Small GTPases and tyrosine kinases coregulate a molecular switch in the phosphoinositide 3-kinase regulatory subunit

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Summary

Phosphoinositide 3-kinase (PI3K) type IA is a heterodimer of a catalytic subunit, p110, and a regulatory subunit, p85. Here we show that p85 contains a GTPase-responsive domain and an inhibitory domain, which together form a molecular switch that regulates PI3K. H-Ras and Rac1 activate PI3K by targeting the GTPase-responsive domain. The stimulatory effect of these molecules, however, is blocked by the inhibitory domain, which functions by binding to tyrosine-phosphorylated molecules and is neutralized by tyrosine phosphorylation. The complementary effects of tyrosine kinases and small GTPases on the p85 molecular switch result in synergy between these two classes of molecules toward the activation of the PI3K/Akt pathway.

Introduction

Phosphoinositide 3-kinase (PI3K) type IA, a heterodimer of a regulatory subunit (p85) and a catalytic subunit (p110), catalyzes the phosphorylation of PtdIns-4,5-P_2 to form PtdIns-3,4,5-P_3 (Wymann and Pirola, 1998). PtdIns-3,4,5-P_3, in turn, regulates a diverse array of cellular functions including apoptosis, proliferation, differentiation, and intermediary metabolism (Chan et al., 1999; Rameh and Cantley, 1999). PI3K is activated rapidly following growth factor stimulation and crosslinking of cell adhesion molecules (Rameh and Cantley, 1999). Despite extensive studies on the mechanism of PI3K activation by these signals, however, significant questions remain unanswered.

Earlier studies suggested that the binding of the regulatory subunit of PI3K to tyrosine-phosphorylated molecules is directly responsible for PI3K activation (Backer et al., 1992; Carpenter et al., 1993). However, recent in vitro studies (Layton et al., 1998) and earlier in vivo studies (Fukui and Hanafusa, 1989; Varticovski et al., 1991) suggested that binding is not sufficient to activate PI3K. Other studies showed that PI3K is also activated by small GTPase molecules such as Ras and Rac1 (Nishida et al., 1999; Rodriguez-Viciana et al., 1994). These molecules activate PI3K by diverse, although not clearly defined, mechanisms. Thus, PI3K activation by Ras has been correlated with direct binding between Ras and p110 (Rodriguez-Viciana et al., 1994), while PI3K activation by Rac1 may be mediated by binding of Rac1 to p85 (Tolias et al., 1995; Zheng et al., 1994). However, we and others have recently found that direct interaction of PI3K with either H-Ras or Rac1 is not required for PI3K activation (Karasarides et al., 2001; see Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/1/2/181/DC1). Moreover, constitutively active Ras activates the PI3K/Akt pathway in synergy with Src (Datta et al., 1996), suggesting that signals transduced via small GTPases and tyrosine kinases regulate this pathway cooperatively.

The studies in this manuscript reexamine the role of tyrosine kinases and small GTPases in PI3K activation in vivo. The reason we focus on the regulation of PI3K in vivo is that critical factors involved in its regulation are lost upon purification. Thus, in the absence of growth factor or integrin stimulation, PI3K fails to catalyze PtdIns-3,4,5-P_3 synthesis in vivo (Wymann and Pirola, 1998). However, the enzyme becomes constitutively active upon purification. Moreover, Carpenter et al. found that higher PI3K-specific activity in vitro correlates with increasing purity of PI3K preparations (Carpenter et al., 1990). Finally, PI3K immunopurified from serum-starved cells has the same specific activity as PI3K from PDGF-stimulated cells (Hu et al., 1992). These studies indicate that inhibitory factors, lost upon purification, block PI3K

