Inhibition of PI3K binding to activators by serine phosphorylation of PI3K regulatory subunit p85α Src homology-2 domains

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Class IA PI3Ks are activated by growth factor receptors and generate lipid second messengers that mediate downstream responses including cell growth, cell migration, and cell survival. The p85 regulatory subunit of PI3K contains Src homology-2 (SH2) domains that mediate binding to tyrosine-phosphorylated receptors or adaptor proteins to facilitate localization and activation of PI3K at the plasma membrane. We report here that persistent activation of PKC family members by phorbol ester stimulation in cells leads to phosphorylation of two serine residues at analogous sites on both SH2 domains of p85α (S361 and S652). The modified serine residues are located in the phospho-tyrosine binding pockets of the two SH2 domains, and in the crystal structures the phosphate moieties are predicted to occupy the same space as the phosphate moieties of bound phospho-tyrosine peptides. Consistent with this prediction, phosphorylation at these serine residues or mutation to aspartate inhibits binding of p85α to tyrosine-phosphorylated peptides. We provide evidence that protein kinase D, which is phosphorylated and activated by PKCs, mediates phosphorylation of S652 in the C-terminal SH2 domain. These results reveal cross talk between PKC signaling and PI3K signaling that impairs PI3K pathway activation under conditions of persistent PKC (and protein kinase D) activity.

PI3K plays an important role in regulating many cellular processes including cell growth, survival, proliferation, and motility (reviewed in ref. 1). Class IA PI3K, which comprises a p85 regulatory subunit and a p110 catalytic subunit, is localized primarily in the cytoplasm and is activated by growth factor receptor tyrosine kinases at the plasma membrane. Upon growth factor stimulation, receptor tyrosine kinases dimerize and autophosphorylate on tyrosine residues, creating binding sites for the Src homology-2 (SH2) domains of the p85 subunit. As a result, PI3K is localized to the plasma membrane where it catalyzes the generation of the lipid second messenger phosphoinositol-3,4,5-trisphosphate (PIP3). PIP3 formation recruits pleckstrin homology domain-containing proteins, most notably the protein kinase Akt, to the plasma membrane and initiates downstream signaling to modulate cellular processes such as survival and proliferation.

SH2 domains are conserved protein modules of ~100 amino acids that bind to phosphorylated tyrosine residues within specific sequence contexts (2). In humans, 120 SH2 domains have been identified in 110 distinct proteins involved in diverse cellular processes (3). SH2 domains share a common protein fold and phospho-tyrosine binding region, the conserved FLVR (F, Phe; L, Leu; V, Val; R Arg) sequence (4). The PI3K p85 subunit contains two SH2 domains, an N- and a C-terminal SH2 domain, that bind to two closely spaced pYXXM motifs (pY, phospho-Tyr; X, any amino acid; M, Met) (2) located on receptor tyrosine kinases or adaptor proteins such as growth factor receptor–bound protein 2–associated binding protein 1 or insulin receptor substrate 1 (IRS1). The simultaneous engagement of the two SH2 domains with doubly Tyr-phosphorylated peptides mediates a conformational change that relieves inhibition of PI3K activity (5).

Recent structural studies with p85α-p110α and p85β-p110β have elucidated interactions and modes of regulation of PI3K activity (6–10). In the absence of Tyr-phosphorylated peptides, the N-terminal SH2 domain associates with the helical domain of p110α in a conformation that precludes phospho-tyrosine binding and in this conformation access of the catalytic moiety of p110α to membrane-bound substrate is impaired (6–9). Taken together, the studies suggest a model in which engagement of both SH2 domains of p85 with phospho-Tyr peptides may be necessary to dissociate the SH2 domains from their interactions with the helical and catalytic domains of p110α (without disrupting the interaction between the N terminus of p110α and the inter-SH2 domain of p85α). This model would allow the p85 subunit to localize p110 at activated receptors on the plasma membrane in an "open" and active conformation. In support of this model, one of the most frequently observed mutations in human cancers is mutation of Glu545 in the helical domain of p110α to Lys. Glu545 forms a salt bond with Lys379 near the phospho-Tyr binding pocket of the N-terminal SH2 domain of p85 (6, 9), and the charge reversal by the oncogenic mutation is predicted to destabilize the interaction between the N-terminal SH2 domain and the helical domain, allowing an open, active conformation analogous to that induced by binding to Tyr-phosphorylated peptides. This open state also is predicted to have higher affinity for Tyr-phosphorylated receptors and adaptor proteins because, in this state, binding to the N-terminal SH2 domain is not impaired by inhibitory contact with the p110α subunit.

