Direct positive regulation of PTEN by the p85 subunit of phosphatidylinositol 3-kinase

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The phosphatidylinositol 3-kinase (PI3K) signaling pathway is deregulated in many human diseases including cancer, diabetes, obesity, and autoimmunity. PI3K consists of a p110 catalytic protein and a p85α regulatory protein, required for the stabilization and localization of p110-PI3K activity. The p110-PI3K enzyme generates the key signaling lipid phosphatidylinositol 3,4,5-trisphosphate, which is dephosphorylated by the PI3-phosphatase PTEN. Here we show another function for the p85α regulatory protein: it binds directly to and enhances PTEN lipid phosphatase activity. We demonstrate that ectopically expressed FLAG-tagged p85 coimmunoprecipitates endogenous PTEN in an epidermal growth factor dependent manner. We also show epidermal growth factor dependent coimmunoprecipitation of endogenous p85 and PTEN proteins in HeLa cells. Thus p85 regulates both p110-PI3K and PTEN-phosphatase enzymes through direct interaction. This finding underscores the need for caution in analyzing PI3K activity because anti-p85 immunoprecipitations may contain both p85:p110-PI3K and p85:PTEN-phosphatase enzymes and thus measure net PI3K activity. We identify the N-terminal SH3-BH region of p85α, absent in the smaller p55α and p50α isoforms, as the region that mediates PTEN binding and regulation. Cellular expression of p85ΔSH3-BH results in substantially increased magnitude and duration of pAkt levels in response to growth factor stimulation. The ability of p85 to bind and directly regulate both p110-PI3K and PTEN-PI3-phosphatase allows us to explain the paradoxical insulin signaling phenotypes observed in mice with reduced PI3K or PTEN proteins. This discovery will impact ongoing studies using therapeutics targeting the PI3K/PTEN/Akt pathway.

lipid phosphatase | PTEN tumor suppressor | protein:protein interactions | explains mouse phenotypes | insulin signaling

PTEN is a tumor suppressor protein lost or mutated in as many as 30% of human cancers (1–3). PTEN dephosphorylates the D3 position of phosphatidylinositol 3,4,5-trisphosphate ($PI3,4,5P_3$), the product of activated phosphatidylinositol 3-kinase (PI3K). PI3K consists of a p110 catalytic subunit and a p85α regulatory subunit and is activated in response to receptor tyrosine kinases, including the platelet-derived growth factor (PDGF) receptor (PDGFR) and epidermal growth factor (EGF) receptor (EGFR). The PI3K pathway provides proliferative and anti-apoptotic signals and is frequently deregulated and/or activated in human cancers (1–3). PTEN acts to attenuate PI3K signaling and switch off proliferative and antiapoptotic signals.

The PI3K pathway also plays a central role in mediating insulin responses via the insulin receptor, a receptor tyrosine kinase that phosphorylates insulin receptor substrate proteins (e.g., IRS-1 and IRS-2) that nucleate downstream signaling proteins including PI3K. Animal studies have found that mice with reduced p85 levels have insulin signaling phenotypes like PTEN($^{+/-}$) mice, rather than like p110($^{+/-}$) mice as expected (4–8). Further animal work has suggested that p85 may be able to modulate the tumorigenicity of PTEN($^{+/-}$) mice (9), as well as the observed insulin sensitivity (10) via effects on PTEN. Our previous work suggested p85α may bind a negative regulator of PI3K activity (11). Therefore, we set out to determine if p85 could bind to and directly regulate PTEN. During the course of our studies, this connection was also speculated about in a Sci STKE paper (12). In this report, we demonstrate that p85α can directly bind and enhance the lipid phosphatase activity of PTEN, making it a dual regulatory protein for both the p110-PI3-kinase and the PTEN-PI3-phosphatase, performing a critical regulatory function in maintaining the balance of PI3K/PTEN signaling.