SIGNIFICANCE

Upon stimulation by many extracellular stimuli, PI3K type IA catalyzes the synthesis of phosphatidylinositol-3,4,5-P_3, a second messenger that regulates cell growth, proliferation, apoptosis, and intermediary metabolism. Interestingly, the cellular levels of phosphatidylinositol-3,4,5-P_3 are elevated in almost all cancers through the acquisition of mutations in tyrosine kinases, small GTPases, and the phosphatidylinositol-3 phosphatase, PTEN. Data in this report suggest a unifying model of PI3K activation by tyrosine kinases and small GTPases, according to which these two classes of signaling molecules act in concert to trigger a molecular switch that regulates PI3K. Given the importance of phosphatidylinositol-3,4,5-P_3 and its target Akt, in human cancer, the molecular switch described in this paper defines an important target for future chemotherapeutic drugs.

activity in vivo (Carpenter et al., 1990). Though phosphotyrosine peptides have been shown to modestly enhance type IA PI3K activity in vitro (Backer et al., 1992; Carpenter et al., 1993), Layton et al. showed, and we confirmed, that this enhancement depends on conditions used for the in vitro assay (Layton et al., 1998; see Supplemental Figure S2 at http://www.cancercell.org/cgi/content/full/1/2/181/DC1) and that phosphotyrosine binding alone is not sufficient to activate PI3K in vivo. Based on the results of the studies described in this report, we propose a unifying model according to which all tyrosine kinases and small GTPases activate PI3K by acting synergistically on a molecular switch in p85.

Results

Wild-type p85 and carboxy-terminally truncated p85 exert opposite effects on PtdIns-3,4,5-P_3 synthesis induced by H-Ras or Rac1

Earlier studies had shown that a truncated p85 molecule lacking the carboxy-terminal SH2 domain and 52 adjacent amino acids from the inter-SH2 (iSH2) region, referred to as p85Δonc in this communication, activates PI3K constitutively and transforms cells in culture (Jimenez et al., 1998). Wild-type p85, on the other hand, blocks H-Ras-induced PI3K activation and cellular transformation (Rodriguez-Viciana et al., 1997). This raised the question whether p85Δonc and p85 differ in their ability to regulate PI3K activation by H-Ras. To address this question, we used a thin-layer chromatography-based assay (Maehama and Dixon, 1998) to separate PtdIns-3,4,5-P_3 from lipid extracts of ^{32}P-labeled NIH3T3 cells transfected with Hemagglutinin (HA)-tagged constructs of wild-type p85 (HA-p85-WT) or p85Δonc (HA-p85Δonc) and constitutively-active H-Ras (H-Ras G12V) in the combinations shown in Figure 1B. HA-p85-WT and HA-p85Δonc were both expressed at similar levels (Figure 1A). Consistent with the findings of Jimenez et al. (1998), HA-p85Δonc alone weakly activated PI3K in vivo (Figure 1B, upper gel). p85 and p85Δonc, however, had diametrically opposite effects when cotransfected with Ras in that whereas p85 inhibited the activation of PI3K, p85Δonc enhanced it dramatically. Figure 1C shows that the synergy between H-Ras and p85Δonc is wortmannin sensitive, and therefore, PI3K dependent.

PtdIns-3,4,5-P_3 synthesized by the activated PI3K is required for activation of the Akt kinase (Chan et al., 1999). This suggests that Akt phosphorylation and activity may correlate with the intracellular levels of PtdIns-3,4,5-P_3. To address this question, we probed an immunoblot of cell lysates derived from the cells in the preceding experiment, with an antibody that recognizes the phosphorylated Thr308 motif of Akt. The results (Figure 1B, lower gel) confirmed the predicted correlation between Akt phosphorylation and PtdIns-3,4,5-P_3 levels. The fact that Ras and p85Δonc activate Akt via PI3K and not via direct phosphorylation of Akt was further confirmed by experiments showing that the combination of Ras and p85Δonc does not activate Akt in PDK1$^{-/-}$ cells (see Supplemental Figure S3 at http://www.cancercell.org/cgi/content/full/1/2/181/DC1).