Many of the growth factors and cellular regulators that activate PI3K also activate plasma membrane phospholipase C (PLC) and thereby elevate cytosolic calcium and plasma membrane diacylglycerol levels, conditions that result in activation of PKC family members. In some tissues, especially lymphocytes, activation of PI3K facilitates activation of PLC-γ (11, 12). PKC family proteins also can be activated by treatment of cells with phorbol esters that mimic diacylglycerol. In addition, protein kinase D (PKD), also known as PKCμ, can be activated by phorbol esters either directly or indirectly through phosphorylation by other PKCs. PKD plays roles in diverse cellular processes, including protein transport from the Golgi network, transcription, cell motility, invasion, adhesion, proliferation, and oxidative stress response (13). In this study we report that phorbol ester stimulation leads to serine phosphorylation at the phospho-tyrosine binding pockets of the p85 SH2 domains, resulting in decreased interaction with Tyr-phosphorylated pep-

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tides in vitro as well as abrogation of PI3K signaling in cells. We show that PKD is required for phosphorylation of the C-terminal SH2 domain. These findings reveal a mechanism for negative regulation of the PI3K pathway through cross talk from the PKC/PKD pathway.

Results

Phosphorylation of PI3K p85α SH2 Domains upon Phorbol Ester Stimulation. Protein kinases can recognize their substrates in the context of the protein sequence surrounding the phosphoacceptor site, and the optimal substrate motifs have been determined for a number of kinases by our laboratory and others (14, 15). For example, it has been found that kinases in the Ca^{2+}/calmodulin-dependent kinase group (16) such as AMP-activated protein kinase (AMPK) and PKD prefer to phosphorylate serine sites that contain a basic amino acid three residues N-terminal to the phosphoacceptor site (−3 position) and a hydrophobic amino acid (usually Leu or Met) five residues N-terminal to the phosphoacceptor site (−5 position) (17, 18). Based on these criteria, we identified three serine residues on PI3K p85α that are potential phosphorylation sites for AMPK and/or PKD or related kinases: S154, S361, and S652 (Fig. 1*A*).

To investigate whether these sites are phosphorylated by endogenous kinases in vivo, we stimulated COS-7 cells transiently expressing tandem affinity purification (TAP)-tagged p85α with insulin to activate PI3K, 2-deoxyglucose (2-DG) to activate AMPK, or the phorbol ester phorbol 12-myristate 13-acetate (PMA) to activate PKC and PKD family members and monitored phosphorylation on p85α immunoprecipitates by immunoblot using a phospho-specific antibody that recognizes phosphorylation in the sequence context LXRXXpS (L, Leu; X, any amino acid; R, Arg; pS, phospho-Ser) that is common to all three sites. PMA stimulation led to an increase in p85α phosphorylation at sites recognized by this anti–phospho-motif antibody, but insulin and 2-DG stimulation did not (Fig. 1*B*).

Next, we generated phosphorylation-deficient Ser-to-Ala point mutants of each of the serine residues (S154A, S361A, and S652A) to determine which of the sites was phosphorylated upon PMA stimulation. The S154A mutation had no effect on PMA-stimulated immunoblotting with the anti–phospho-motif antibody, but the S361A mutation showed some reduction in immunoblotting, and the S652A mutation resulted in a major decrease in immunoblotting (Fig. 1*C*). Moreover, phosphorylation, as detected by this antibody, was completely abolished in the S361/652A double mutant, indicating that these are the sites of phosphorylation detected by the anti–phospho-motif antibody upon PMA stimulation.

We also analyzed the phosphorylation on p85α S361 and S652 by microcapillary liquid chromatography/tandem mass spectrometry (LC/MS/MS). Although trypsin commonly is used for digestions for mass spectrometry analysis, the peptides containing S361 and S652 generated from a tryptic digest were expected to be too small for efficient detection by LC/MS/MS, so we used a chymotryptic digest. Trypsin typically is used for protein digestion before mass spectrometry, perhaps explaining why phosphorylation at S361 and S652 has not been reported previously. We were able to detect chymotryptic phosphopeptides containing phospho-S361 and phospho-S652 in both unstimulated cells and PMA-stimulated cells (Fig. S1 *A* and *B*). The quantities of the phosphopeptides containing phospho-S361 and phospho-S652 relative to their unphosphorylated counterparts were analyzed using a label-free method using ratios of the integrated total ion current (TIC) from the full-scan MS profile over the LC elution peaks. This analysis revealed a fourfold increase in S361 phosphorylation and a sevenfold increase in S652 phosphorylation in response to PMA stimulation (Fig. S1*C*). Assuming that the phosphorylation does not change the ionization efficiency of the peptide in the mass spectrometer, we estimated the stoichiometry of phosphorylation to be 44% after PMA stimulation for both S361 and S652. Taken together, these results indicate that both S361 and S652 are basally phosphorylated but that phosphorylation of both sites is increased dramatically in response to PMA stimulation.