Results

p85α Associates with PTEN in Cells, a Direct Interaction That Enhances PTEN Activity. We used a coimmunoprecipitation and immunoblot analysis to demonstrate association between FLAG-p85α and endogenous PTEN (Fig. 1*A*) in COS-1 cells in an EGF-dependent manner (Fig. 1*D*). Further, this interaction was reconstituted in vitro using GST-PTEN-C124S (phosphatase dead mutant) immobilized on glutathione Sepharose beads and purified p85 protein (Fig. 1 *B* and *C*), indicating the binding is direct. The lipid phosphatase activity of purified His_6-PTEN (Fig. 1*E*) toward a fluorescent lipid substrate was enhanced by p85 addition, in contrast to a phosphatase dead His_6-PTEN-C124S mutant that lacks activity (Fig. 1*F*). His_6-PTEN activity was stimulated by p85 at least threefold, in a concentration-dependent manner that peaked at roughly equal molar concentrations (Fig. 1 *G* and *H*).

The BH Domain of p85 Binds PTEN and May Sequester PTEN in Cells. We found that the BH domain of p85, either alone, or together with the SH3 domain (SH3+BH) were able to bind PTEN using an in vitro pull-down assay (Fig. 2). In cells stably overexpressing FLAG-p85BH, association with PTEN was growth factor independent (Fig. S1*A*), in contrast to the EGF-dependent p85:PTEN association (Fig. 1*D*) and suggests regions outside the p85 BH domain may play a role in the EGF-dependency of this interaction. As expected, FLAG-p85BH was incapable of associating with p110 (Fig. S1*B*) or activated PDGFR (Fig. S1*C*) because it lacks the necessary p110-binding and SH2 domains. We also show these control experiments to verify the expected binding characteristics for two p85 mutants (p85Δ110 and p85ΔSH3-BH), with the p85Δ110 mutant binding to activated PDGFRs but not p110 and the p85ΔSH3-BH mutant retaining binding to both proteins (Fig. S1 *B* and *C*). The p85ΔBH mutant has been previously characterized (13). Cells expressing FLAG-p85ΔSH3-BH did not show enhanced PI3K activity in the FLAG immunoprecipitates (Fig. S1*B*), likely because this series of samples were not stimulated with growth factor necessary to induce PTEN binding to wild-type FLAG-p85 (Fig. 1*D*). The

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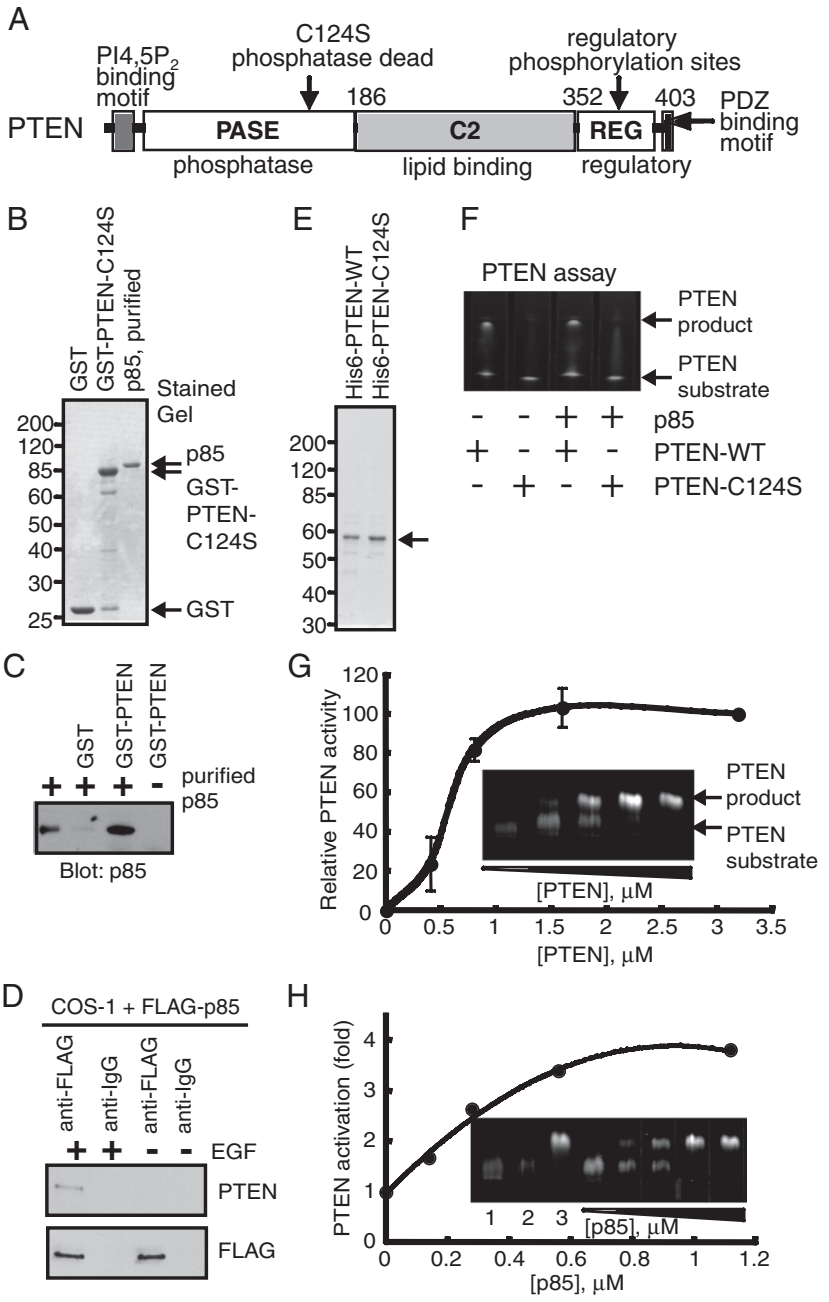