We next examined whether p85Δonc synergizes with small GTPases other than Ras to activate the PI3K pathway. Figure 1D (upper gel) shows that the combination of transiently transfected, constitutively active Rac1 (Rac1 Q61L) and p85Δonc in NIH3T3 cells dramatically enhanced the intracellular levels of PtdIns-3,4,5-P_3. The lower gel of the same figure shows again that the levels of PtdIns-3,4,5-P_3 correlate with the phosphorylation of Akt at Thr308. Based on these observations, we decided to use the in vitro kinase activity of Akt to monitor the in vivo activity of PI3K. The results of critical experiments were confirmed by direct measurement of the intracellular levels of PtdIns-3,4,5-P_3.

PI3K activation via the synergistic actions of Rac1 and

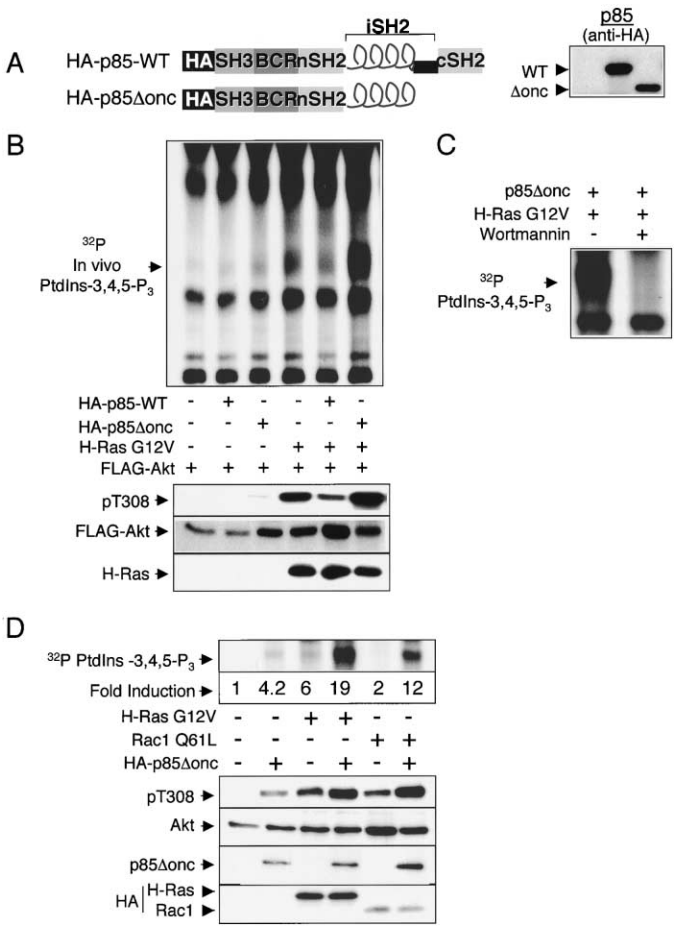

Figure 1. p85 inhibits while p85Δonc enhances PI3K activation by small GTPases

A: Schematic diagrams and expression of transiently transfected HA-tagged p85-WT and p85Δonc constructs. **B:** Top: p85-WT inhibits while p85Δonc enhances H-Ras-induced PtdIns-3,4,5-P_3 production. The indicated constructs were transiently transfected into NIH3T3 cells. Sixteen hours after transfection, the cells were cultured in serum-free media. Five hours later, they were labeled with ^{32}P-orthophosphate for 1.5 hr. Total phospholipids were analyzed by thin-layer chromatography. Bottom: p85-WT inhibits while p85Δonc enhances H-Ras-induced Akt phosphorylation. Immunoblots of cell extracts from duplicate transfections were probed with an antibody that recognizes Akt phosphorylated at Thr308 or with antibodies that recognize the proteins expressed from the transfected constructs. **C:** PtdIns-3,4,5-P_3 induction by p85Δonc and constitutively active H-Ras is inhibited by the PI3K inhibitor, wortmannin. NIH3T3 cells were transiently transfected with the indicated constructs, and they were labeled with ^{32}P-orthophosphate as described in **B**. 45 minutes prior to lipid extraction, the cells were treated with 100 nM wortmannin or DMSO. **D:** p85Δonc synergizes with Rac1 to induce PtdIns-3,4,5-P_3 synthesis and Akt activation. NIH3T3 cells were transiently transfected with the indicated constructs. ^{32}P-labeled PtdIns-3,4,5-P_3 was measured as described in **B**. Akt phosphorylation at Thr308 and expression of transfected constructs were determined by probing immunoblots with the corresponding antibodies.