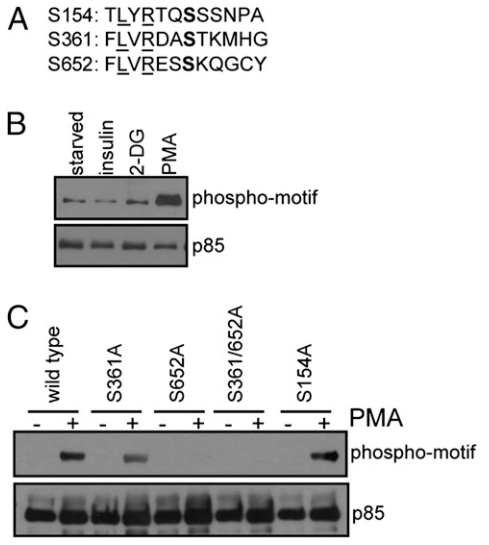

Fig. 1. Phorbol ester stimulation results in p85α phosphorylation on S361 and S652. (*A*) Amino acid sequence surrounding potential serine phosphorylation sites on human p85α. Potential phosphoacceptor sites are in bold, and the amino acids at the −3 and −5 positions are underlined. (*B*) COS-7 cells transfected with TAP-tagged p85α were serum starved overnight and stimulated with insulin (10 nM, 10 min), 2-DG (25 mM, 25 min), or PMA (100 nM, 15 min). TAP-p85α was precipitated from cell lysates and immunoblotted with an anti–phospho-motif antibody that recognizes the sequence LXRXXpS. (*C*) HEK293T cells transfected with either wild-type p85α or Ser-to-Ala mutants (S154A, S361A, S652A, or S361/652A) were stimulated with 100 nM PMA or DMSO vehicle for 4 h. p85α was precipitated from cell lysates and immunoblotted using the anti–phospho-motif antibody.

Time Course of Phorbol Ester-Induced Phosphorylation on PI3K p85α. To gain further insight into the dynamics of this phosphorylation, we monitored the time course of p85α phosphorylation as detected with the anti–phospho-motif antibody. Interestingly, the p85α phosphorylation increased linearly over several hours with PMA stimulation (Fig. 2). Phosphorylation of the activation loop

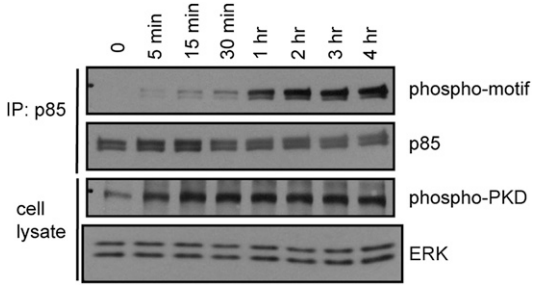

Fig. 2. Phosphorylation of p85α upon phorbol ester stimulation increases linearly over several hours. HEK293T cells transfected with TAP-p85α were stimulated with 100 nM PMA for the indicated times (0, 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, or 4 h). TAP-p85α was precipitated from cell lysates and immunoblotted with the anti–phospho-motif antibody, and cell lysates were immunoblotted with an anti–phospho-PKD and anti-ERK antibody. IP, immunoprecipitation.

of PKD, indicative of PKD activation, was monitored in the same experiment and was found to be maximally phosphorylated within 15 min and to remain phosphorylated throughout the time course. Because PKC is known to phosphorylate the activation loop of PKD, these results indicate that phosphorylation of p85α at S361 and S652 proceeds much more slowly than activation of PKC or PKD and is consistent with a model in which access of these sites on p85α to PKC or PKD or another downstream kinase is limiting.

Phosphorylation of the PI3K p85α/p110α Heterodimer upon Phorbol Ester Stimulation. Although p110 is unstable when not bound to p85 (19), there has been evidence for roles for p85 that is not bound to p110, the so-called "free p85" or "p85 monomer" (20–27). Some of these roles involve the negative regulation of PI3K (24, 26, 27). Therefore, it was important to determine whether p85α phosphorylation upon PMA stimulation was occurring on the p85α/p110α heterodimer or only on the p85α monomer. To address this question, we transiently expressed FLAG-tagged wild-type p85α or a mutant that contains a deletion in the region that is important for interaction with p110 (Δp85) and HA-p110α in HEK293T cells and examined the phosphorylation status of p85 (Fig. 3). The Δp85 was not phosphorylated upon PMA stimulation, indicating that PMA stimulation leads to preferential phosphorylation of the p85α/p110α heterodimer rather than of free p85α.