Fig. 1. p85 protein associates with PTEN in a growth factor-dependent manner in COS-1 cells, binds directly to PTEN and stimulates PTEN activity in vitro. (*A*) Domain structure of PTEN protein. (*B*) Coomassie blue stained SDS/PAGE of GST and GST-PTEN-C124S proteins immobilized on glutathione Sepharose beads and purified p85 protein used in pull-down experiments. (*C*) GST and GST-PTEN (C124S) were immobilized on glutathione Sepharose beads and mixed with purified p85 protein in a pull-down experiment. Bound p85 protein was detected by immunoblotting with anti-p85 antibody. As controls, pure p85 protein was loaded (0.05 μg) and GST-PTEN-C124S was incubated in the absence of p85. (*D*) Lysates from COS-1 cells transfected with FLAG-p85 ± EGF stimulation were immunoprecipitated (IP) and immunoblotted with the indicated antibodies. (*E*) Coomassie blue stained SDS/PAGE of purified His_6-PTEN wild-type (WT) and phosphatase dead (C124S) proteins used for PTEN assays. (*F*) PTEN lipid phosphatase activity was assayed ± added p85, using a fluorescent lipid substrate and by resolving the reaction products by TLC. The His_6-PTEN-C124S mutant lacks phosphatase activity. (*G*) Phosphatase activity was assayed over a range of PTEN (WT) concentrations. Results shown are the mean of three independent experiments ± SD (*H*) PTEN (0.56 μM) lipid phosphatase activity was determined with increasing concentrations of p85. (*Inset*) Lipid substrate alone (lane 1), lipid with p85 (1.12 μM; lane 2), and lipid with a high concentration of PTEN (3.2 μM; lane 3). Similar results were obtained in replicate experiments.

PDGFR-associated PI3K activity was also not significantly different in any of the cell extracts prepared from cells expressing various FLAG-p85 mutants (Fig. S1*C*). These experiments were carried out to test the functionality of the different FLAG-p85 mutants for PDGFR binding. Therefore, samples were prepared in the presence of phosphatase inhibitors to promote phosphotyrosine-dependent binding between p85/FLAG-p85 and the activated PDGFR, perhaps negating effects of PTEN binding.

We expressed a dominant negative phosphatase dead PTEN-C124S in cells to displace endogenous PTEN from the p85:PTEN complex, sequestering p85 in a PTEN-C124S:p85 complex. This resulted in a more robust activation of the downstream target of PI3K signaling, Akt (14) (Fig. S2). These enhanced pAkt (pS473) levels were blunted by coexpression of additional p85, consistent with a dominant negative mechanism and the ability to restore p85:PTEN (endogenous) complexes.

Deletion of Both the N-Terminal SH3 and BH Domains of p85 Is Required to Abrogate Binding to PTEN. Deletion of the BH domain (ΔBH) of p85 was sufficient to decrease but not abolish interaction with PTEN, whereas deletion of the p110-binding domain (Δ110) of p85 had no effect on PTEN binding (Fig. S3 *Left*). Thus, the BH domain of p85 is important for PTEN binding and p85:PTEN interaction does not require p110 binding. (Myc-) p110 did not associate with GST-PTEN-C124S unless FLAG-p85 protein with an intact p110-binding domain was present (Fig. S3 *Right*), suggesting p110 does not directly bind PTEN, but can associate with a PTEN-containing complex via interaction with p85. The small amount of Myc-p110 associated with GST-PTEN-C124S in the FLAG-p85Δ110 lane was attributed to the presence of the endogenous p85. Similar low levels of Myc-p110 associated with GST-PTEN-C124S in the FLAG-p85ΔBH sample, a p85 mutant with reduced PTEN binding (Fig. S3 *Right*).