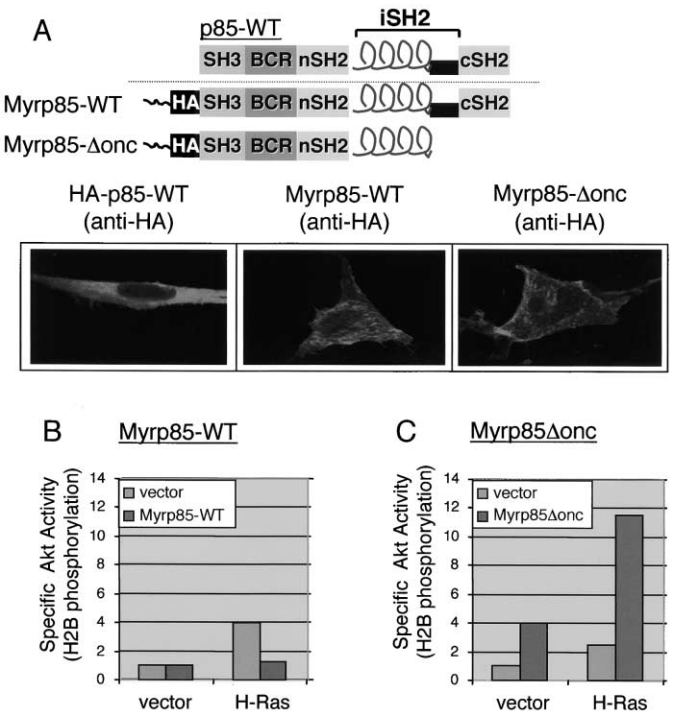

Figure 2. Membrane-targeted p85 continues to block PI3K/Akt activation by H-Ras

A: Schematic diagrams of HA-tagged Myrp85-WT and Myrp85Δonc. NIH3T3 cells transfected with these constructs were fixed with paraformaldehyde, and they were incubated with the anti-HA antibody. Bound antibody was visualized by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antiserum and fluorescence confocal microscopy. **B:** Myrp85-WT inhibits PI3K activation by H-Ras. Constitutively active H-Ras and FLAG-c-Akt were transfected into NIH3T3 cells in combination with Myrp85-WT or vector. The in vitro phosphorylation of Histone 2B (H2B) by FLAG-Akt immunoprecipitates was measured by phosphorimager. Akt levels in the immunoprecipitates were measured by immunoblotting and laser densitometry scanning (Molecular Dynamics). The bar graphs show the specific activity of Akt as calculated from these two values. H2B phosphorylation is presented in arbitrary units. **C:** Myrp85Δonc enhances PI3K activation by H-Ras. Constitutively active H-Ras was transfected in combination with Myrp85Δonc or vector into NIH3T3 cells. The specific activity of Akt was determined as in **B**.

p85Δonc does not depend on the binding of Rac1 to p110, because these proteins do not interact (Tolias et al., 1995; Zheng et al., 1994). Therefore, if Rac1 and H-Ras synergize with p85Δonc via similar mechanisms, the synergy of H-Ras with p85Δonc should also be independent of the interaction between H-Ras and p110. Recent studies by us and others revealed that this is indeed the case. Karasarides et al. (2001) recently showed that an activated H-Ras mutant (Y64G/Y71G/F156L), which fails to bind p110, activates the PI3K/Akt pathway in vivo. Similarly, we found that both H-Ras G12V/E37G and H-Ras G12V/Y40C, two activated H-Ras mutants of which only the latter interacts with p110 (Rodriguez-Viciana et al., 1997; White et al., 1995), synergize equally well with p85Δonc to activate PI3K (see Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/1/2/181/DC1). The same figure shows that overexpression of the BCR domain of p85, which is known to bind Rac1 (Tolias et al., 1995; Zheng et al., 1994), does not inhibit PI3K activation by Rac1. Therefore, the Rac1/p85 interaction is also not required for PI3K activation.