Phosphomimetic Mutations at S361 and S652 Impair Binding of PI3K p85α to Phospho-Tyr Peptides. Crystal and solution structures of the N- and C-terminal SH2 domains of p85α bound to Tyr-phosphorylated peptides reveal that the S361 and S652 residues are at analogous positions in the N- and C-terminal SH2 domains (28, 29). In both SH2 domains, these residues are in a loop between two beta strands with the hydroxy moiety of the Ser pointing into the phospho-Tyr binding pocket and forming a hydrogen bond with an oxygen of the bound phospho-Tyr phosphate (Fig. 4*A*). The Arg at the −3 position is part of the signature FLVR motif of all SH2 domains and participates in a salt bond with the bound phospho-Tyr phosphate. These structures indicate that phosphorylation of S361 or S652 is unlikely to occur when p85 is bound to an activating receptor/adaptor protein. The recently identified structure of the p85α/p110α heterodimeric complex reveals that S361 is proximal to D1029 of p110α (8). This structure indicates that phosphorylation of S361 is unlikely to occur when PI3K is in the "closed," low-activity state and raises the possibility that phosphorylation at S361 occurs only when the protein is in the open, active state but unbound to a Tyr-phosphorylated receptor/adaptor protein. In contrast, access to S652 in the p85α /p110b crystal structure is not impaired (10).

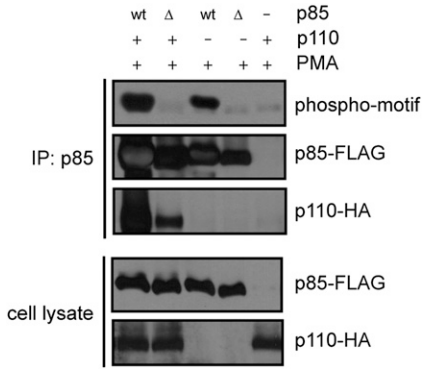

Fig. 3. The p85α/p110α heterodimer is phosphorylated upon phorbol ester stimulation. HEK293T cells cotransfected with FLAG-tagged wild-type p85α or a mutant containing a deletion in the region that is important for interaction with p110 (Δp85) and HA-p110α were stimulated with 100 nM PMA for 4 h. The levels of p85α phosphorylation in FLAG-p85α immunoprecipitates were determined by immunoblot with the anti–phospho-motif antibody.

Based on the structures discussed above, we hypothesized that phosphorylation of S361 and S652 would impair the ability of p85α to bind to phospho-Tyr peptides. We tested this hypothesis with a phospho-Tyr peptide pull-down assay using lysates from cells transiently expressing either wild-type p85α or phosphomimetic mutants. When both S361 and S652 were mutated to Asp, the interaction with the phospho-Tyr peptide beads was decreased dramatically (Fig. 5, lanes 5 and 6). These results indicate that an extra negative charge in the phospho-Tyr binding pocket, such as that caused by phosphorylation of S361 or S652, renders the SH2 domains less capable of binding to phospho-Tyr.

In addition, we examined whether PMA-dependent phosphorylation of wild-type p85α or the S652D mutant impaired binding to phospho-Tyr peptides. Although PMA stimulation of cells did not cause a significant decrease in binding of the wild-type or S652D mutant of p85α to phospho-Tyr peptides, it did cause a decrease in p110α binding to the peptides (Fig. 5, lanes 3 and 4). These results argue that only the minor subfraction of the wild type and S652D mutant that was in complex with endogenous p110α became phosphorylated at S361. This result is consistent with the result in Fig. 3 showing preferential phosphorylation of the p85α/p110α complex compared with monomeric p85α. In the case of wild-type p85α, we did not observe a significant difference in binding to phospho-Tyr peptides upon PMA stimulation (Fig. 5, lanes 1 and 2), presumably because under the conditions of this experiment only a small fraction of the protein was phosphorylated at both sites, and the singly phosphorylated forms of p85α still are capable of binding to the phospho-Tyr peptides.

Phosphorylation of PI3K p85α S652 by PKD. Because PKD phosphorylates substrates in the sequence context LXRXXS and also is activated by PMA stimulation, we investigated whether PKD can phosphorylate p85α on S361 and/or S652. We transiently coexpressed FLAG-p85α (wild type or S361A, S652A, or S361/652A point mutants) with various PKD constructs (constitutively active S744/748E mutant or catalytically inactive K612W mutant) in HEK293T cells that were not stimulated with PMA and examined phosphorylation on precipitated p85α. Expression of the constitutively active PKD resulted in phosphorylation of wild-type p85α and the S361A mutant but not the S652A or S361/652A mutants, indicating that PKD phosphorylates p85α at S652 (Fig. 6*A*). By quantitative mass spectrometry analysis as described above, a threefold increase in phosphorylation of S652 was observed with activated PKD expression (Fig. 6*B*). Knockdown of PKD1 expression by shRNA (Fig. 6*C*) or inhibition by PKC inhibitors (Fig. 6*D*) resulted in a decrease in PMA-dependent p85α phosphorylation, providing further support for a role for PKD in PMA-induced phosphorylation of p85α. Direct phosphorylation of purified p85α by recombinant PKD was demonstrated also (Fig. 6*E*).