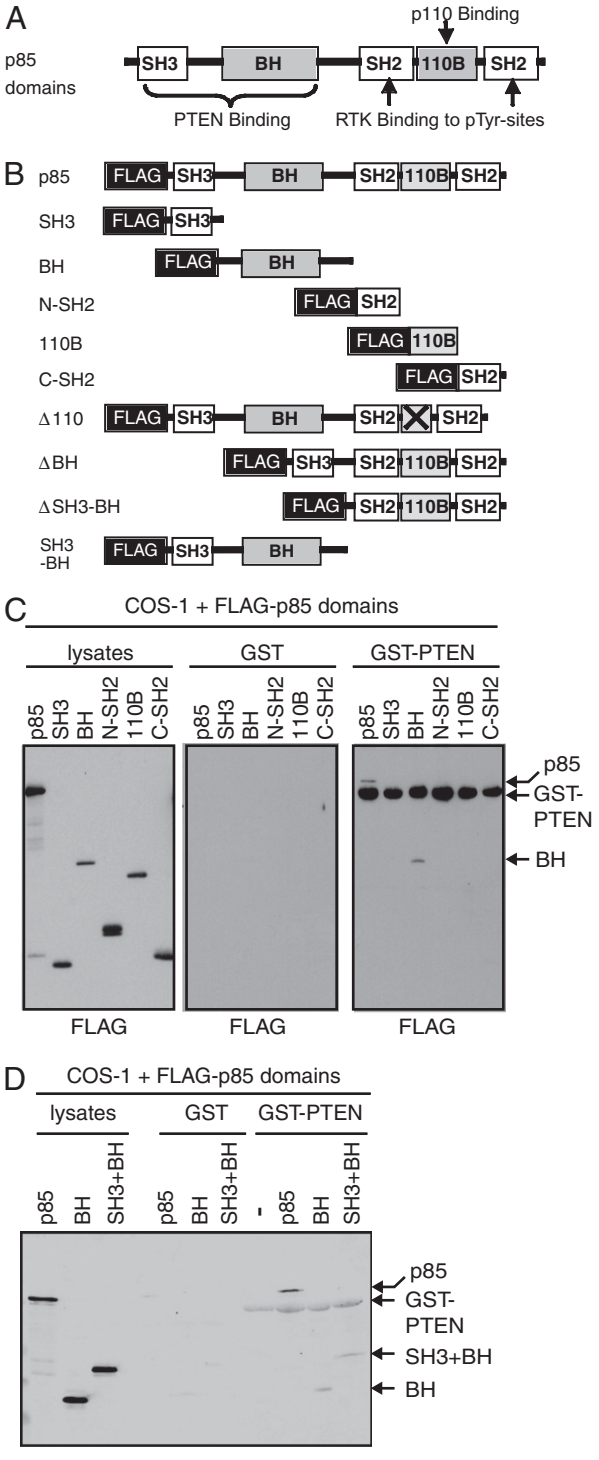

Fig. 2. The BH domain of p85 binds PTEN. (*A*) Domain structure of p85 protein. (*B*) Schematic representation of the domains present in the FLAG-tagged p85 fusion proteins. (*C* and *D*) Lysates from COS-1 cells transfected with different FLAG-tagged domains of p85 were used in pull-down experiments with immobilized GST and GST-PTEN (C124S) as indicated.