Membrane localization of p85 is not sufficient to activate PI3K

Truncated p85 is thought to activate PI3K by directing it to the plasma membrane (Jimenez et al., 1998). We, therefore, tested whether membrane-associated full-length p85 is functionally similar to the carboxy-terminally truncated protein. To this end, p85 fused at its amino terminus to a peptide encoding the Src myristoylation signal (Myrp85-WT; Figure 2A) was transiently expressed in NIH3T3 cells in combination with FLAG-Akt or FLAG-Akt plus H-Ras. Myrp85Δonc was used as a control. The cells were lysed 48 hr later, following overnight serum starvation. Akt activation in this and subsequent experiments was determined by measuring the phosphorylation of histone 2B (H2B) by immunoprecipitated Akt.

In vitro kinase assays of FLAG-Akt revealed that membrane-targeted p85 is similar to the wild-type protein in that it does not activate the PI3K/Akt pathway by itself and in that it blocks activation of the pathway by H-Ras (Figure 2B). Myrp85Δonc, on the other hand, was similar to p85Δonc in that it activated the PI3K/Akt pathway both alone and in synergy with H-Ras (Figure 2C). We conclude that association of p85 with the plasma membrane is not sufficient to activate PI3K. Therefore, the carboxy-terminal truncation of p85 is unlikely to activate PI3K by targeting the enzyme to the plasma membrane. A more likely possibility is that truncation unveils a domain in p85 that responds to Ras- or Rac1-transduced signals to activate PI3K (GTPase-responsive domain, GRD). Subsequent experiments were designed to map the GRD within p85.

A protein motif within the iSH2 domain of p85 synergizes with H-Ras and Rac1 to activate the PI3K/Akt pathway

The p85 regulatory subunit of PI3K contains several protein domains, including SH3, BCR homology, two SH2 domains, and an inter-SH2 domain (iSH2; Figure 3A). Computer modeling of the iSH2 domain suggested that this region resembles a coiled-coil structure (Wymann and Pirola, 1998). A motif (TIF), previously mapped within this structure, binds the PI3K catalytic subunit, p110 (Dhand et al., 1994). The TIF motif is highly conserved across p85 subtypes (α, β, and γ) and across species (bovine, rat, and *Drosophila*; Figure 3A). Other conserved motifs include a motif we named IKR, which is rich in isoleucine, lysine, and arginine residues, and a motif we named LED, which is rich in leucine, glutamic acid, and aspartic acid residues. The LED motif is located downstream from the IKR motif and corresponds to the carboxyl terminus of the iSH2 domain. The carboxyl terminus of the IKR motif corresponds to the breakpoint of the p85 oncogenic truncation.