Negative Regulation of PI3K Signaling by Phorbol Ester Stimulation. Next, we examined the effects of phorbol ester stimulation on downstream PI3K signaling. Phorbol ester treatment resulted in decreased Akt activation with a time course that correlated with increased anti–phospho-motif antibody immunoblotting (Fig.7*A*). This inhibition of Akt activation was reduced when PKD1 expression was knocked down by siRNA (Fig. 7*B*). These results demonstrate that, under the conditions in which we observe p85α serine phosphorylation induced by PMA stimulation, we observe decreases in PI3K signaling to Akt, and this effect is mediated by PKD.

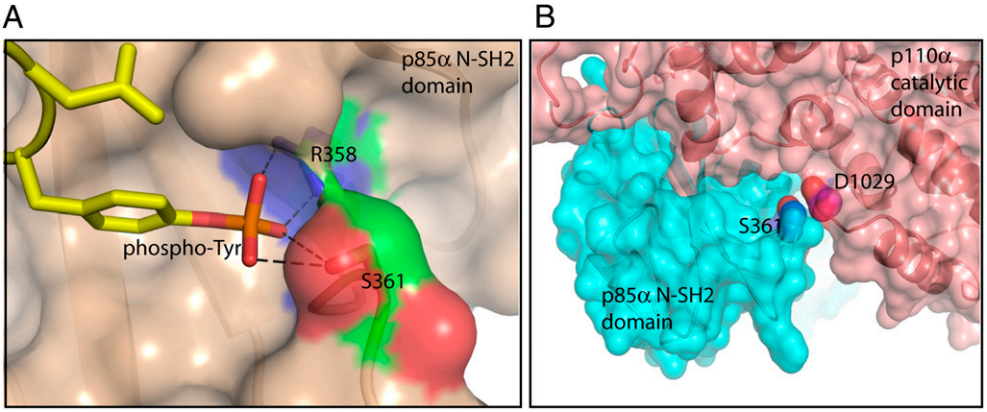

Fig. 4. S361 resides in the phospho-tyrosine binding pocket of the p85α N-terminal SH2 domain; in the p85α/p110α heterodimer, S361 is at the interface with the p110α catalytic domain. (*A*) p85α N-terminal SH2 domain bound to PDGF phospho-Tyr peptide (yellow). Interactions between S361 and R358 and the phosphate moiety of the phospho-Tyr are shown by dashed lines (Protein Data Bank ID: 2IUG). (*B*) Interface between the p85α N-terminal SH2 domain (cyan) and the p110α catalytic domain (pink), illustrating the proximity of S361 to p110α D1029 (Protein Data Bank ID: 3HIZ).

Discussion

Here we have identified two serine phosphorylation sites (S361 and S652) on the p85α subunit of PI3K. Phosphorylation of these sites is detectable in exponentially growing cells, and treatment with the phorbol ester PMA causes a major increase in phosphorylation at both sites. The S652 site appears to be phosphorylated by PKD. It is within a sequence context known to be optimal for phosphorylation by PKD, phosphorylation is enhanced when PKD is activated by PMA, and knocking down PKD impairs phosphorylation. The kinase responsible for phosphorylating S361 is not yet clear, but it could be a PKC family member or another basophilic protein kinase downstream of PKC. These two sites are at analogous positions on the N- and C-terminal SH2 domains, and crystal structures predict that the phosphate moiety of each phospho-Ser residue is likely to occupy the same position as the phosphate moiety of a bound phospho-Tyr peptide. Consistent with this prediction, we find that phosphorylation of these sites and/or mutation of these sites to Asp impairs binding of p85α to Tyr-phosphorylated peptides. Also, consistent with this prediction, we find that downstream activation of AKT is impaired in parallel with phosphorylation of p85α at these sites.

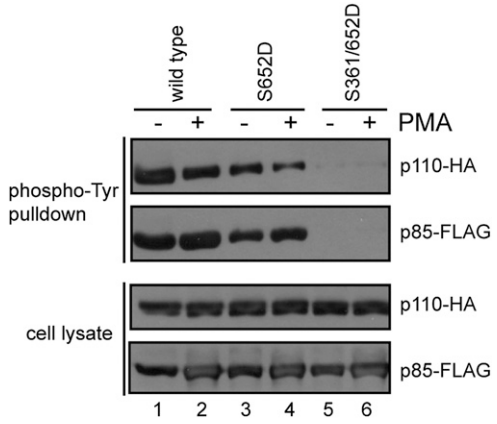

Fig. 5. S361 and S652 phosphomimetic mutations abrogate PI3K binding to phospho-Tyr peptides. HEK293T cells transfected with FLAG-tagged p85α (wild type, S652D, or S361/652D double mutant) and HA-tagged p110α were treated with 100 nM PMA or DMSO vehicle for 4 h, and beads containing Tyr-phosphorylated peptide were used to pull down the various p85α wild-type or mutant proteins from the cell lysates. The amounts of phospho-Tyr–bound p85α and p110α were detected by immunoblot with anti-FLAG and anti-HA antibodies, respectively.