We further showed that deletion of the N-terminal SH3-BH domains of p85 was sufficient to ablate PTEN binding (Fig. 3 *A* and *B*). Expression of p85ΔBH, a mutant with reduced PTEN binding, caused a minor increase in pAkt levels at the later time points (120, 240 min) (Fig. 3*C*). Notably, expression of p85ΔSH3-BH, a mutant unable to bind PTEN, resulted in

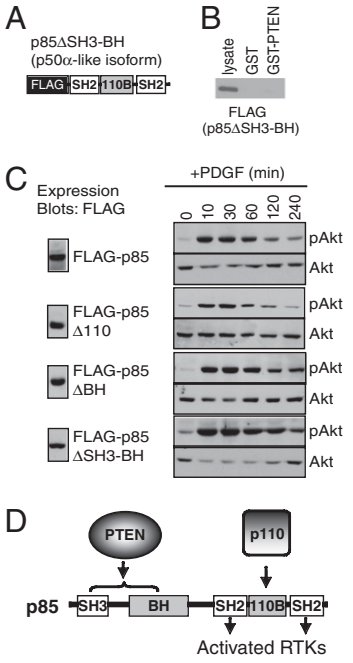

Fig. 3. The p85 protein binds PTEN via its N-terminal SH3-BH domains and p110 via its p110-binding (110B) domain. (*A*) Domains present in p85ΔSH3-BH protein. (*B*) Pull-down experiment with GST-PTEN-C124S and COS-1 lysates containing FLAG-p85ΔSH3-BH, a mutant lacking the N-terminal half of p85, is unable to bind PTEN. (*C*) Lysates (20 μg) from NIH 3T3 cells stably expressing near endogenous levels of different FLAG-p85 proteins ± PDGF were probed for pAkt (pS473) and total Akt. FLAG-p85 mutant expression levels were similar as determined using FLAG immunoblots. (*D*) Diagram illustrating regions of p85 involved in binding PTEN, p110, and activated RTKs.

increased and sustained pAkt levels at all time points tested (Fig. 3*C*). These results are consistent with the absence of p85:PTEN interaction to enhance PTEN activity and promote the clearance of PI3K lipid products necessary for Akt activation. Together, these results suggest p85 can form p85:PTEN and p85:p110 complexes where N-terminal SH3-BH domains of p85 bind PTEN, and the p110-binding domain of p85 binds p110 (Fig. 3*D*).

Growth Factor Dependent Coimmunoprecipitation of Endogenous p85 and PTEN from Cells. Lysates from HeLa cells stimulated with EGF contained endogenous p85:PTEN complexes, as determined by reciprocal coimmunoprecipitation experiments (Fig. 4 *A* and *B*). Mouse embryonic fibroblasts derived from wild-type p85α, heterozygous ($^{+/-}$), and null ($^{-/-}$) mice were used to verify the specificity of the anti-p85α-specific antibodies used (Fig. 4*C*). This suggests that p85 associates with PTEN primarily in a growth factor dependent manner, consistent with an interaction with the open unphosphorylated form of PTEN, as reported recently (15).

Discussion

This newly identified PTEN association with p85 has significant implications for interpretation of experiments that measure PI3K activity from anti-p85 immunoprecipitates that may contain both associated p110-kinase and PTEN-phosphatase enzymes. Depending upon the lipid substrate used and the presence or absence of inhibitors, such PI3K assays may provide the net activity resulting from p110 phosphorylation and dephosphorylation by associated PTEN. Thus, some regulatory effects previously ascribed to alter PI3K activity may in fact be due to alterations of PTEN activity.

This discovery that p85 enhances PTEN activity, in addition to regulating p110-PI3K activity, resolves the paradox identified in the regulation of insulin signaling by p85α and p110α subunits of