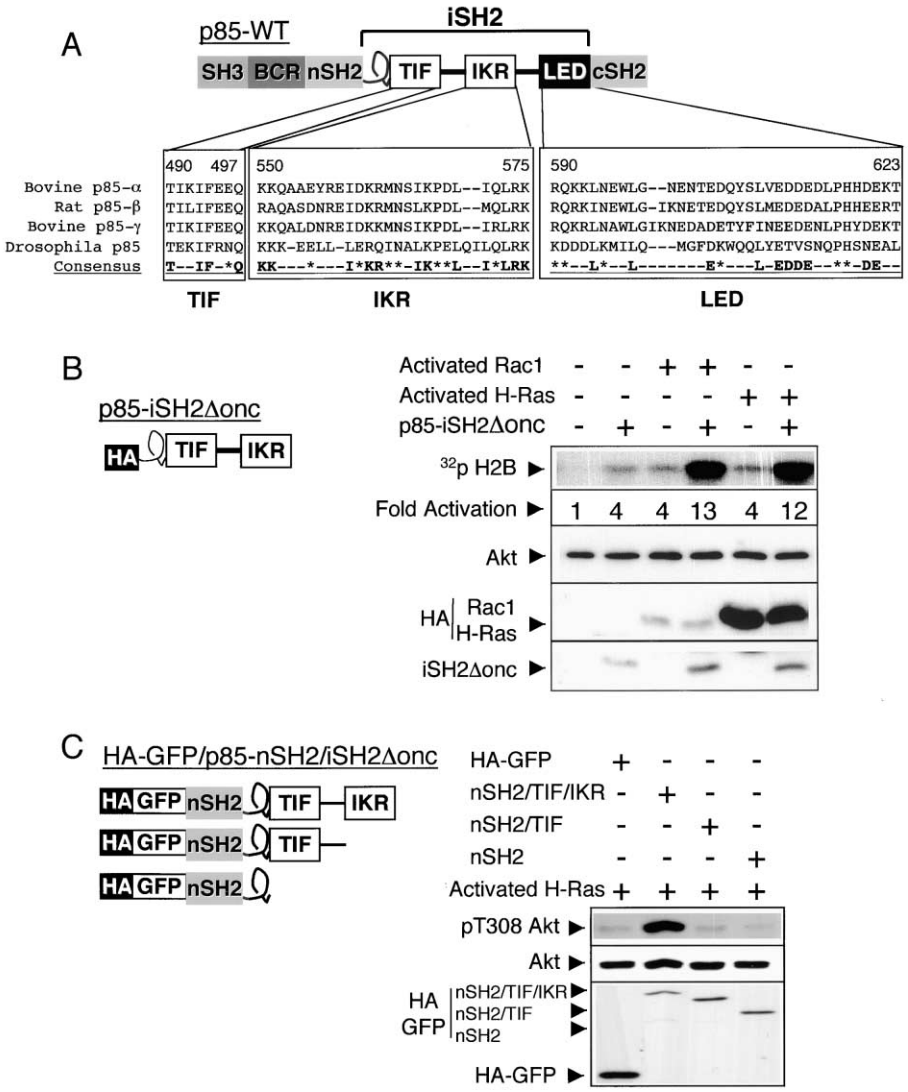

Figure 3. The carboxy-terminally truncated inter-SH2 domain of p85 synergizes with Ras and Rac1 to activate the PI3K/Akt pathway.

A: Schematic diagram of wild-type p85 and sequence comparison of inter-SH2 domains. Lineup of the amino acid sequence of several p85 iSH2 domains, bovine p85-α(M61745), Rat p85-β (NM_022185), bovine p85-γ (AF036256), and *Drosophila* p85(Y11143). Conserved amino acid motifs include TIF (p110 binding motif), IKR, and LED. **B:** The p85-iSH2 domain lacking 52 amino acids from its carboxyl terminus synergizes with both activated H-Ras and activated Rac1. (Left) Schematic diagram shows an HA-tagged construct of the inter-SH2 domain carrying the same carboxy-terminal truncation as p85Δonc (p85-iSH2Δonc). (Right) The indicated constructs were transiently transfected into NIH3T3 cells. Akt activity was measured by in vitro phosphorylation of H2B. Expression of proteins encoded by the transfected constructs was determined by probing immunoblots of total lysates with the anti-HA antibody. **C:** The p85 IKR motif is a required component of the GRD domain. Schematic diagram on the left shows the domain composition of transfected constructs. These constructs were transfected into NIH3T3 cells together with an expression construct of constitutively active H-Ras. Akt activity was measured by Akt phosphorylation at Thr308. Expression of the transfected constructs were determined by probing immunoblots with the corresponding antibodies.

