of these peptides as EGFR-regulated peptides (See Materials and Methods) and confirmed in preliminary experiments that most of these peptides indeed showed increased phosphorylation when we compared lysates from normal human astrocytes (NHA) engineered to express “high EGFR” levels with a matching “low EGFR” expressing subline (Fig. S3B). We then compared whole cell lysates from three different PTEN knockdown lines (A431, HCC4006, and NHA-EGFR) with lysates from their matching parental cell lines and found that 8/10 EGFR-related peptides showed significantly increased phosphorylation in all three PTEN knockdown lines (Fig. 2C). The other two peptides (DDR2_733_745_Y740 and VEGFR2_944_956_Y951) showed increased phosphorylation in 2/3 PTEN knockdown lines (Table S1). None of the other detectable peptides showed a similarly consistent change in phosphorylation rates in response to PTEN knockdown (P = 0.0173).

PTEN Accelerates Down-Regulation of Activated EGF Receptors. To determine how PTEN might regulate EGFR activity, we first surveyed RNA transcript levels for EGFR, its coreceptors, and its ligands in the A431 and HCC827 isogenic pairs, but did not find any statistically significant differences between PTEN-intact and

![Figure 2](#)

We next examined the effect of PTEN on ligand-induced EGFR activation and subsequent signal termination. Down-regulation of the EGFR protein in response to EGF was blunted in the A431 subline with PTEN knockdown when compared to parental A431 cells (Fig. 3A, compare lanes 8 and 4). We conducted similar experiments in PC3 cells, which are naturally PTEN deficient but in which PTEN expression could be restored acutely using a doxycycline-inducible PTEN allele. Pretreatment of these cells with doxycycline for 36 h resulted in robust PTEN induction and inhibition of pAkt (Fig. 3B). Treatment with EGF resulted in down-regulation of EGFR protein levels only after PTEN expression had been restored by pretreatment with doxycycline (Fig. 3B, lane 12 versus lane 6). Consistent with the accelerated down-regulation of EGFR, phosphorylation of the EGFR substrate Gab1 and the EGFR effector molecule ERK1/2 in response to EGF was markedly shortened in the presence of PTEN (Fig. 3B).

To explore whether PTEN also regulates EGFR signal termination in nonmalignant cells, we used embryonic fibroblasts derived from mice with a “floxed” PTEN gene [PTEN<sup>fl/fl</sup>/loxP mouse embryonic fibroblasts (MEFs)]. Due to the low amount of endogenous EGFR in these cells, we first retrovirally introduced human wild-type EGFR into these cells, derived stable subclones through antibiotic selection, and subsequently inactivated PTEN in a second round of infection with Cre recombinase. Consistent with our observations in the PC3 system, PTEN inactivation markedly blunted down-regulation of EGFR protein levels in response to EGF (Fig. 3C, lanes 6–10 vs. 1–5).

**PTEN Promotes Ubiquitylation of Activated EGF Receptors.** Down-regulation of activated EGFR receptors involves EGFR internalization from the cell surface and subsequent shuttling of the receptors through various intracellular compartments to the lysosome (8). We found no defect in EGFR internalization in PTEN-deficient cells using immunofluorescence and by measuring the shuttling of biotinylated EGFR from the cell surface to the cytoplasm (Fig. S4).