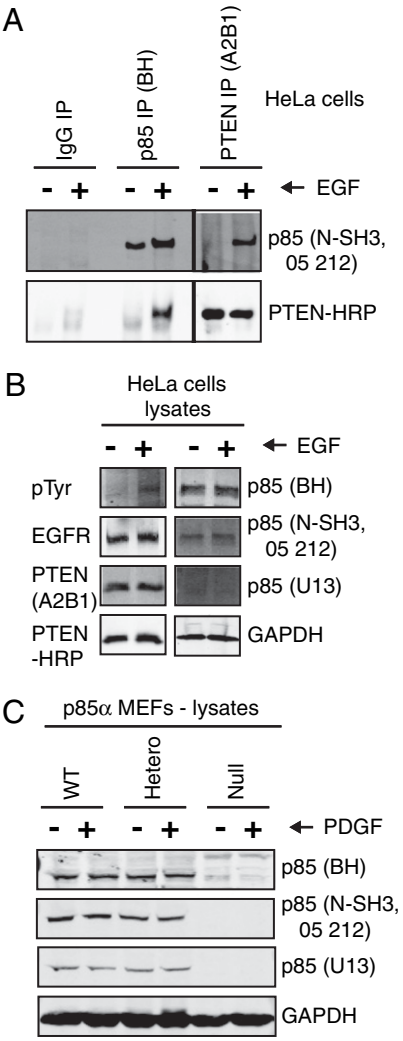

Fig. 4. Growth factor dependent coimmunoprecipitation of endogenous p85 and PTEN from cells. (*A*) HeLa cells were serum-starved in 0.5% serum overnight and treated (+) or not (–) with EGF for 5 min. Cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. (*B*) Control HeLa cell lysates (20 μL) were immunoblotted with the indicated antibodies. (*C*) MEFs derived from p85α wild-type, heterozygous ($^{+/-}$) and null ($^{-/-}$) mice were serum-starved in 0.5% serum overnight and treated (+) or not (–) with PDGF for 5 min. Cell lysates (20 μL) were immunoblotted with the indicated antibodies. The p85 antibodies used were specific for p85α.

PI3K (4–7, 12, 16–18). The *Pik3r1* gene encodes p85α and splice variants, p55α and p50α, lacking N-terminal SH3-BH domains that we have identified as necessary for PTEN binding. Pik3r1 knockout is embryonic lethal in mice, whereas selective p85α knockout mice, retain p55α and p50α expression and are viable (5–7). Both heterozygous Pik3r1($^{+/-}$) mice and knockout p85α ($^{-/-}$) mice show increased glucose uptake and insulin sensitivity, in contrast to heterozygous p110($^{+/-}$) mice that are mildly glucose intolerant and less sensitive to insulin (4, 6). In addition, p85α($^{-/-}$) mice show increased and sustained levels of $PI3,4,5P_3$ in adipocytes, suggesting a defect in $PI3,4,5P_3$ clearance (7).

Mouse embryonic fibroblasts (MEFs) derived from Pik3r1($^{+/-}$) and Pik3r1($^{-/-}$) mice have been used to determine the effects of p85α/p55α/p50α depletion on insulin-like growth factor (IGF1) responses (19). Pik3r1($^{+/-}$) MEFs contain half the p85α yet near normal levels of p110α:p85α complex, and there is a selective reduction in unbound p85α (i.e., not bound to p110α). As a result, PI3K activities (both p85α-associated and p110α-associated) were almost equal to that of wild-type MEFs. Pik3r1($^{+/-}$) cells had substantially higher $PI3,4,5P_3$ levels, increased pAkt, and increased PTEN expression. Despite increased PTEN expression, high $PI3,4,5P_3$ levels were sustained, again suggesting decreased $PI3,4,5P_3$ clearance.

It has been suggested that monomeric p85 may compete with p85:p110 complexes for binding to tyrosine phosphorylated receptor substrates and result in decreased pTyr-associated PI3K activity and reduced Akt activation (5, 17–20). Two mechanisms of negative regulation by monomeric p85 have been proposed: (*i*) competition with p85:p110 for tyrosine phosphorylated sites and (*ii*) sequestration of IRS-1 away from p85:p110 (21). In p85α ($^{-/-}$) mice, the p50α isoform compensates for p85α functions of p110α and IRS-1 binding (7), yet importantly they show increased and sustained PI3K signaling, arguing against a competition or sequestration mechanism for p85α effects. The inhibitory effect of p85α on p110α catalytic activity has been shown to depend upon the p85 N terminus (SH3-BH domains) (20), consistent with our findings that PTEN interacts directly with this region of p85, and as recently speculated (12).

The search for *Pik3r1* mutations in human cancers or other diseases have focused mainly on the exons encoding the C-terminal portion of the p85α protein (N-SH2, iSH2, C-SH2) and the ability of these mutations to release inhibitory effects on p110-encoded PI3K activity (22–26). We speculate that a more rigorous analysis of *Pik3r1* for mutations within exons encoding the N-terminal portion of p85α (SH3 and BH) may reveal another class of mutations defective for regulation of PTEN. In addition, we have previously shown the BH domain of p85α encodes RabGAP activity toward Rab5 and Rab4 GTPases that play important roles in receptor trafficking and degradation (13). We also demonstrated that a RabGAP-defective p85α mutant containing a single point mutation within this domain (R274A) is oncogenic (27). Thus, human cancers could contain mutations within the p85α BH domain contributing to tumorigenesis, either via deregulation of Rab-mediated receptor degradation or via reduced positive regulation of PTEN activity.