Given that the p85 oncogenic truncation separates the TIF and IKR motifs from the LED motif, we examined whether the TIF/IKR portion of iSH2 (p85-iSH2Δonc; Figure 3A) synergizes with Ras or Rac1 to activate the PI3K pathway. To this end, we transfected NIH3T3 cells with FLAG-Akt and activated H-Ras G12V or activated Rac1 Q61L in combination with p85-iSH2Δonc. The Akt kinase activity was measured 48 hr later, following serum starvation for 16 hr. The results showed that iSH2Δonc, indeed, activates the PI3K/Akt pathway, alone and in synergy with H-Ras or Rac1 (Figure 3B). However, iSH2Δonc did not bind either Ras or Rac1 (data not shown), suggesting that the functional interaction between iSH2Δonc and these molecules is likely to be indirect. Next, we examined whether the IKR motif is necessary for iSH2Δonc function. To this end, the intact nSH2/iSH2Δonc and nSH2/ iSH2Δonc molecules with carboxy-terminal deletions of the IKR or the IKR plus TIF motifs were fused to Hemagglutinin-tagged green fluorescence protein (HA-GFP). The GFP fusion constructs were transiently transfected in combination with FLAG-Akt into NIH3T3 cells. Probing an immunoblot of cell lysates with an antibody that recognizes the phosphorylated Thr308 motif of Akt revealed that the deletion of IKR abolishes iSH2Δonc function (Figure 3C). We conclude that the IKR motif is a required component of the GRD domain.

The LED and cSH2 motifs of p85 define a modular inhibitory domain

The blockage of Ras signal by wild-type p85, but not by p85Δonc, suggests that the carboxy-terminal region of this molecule may contain an inhibitory domain. To test this hypothesis, we transfected NIH3T3 cells with H-Ras G12V in combination with expression constructs of the carboxyl terminus of p85 (p85-CT, amino acids 514–725) or p85-DN, a known dominant-negative mutant of p85 (deletion of amino acids 479–513) (Dhand et al., 1994). After overnight serum starvation, the activity of cotransfected FLAG-Akt was examined. The results showed that p85-CT is as efficient as p85-DN at inhibiting the Ras signal (Figure 4B) and confirmed that the carboxyl terminus of p85 contains a modular inhibitory domain.

To map the p85-CT motifs required for the inhibitory function, we fused p85-CT or portions of it with HA-GFP (Figure

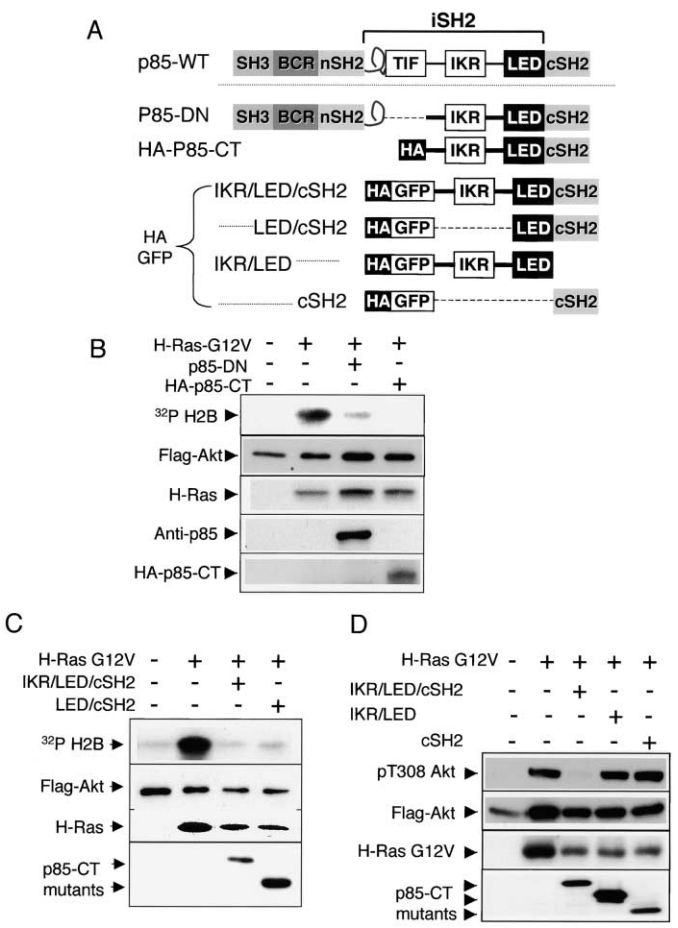

Figure 4. The carboxy-terminal p85 inhibitory domain is modular and consists of the LED and cSH2 motifs