The data presented here lead to a model in which prolonged activation of PKC family members (and PKD) because of persistent signaling through PLC ultimately leads to down-regulation of PI3K signaling. This cross talk between PLC signaling and PI3K signaling is not surprising because PI3K is known to facilitate PLC activation in a number of cell types, especially lymphocytes (11, 12). Interestingly, the phosphorylation of p85α is relatively slow compared with the rate of activation of PKC and PKD by PMA. This result suggests that only a subset of total cellular p85α is capable of being phosphorylated at the inhibitory sites at a given time and is consistent with our observation that p85α that is in complex with p110α is preferentially phosphorylated in response to PMA stimulation of cells. One explanation for this model is that p110α facilitates localization of the p85α/p110α heterodimeric complex at sites where PKC and/or PKD are activated. p110α has a number of domains, including a C2 domain and a Ras-binding domain that could facilitate localization of the heterodimeric complex to regions of the membrane where phosphorylation at the inhibitory sites could occur. Recent structural studies with the p85β–p110β complex revealed that the phospho-Tyr–binding region of the C-terminal SH2 domain is exposed (10), and therefore S652 would be accessible for phosphorylation by PKD.

Also of interest, S361 is in close proximity with D1029 of the catalytic domain of p110α (Fig. 4). It seems unlikely that a protein kinase could phosphorylate S361 when it is in the conformation observed in the crystal structure of the p85α/p110α heterodimer. This observation raises the possibility that the heterodimer must be in an open active conformation to be phosphorylated at S361. Several structural studies have suggested that activation of PI3K involves a conformational change in which the N-terminal SH2 domain dissociates from the helical (and nearby catalytic) domains of p110α to allow substrate access to the catalytic site (6, 8, 9). Thus, phosphorylation of S361 might occur preferentially on a subfraction of PI3K that is in the active conformation but not bound to a Tyr-phosphorylated protein (which would block access to both S361 and S652). Phosphorylation of S361 probably would cause a repulsive interaction with D1029 but possibly could stabilize another interaction with p110α (e.g., formation of a salt bond with the adjacent K1030 of p110α). Thus, it is possible that phosphorylation of S361 not only affects phospho-Tyr–dependent interactions but also has a direct effect on catalytic activity of p110α. Interestingly, D1029 was found mutated to Tyr in one lung cancer cell line (30), suggesting