As further evidence of the positive regulation of p85 on PTEN function, PTEN($^{+/-}$) mice develop a variety of tumors (28–31), whereas double heterozygous PTEN($^{+/-}$)/Pik3r1($^{+/-}$) mice have increased intestinal polyp incidence (approximately twofold) and number (more than 3, versus 1–2 per animal) (9). These results support a role for p85 in the regulation of the tumor suppression by PTEN in mice. Significantly, PTEN($^{+/-}$) mice show the same phenotype of increased glucose uptake and increased insulin sensitivity as Pik3r1($^{+/-}$) and p85α($^{-/-}$) mice (8). In addition, Taniguchi et al. (10) observed that cells with reduced p85α expression as a result of p85α knockout, have decreased PTEN activity.

Our pull-down binding data indicates that GST-PTEN:FLAG-p85:Myc-p110 can form a trimeric complex in vitro, however, there may be additional constraints present that restrict or prevent such interactions in cells. Attempts to carry out reciprocal pull-downs using bacterially expressed GST-p85 and ectopically expressed HA-PTEN-C124S from mammalian cell extracts did not show binding. We suspect this is due to the interaction requiring the open conformation of PTEN (not phosphorylated at the regulatory casein kinase II phosphorylation sites). PTEN becomes dephosphorylated and is thought to adopt an open conformation upon growth factor stimulation to facilitate plasma membrane association (32). During our revisions, a report was published showing unphosphorylated PTEN associated with p85 in a high molecular weight PTEN-associated complex (PAC) (15). Previous fractionation experiments demonstrated that high molecular weight PAC complexes may also contain NHERF2 and PDGFRβ (33). Thus, it is possible that in cells p85 forms primarily separate complexes, with p85:p110 binding to activated RTKs and p85:PTEN also binding through PDZ-domain containing scaffolds, like NHERF2, to RTKs to bring PTEN, and its