We next examined the effect of PTEN on the ligand-induced ubiquitylation of EGFR, a process that tethers the endocytosed receptor to the degradative, lysosomal pathway (i.e., rather than receptor recycling to the cell surface) (9). EGF stimulation of PTEN<sup>fl/fl</sup>/loxP MEFs for 5 minutes resulted in EGFR ubiquitylation, seen as high-molecular weight bands on ubiquitin immunoblots of EGFR immunoprecipitates. EGFR ubiquitylation was markedly reduced in PTEN<sup>−/−</sup> MEFs (Fig. 4A). We observed a similar effect of PTEN on EGFR ubiquitylation in all examined cancer cell lines. In A431 cells, EGF stimulation for 5 minutes resulted in EGFR ubiquitylation, seen again as upward smearing on ubiquitin immunoblots of EGFR immunoprecipitates and also, less dramatically, by the upward smearing on EGFR immunoblots. EGF-induced EGFR ubiquitylation in A431 cells was markedly reduced in the PTEN knockdown line (Fig. 4B). Conversely, acute PTEN restoration in PTEN-deficient PC3 cells (Fig. S5A) or in PTEN-deficient U87 glioblastoma cells (Fig. S5B) augmented EGF-induced ubiquitylation of EGFR (Fig. S5A). The endogenous del746-750 EGF mutant expressed in HCC827 cells showed less ubiquitylation than wild-type EGF in response to EGF, as previously reported (10), but this ubiquitylation defect was further enhanced by PTEN knockdown (Fig. S5C), suggesting that PTEN inactivation and EGFR mutation may affect receptor ubiquitylation through distinct molecular mechanisms. Of note, we did not observe any impairment in HGF-induced ubiquitylation of the Cbl client MET in our PTEN-isogenic cell lines, suggesting that the effect of PTEN on Cbl-mediated EGFR ubiquitylation was not due to a general impairment in Cbl function.

To determine whether the effect of PTEN on EGF-induced ubiquitylation of EGFR was mediated by the lipid phosphatase

![Fig. 4](https://www.pnas.org/cgi/doi/10.1073/pnas.0911188107) Vivanco et al.
activity of PTEN, we reexamined ligand-induced EGFR ubiquitylation in A431 PTEN shRNA cells with reconstitution of either wild-type or lipid-phosphatase-dead PTEN. Reconstitution of wild-type PTEN, but not the G129E PTEN mutant, was sufficient to restore EGFR ubiquitylation in response to EGF (Fig. 4C). Overexpression of a constitutively active mutant of Akt1 found in sporadic human cancers (Akt1 E17K) blunted the EGFR-induced ubiquitylation of EGFR (Fig. S6A) and conferred resistance to EKB-569 (Fig. S6B). Together, these data suggest that PTEN regulates EGFR ubiquitylation through PtdIns(3,4,5)P3 and Akt-dependent mechanisms.

PTEN Stabilizes the EGFR–CBL Complex. EGFR ubiquitylation is mediated by the Cbl family of RING domain E3 ubiquitin ligases, which bind to the activated receptor and assemble a multiprotein complex including hepatocyte growth factor-regulated substrate (Hrs) and other members of the endosomal sorting complex required for transport (ESCRT) (9). Because both c-Cbl and Cbl-b are recruited to the activated EGFR and serve redundant functions in receptor ubiquitylation, we examined the effect of PTEN on EGFR association with these Cbl family members. In parental A431 cells, immunoprecipitation of EGFR under non-denaturing conditions showed rapid recruitment of both Cbl family members to EGFR (Fig. 4D) with a more sustained association between EGFR and Cbl-b as previously reported (11). A431 cells with PTEN knockdown showed similarly rapid recruitment of both Cbl family members to EGFR, but the durability of the EGFR–Cbl complex was substantially shortened with barely detectable EGFR–CBL association beyond 5 min following EGF induction (Fig. 4D).

Consistent with the early disassembly of the EGFR–Cbl complex and incomplete EGFR ubiquitylation in the PTEN knockdown cells, we were unable to detect recruitment of ubiquitin binding protein Hrs to the EGFR complex in these cells (Fig. 4E).

Residual EGFR Activity Determines Cell Death in Response to EGFR Kinase Inhibition. Our finding that PTEN regulates EGFR signal termination raised the question of whether this function of PTEN might contribute to EGFR kinase inhibitor resistance in cells that require chronic EGFR signaling for survival (“EGFR-dependent cells”). If PTEN inactivation indeed conferred drug resistance through its effects on EGFR activity, i.e., rather than EGFR-independent mechanisms, we would expect that: (i) genetic inactivation of Cbl, the critical mediator of the PTEN–EGFR crosstalk, should be sufficient to confer EGFR kinase inhibitor resistance, and (ii) more complete EGFR kinase inhibition should overcome EGFR kinase inhibitor resistance associated with PTEN inactivation.