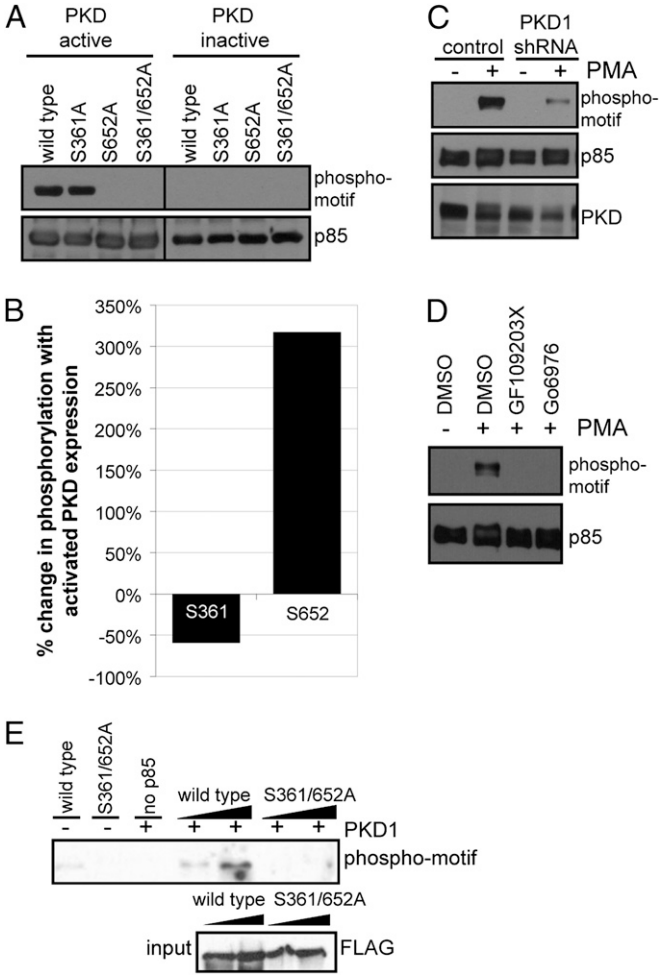

Fig. 6. PKD phosphorylates p85α S652. (*A*) HEK293T cells were cotransfected with FLAG-tagged p85α (wild type, S361A, S652A, or S361/652A) and PKD (constitutively active S744/748E or catalytically inactive K612W). FLAG-p85α was immunoprecipitated from cell lysates with anti-FLAG M2 agarose and immunoblotted with the anti–phospho-motif antibody. (*B*) HEK293T cells were transfected with TAP-p85α and constitutively active or catalytically inactive PKD. TAP-p85α was precipitated with streptavidin beads and separated by SDS/PAGE, and a Coomassie-stained band containing p85α was excised for quantitative mass spectrometry ratio analysis as described in Fig. S1. The results are shown in the bar graph. (*C*) HEK293T cells stably expressing PKD1 shRNA were transfected with TAP-p85α and treated with 100 nM PMA or DMSO vehicle for 4 h. TAP-p85α was precipitated from cell lysates and immunoblotted with the anti–phospho-motif antibody. Cell lysates were immunoblotted with anti-PKD antibody. (*D*) HEK293T cells transfected with TAP-p85α were pretreated with GF109203X (5 μM) or Go6976 (1 μM) for 30 min, followed by stimulation with 100 nM PMA for 4 h. TAP-p85α was precipitated from cell lysates and immunoblotted with the anti–phospho-motif antibody. (*E*) Wild-type p85α or S361/652A double mutant was expressed in and purified from HEK293T cells. The purified p85α was incubated with recombinant PKD1 and ATP, and in vitro phosphorylation of p85α was detected by immunoblot with the anti–phospho-motif antibody.

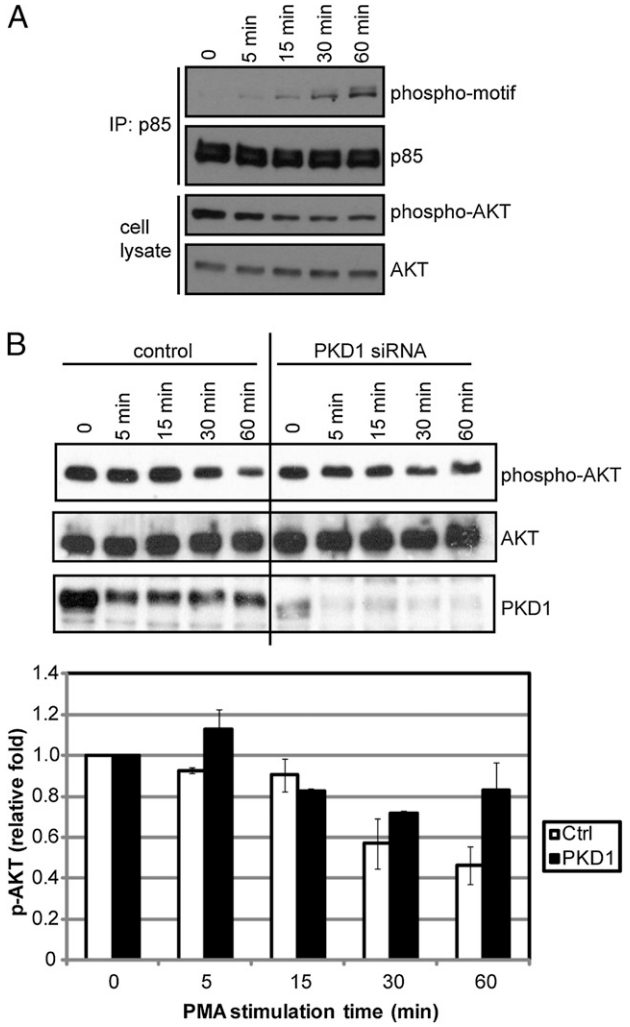

Fig. 7. Phorbol ester stimulation results in decreased Akt activation. (*A*) HEK293T cells transfected with TAP-p85α were stimulated with 100 nM PMA for the indicated times. TAP-p85α was precipitated from cell lysates and immunoblotted with the anti–phospho-motif antibody. Cell lysates were immunoblotted with anti–phospho-Akt (S473) and anti-Akt antibodies. (*B*) (*Upper*) HEK293T cells expressing PKD1 or nontargeting siRNA were treated with PMA for the indicated times, and cell lysates were immunoblotted with anti–phospho-Akt (S473), anti-Akt, and PKD1 antibodies. (*Lower*) Quantitation of results in *Upper* panel. Error bars indicate SEM from three independent experiments. The decrease in phospho-Akt from 0 to 60-min PMA stimulation in the control cells was statistically significant by student's *t* test ($P = 0.02$), but the change in the PKD1 siRNA-treated cells was not statistically significant ($P = 0.24$).

that interaction between S361 and D1029 of p110α could participate in suppressing activity. Additional structural studies with mutations at S361 and S652 should help elucidate the role of these residues in the p85α/p110α heterodimer interactions.