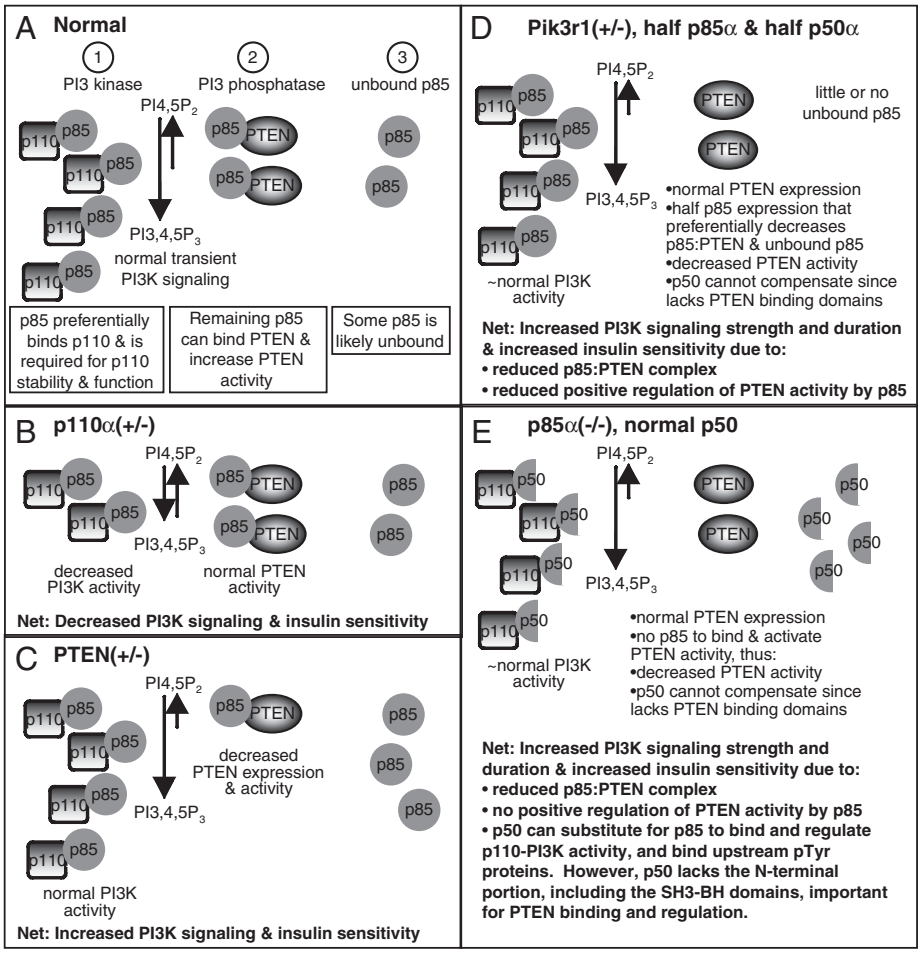

Fig. 5. Resolving the paradox that both Pik3r1($^{+/-}$) mice and p85α($^{-/-}$) mice have insulin signaling phenotypes resembling PTEN($^{+/-}$) mice rather than p110α ($^{+/-}$) mice. (*A*) PI3K signaling is transient in normal mice as a result of a balance between p85:p110-mediated generation of $PI3,4,5P_3$ and p85:PTEN-mediated dephosphorylation of $PI3,4,5P_3$. (*B*) p110α($^{+/-}$) mice contain half the normal amount of p110α. (*C*) PTEN($^{+/-}$) mice contain half the normal amount of PTEN. (*D*) Pik3r1($^{+/-}$) mice contain half the levels of p85α and p50α, both products of the *Pik3r1* gene and both able to regulate p110-PI3K (and not p55α) as well as bind upstream activators. Decreased p85α levels do not, however, reduce p85:p110 and p85:PTEN complexes equally, with near normal levels of p85:p110 (19). The selective reduction of the p85 not associated with p110 includes p85:PTEN, specifically impairing PTEN-mediated $PI3,4,5P_3$ dephosphorylation and leading to increased and sustained PI3K signaling. (*E*) Selective isoform-specific p85α($^{-/-}$) knockout mice do not express p85α but have normal levels of p50α, containing both the p110-binding domain and the pTyr-binding SH2 domains. Thus, p50α substitutes for p85α in its ability to link p110-PI3K to upstream pTyr activation signals. However, p50α lacks the N-terminal half of p85 (i.e., SH-BH domains), we have shown to be required for binding and positive regulation of PTEN activity. Thus, p50α is unable to bind and positively regulate PTEN resulting in increased and sustained PI3K signaling.

activator p85, to sites of newly generated PI3K lipid products for rapid dephosphorylation and transient signaling.

We propose a model (Fig. 5*A*) where p85α positively regulates both p110α-PI3-kinase and PTEN-PI3-phosphatase activities. Using this model, we suggest a detailed explanation for the observed PI3K signaling phenotypes in various transgenic mice (Fig. 5 *A–E*). The p85α protein preferentially binds and stabilizes p110α (34), so p85α is sequestered preferentially by p110α when p85α levels are low (19) (Fig. 5*A*). Once p110α is saturated, excess p85α can bind PTEN and positively regulate its lipid phosphatase activity. Therefore when p85α levels are reduced, the p85α:PTEN complex is selectively reduced, giving rise to increased and sustained $PI3,4,5P_3$ levels (Fig. 5*D*). If p85α is absent, p50α can substitute and regulate p110α-PI3 kinase activity but is unable to regulate PTEN because it lacks the necessary SH3-BH domains (Fig. 5*E*). Therefore, the function of p85α needs to be re-evaluated because it is more than solely a regulator of $PI3,4,5P_3$ production via the p85α:p110α complex but is also a regulator of $PI3,4,5P_3$ dephosphorylation via binding and activating PTEN PI3-phosphatase activity.

Materials and Methods

PTEN Assays His_6-PTEN protein was assayed using a fluorescent lipid substrate (1.5 μg from Echelon Biosciences) in PTEN assay buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl), with DTT (2 mM) in 20 μL at 37 °C for 30 min. Dried lipids were resuspended in methanol: 2-propanol: glacial acetic acid (5:5:2) and were spotted onto Silica gel 60 F254 TLC plates pretreated with 1% potassium oxalate, resolved in 1-propanol: glacial acetic acid: water (17.4:7.9:1). Fluorescent lipids were visualized under UV light using a Gel doc system (Biorad Laboratories) and were quantified using Quantity One software (Biorad Laboratories).

Additional experimental procedures and associated references are available in *SI Text*.

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