We first examined whether ectopic expression of 70Z-Cbl (12), a naturally occurring c-Cbl mutant, which lacks RING domain activity and functions as a dominant negative allele, rendered EGFR mutant cells resistant to cell death induction by EKB-569. Overexpression of 70Z-Cbl was sufficient to confer resistance to EKB-569 (Fig. 5A and Fig. S7).

We next examined whether PTEN-associated resistance to EGFR kinase inhibitors could be overcome by more complete EGFR kinase inhibition. Higher concentrations of EKB-569 were indeed able to completely overcome the protective effects of PTEN inactivation on cell death induction by this drug (Fig. 5B Left). To ascertain that cell death at these increased EKB-569 concentrations was due to inhibition of the EGFR/HER2 signaling unit rather than EKB-569 effects on other kinases, we engineered a HER2 mutant, which was constitutively active due to an insertion in the HER2 kinase domain (YVMA-HER2) and was also resistant to EKB-569 due to a second mutation in a drug-interaction residue (Cys805Ser). Stable expression of this double mutant (HER2 YVMA-HER2) in HCC827-PTENshRNA cells restored phosphorylation of EGFR and HER2 in the presence of EKB-569 (Fig. 5B Right) and rescued cell death induction by EKB-569, even at increased EKB-569 concentrations (Fig. 5B Middle).

Finally, we acutely knocked down EGFR in A431-PTEN shRNA cells and in MDA-MB-468 cells which are naturally PTEN deficient and harbor EGFR amplification. In both cell lines, acute EGFR knockdown triggered massive cell death (Fig. 5C).

**Discussion**

Our study describes a previously unrecognized role of the PTEN tumor suppressor in EGFR signal termination. Several mutant growth factor receptors found in human cancer have been shown to derive their oncogenic properties by escaping Cbl-mediated receptor down-regulation (13). It is intriguing to speculate that the strong genetic association between EGFR amplification and PTEN inactivation in glioblastoma (Fig. S8) and perhaps other human cancers (14) is, at least in part, due to the effects of PTEN on EGFR signal output.

Several EGFR kinase domain mutants found in lung cancer and the EGFRVIII mutant (Fig. S9) are substrates of the Cbl E3 ligase,
albeit with reduced affinity relative to wild-type EGFR. In contrast to mutations in EGFR which impair Cbl recruitment to the mutant receptor (15), PTEN inactivation impairs EGFR signal termination at a later step during receptor down-regulation, namely the continued association between EGFR and Cbl family members (Fig. 4D). Maintenance of the EGFR–Cbl complex is critical to counteract the activity of deubiquitylating enzymes in the early endosomal compartments and allow efficient interaction between fully ubiquitylated EGFR receptors with members of the ESCRT complex (16). Whereas our results with mutant alleles of PTEN and AktI suggest that PTEN regulates EGFR signal termination, through its lipid phosphorylation activity, we cannot formally exclude the possibility that PtdIns(3,4,5)P3-dependent and Akt-independent effects further contribute to the effects of PTEN inactivation on EGFR. Further work is required to dissect whether changes in the phosphoinositide pool caused by other oncogenic perturbations of the PI3K pathway may also affect the stability of the EGFR–Cbl interaction.