Finally, although phosphorylation of both S361 and S652 on the two SH2 domains of p85α may be needed to block binding of the p85/p110 heterodimer to Tyr-phosphorylated adaptors such as IRS1, it is necessary to phosphorylate only one SH2 domain to impair activation of PI3K enzymatic activity. The well-characterized activators of class IA PI3Ks invariably have at least two closely spaced pYXXM motifs that engage both SH2 domains simultaneously. Simultaneous engagement of both SH2 domains not only provides additional avidity between the activator and PI3K but also appears to induce a conformational change that converts PI3K from the low-activity closed state into a high-activity open state (5). Although singly phosphorylated peptides can bind to PI3K, they have a minor effect on activity compared with multiphosphorylated peptides. This concept is critical because it argues that phosphorylation of both S361 and S652 simultaneously would be required to dissociate PI3K from upstream activators (consistent with results in Fig. 5) but that phosphorylation of either S361 alone or S652 alone would be sufficient to turn down the activity of PI3K. Although the ab-

solute stoichiometry of phosphorylation of endogenous p85α (or p85β) at the S361 and S652 sites has been difficult to estimate using mass spectrometry, our studies with transfected p85α suggest that a significant fraction of the p85α/p110α heterodimer is phosphorylated at either S361 or S652 (if not both) in response to PMA and that this phosphorylation can account for the decreased AKT activation.

In conclusion, we have demonstrated that phorbol ester stimulation leads to serine phosphorylation in the SH2 domains of PI3K p85α. Through the use of overexpression and shRNA knockdown, we have determined that PKD plays a role in this phosphorylation event. Importantly, serine phosphorylation abrogates the SH2 domain interaction with phospho-tyrosine. We have identified a mechanism for negative regulation of the long-standing function of PI3K SH2 domains and reveal a mechanism of negative feedback to the PI3K pathway through cross talk from the PKC pathway.

Materials and Methods

Plasmids, antibodies, other reagents, and cell culture methods are described in *SI Materials and Methods*.

Immunoprecipitation and Immunoblotting. Cells were lysed in lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% (vol/vol) Nonidet P-40, 0.25% (wt/vol) sodium deoxycholate, 1 mM EDTA, 10% (vol/vol) glycerol, sodium fluoride, sodium pyrophosphate, β-glycerophosphate, calyculin A, sodium orthovanadate, aprotinin, peptstatin, leupeptin, and PMSF. The clarified supernatant was subjected to immunoprecipitation for 2 h at 4 °C, and the immunoprecipitate was washed three times with lysis buffer. Immunoprecipitates and lysates were separated on SDS/PAGE gels and subjected to Western blotting. Antibodies were diluted in 5% (wt/vol) BSA in Tris-buffered saline Tween-20 (Sigma) according to manufacturer's instructions.

Peptide Pull-Down Assay. Biotinylated phosphotyrosine-FLT3 or nonphosphorylated FLT3 peptide was immobilized onto streptavidin beads. Cell lysates from cells expressing FLAG-p85α were incubated with the beads for 2 h at 4 °C, beads were washed three times with lysis buffer, and samples were analyzed by SDS/PAGE and Western blotting.

In Vitro Kinase Assay. Wild-type and mutant p85α proteins were purified from HEK293T cells transfected with FLAG-tagged full-length mouse p85α constructs. Purified p85α proteins were incubated with PKD1 (6 ng/μL) (Millipore) and 0.1 mM ATP in reaction buffer [20 mM Hepes-OH (pH 7.5), 20 mM NaCl, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, and 10 mM $MgCl_2$] at 30 °C for 30 min.

Microcapillary MS/MS. Coomassie-stained SDS/PAGE gels bands containing TAP-p85α from cells treated with PMA or coexpressing PKD were excised and subject to reduction with 10 mM DTT for 30 min, alkylation with 55 mM iodoacetamide for 45 min, and in-gel digestion with chymotrypsin overnight at pH 8.3, followed by reversed-phase microcapillary LC/MS/MS. LC/MS/MS was performed using an EASY-nLC splitless nanoflow HPLC (Proxeon Biosciences) with a self-packed 75-μm i.d. × 15 cm C_{18} Picofrit column (New Objective) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific) in the data-dependent acquisition and positive ion mode at 300 nL/min with one full MS-FT scan followed by six MS/MS spectra. Analysis of the results was performed as described in the *SI Materials and Methods*.

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