Our results have several important implications for therapeutic targeting of growth factor receptors in cancer. Our finding that PTEN inactivation does not impair the cell death response of MET- or PDGFRα-amplified cancer cells to MET and PDGFR kinase inhibitors, respectively, is encouraging for future therapeutic attempts to target these growth factor receptors in cancer types with constitutive mutations in PTEN or PI3K. In terms of EGFR, we show that activation of the PI3K pathway does not relieve EGFR-driven cancer cells from their dependence on EGFR for survival but, instead, shifts the EGFR kinase inhibitor response curve toward drug concentrations, which may be difficult to reach in tumor tissue, in particular in the central nervous system (17). Our finding that the modest boost in EGFR activity conferred by PTEN inactivation is sufficient to confer resistance to EGFR kinase inhibitors is reminiscent of the observation that the T790M-L858R EGFR mutant, responsible for about 50% of cases with acquired EGFR kinase inhibitor resistance in lung cancer (18), confers resistance even when present at an allelic frequency of only ~3% (19). Collectively, these results indicate that maximal EGFR kinase inhibition, perhaps only achievable by pulsatile kinase inhibitor therapy (20) or multipronged attacks on the EGFR life cycle, remains a therapeutic goal for the treatment of EGFR mutant cancer harboring activating lesions in the PI3K pathway.

Materials and Methods

Generation of Stably Transduced Cell Lines. To generate EGFR-expressing Ptenlox/+ and Pten−/− MEFs, EGFR retrovirus was made by transfecting pLXSN-Neo-EGFR and pCL-ampho packaging plasmid into 293T cells. Virus was collected and Ptenlox/+ MEFs were infected and selected with 800 μg/mL G418. To knockout PTEN, G418-resistant cell populations were infected with retrovirus generated in 293T cells with pMSCV-puro-Cre and pCL-ampho and selected with 5 μg/mL puromycin. A431-WT-PTEN-PTEN-shRNA and A431-PTEN-G129E-PTEN-shRNA cells were generated by sequential stable infections. First, A431 cells were infected with either pLNCX2-mPTEN or pLNCX2-mPTEN-G129E and selected with 800 μg/mL G418. Subsequently, G418-resistant cells were infected with shSIREN-Retroq-PTEN and selected with 5 μg/mL puromycin. All other stable lines were generated similarly.

Western Blot and Immunoprecipitations. Cells were harvested directly on tissue culture plates. When only Western blots were carried out on cell lysates, 1% triton lysis buffer (Cell Signaling Technologies) supplemented with protease and phosphatase inhibitors was used. Lysates were sonicated, cleared by centrifugation, and normalized to equal amounts of total protein using the DC protein assay (BioRad). Immunoprecipitations to determine EGFR ubiquitylation were done under denaturing conditions. Cells were serum starved and treated with 10 μM lactacystine 30 min before ligand stimulation. Cells were then washed with cold PBS containing 10 μM N-ethylmaleimide and harvested in lysis buffer supplemented with 1% SDS, protease inhibitors, and phosphatase inhibitors. After addition of lysis buffer, lysates were incubated on ice for 30 min without sonication before they were cleared by centrifugation. Cleared lysates were subjected to immunoprecipitation as described elsewhere (21).

Phosphopeptide Array. We examined phosphorylation of 144 peptides spotted on a three-dimensional porous matrix produced and commercialized by Pamgene (www.pamgene.com). Phosphorylation on these arrays is measured kinetically, using a microfluidic device with an integrated CCD camera as described elsewhere (21).

ACKNOWLEDGMENTS. We thank Charles Sawyer (Memorial Sloan-Kettering Cancer Center, MSKCC) and Neal Rosen (MSKCC) for helpful discussions in the course of this work. Barbara Tanos (MSKCC) for experimental insight, and Nicolaas Vannucci for technical support. This work was supported by National Institutes of Health-sponsored fellowships (to I.V. and N.P.), the American Italian Cancer Foundation (to B.O.), the Department of Defense (to I.K.M.), the Brain Tumor Funders’ Collaborative (to P.S.M., T.F.C., and I.K.M.), the Sidney Kimmel Foundation (to I.K.M.), the National Cancer Institute (U54CA143798-01-to I.K.M.) and the Doris Duke Foundation (to I.K.M.).