A: Schematic diagrams of p85 constructs used in the experiments presented here. **B:** p85-CT and p85-DN inhibit the activation of the PI3K/Akt pathway by H-Ras-G12V. The indicated constructs, in combination with a FLAG-cAkt construct, were transiently transfected into NIH3T3 cells. The Akt kinase activity was measured in anti-FLAG immunoprecipitates. Expression of FLAG-Akt was determined by probing immunoblots of the immunoprecipitates with a rabbit polyclonal anti-Akt antibody. Expression of H-Ras, p85-DN, and p85-CT was determined by probing immunoblots of total cell lysates with the corresponding antibodies. **C:** The PI3K inhibitory domain consists of the LED and cSH2 motifs. NIH3T3 cells were transfected with the indicated constructs. Akt activity was measured by in vitro phosphorylation of H2B. Expression of the HA-GFP fusion proteins and H-Ras-G12V was determined by probing immunoblots of total lysates with the anti-HA antibody. **D:** The cSH2 and IKR/LED motifs alone have no inhibitory activity. NIH3T3 cells were transfected with the indicated constructs. The phosphorylation of Akt at Thr308 was measured by immunoblotting. Expression of the transfected constructs was determined by probing immunoblots with the corresponding antibodies.

4A). Expression of the GFP fusion constructs in combination with H-Ras-G12V in NIH3T3 cells showed that the IKR motif is not required for the p85 inhibitory function (Figure 4C). Additional deletions showed that the minimal inhibitory domain contains both the LED and the carboxy-terminal SH2 domains. Deletion of either domain abolished inhibitory function (Figure 4D). Both the inhibitory protein LED/cSH2 and the noninhibiting cSH2 were able to bind phosphotyrosine targets, suggesting that they folded properly (data not shown).

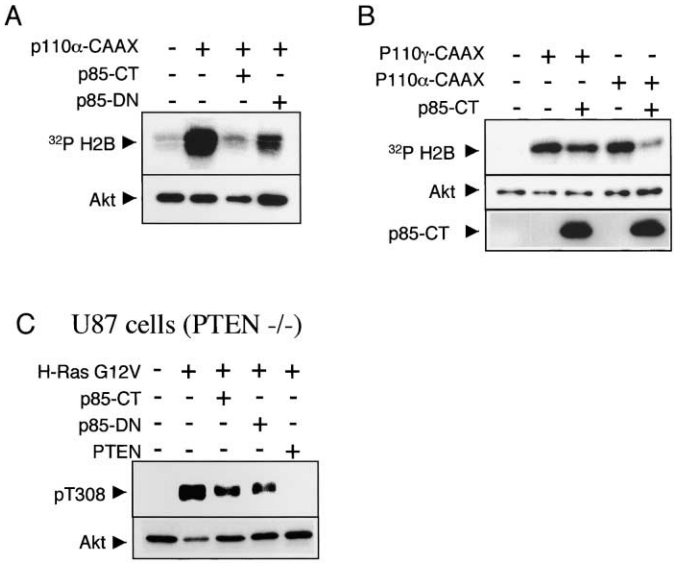

Figure 5. The PI3K inhibitory domain inhibits p110α, but not p110γ

The inhibition is independent of PTEN. **A and B:** p85-CT and p85-DN block Akt activation by constitutively active p110α-CAAX. NIH3T3 cells were transfected with the indicated constructs. Akt activity was measured by in vitro phosphorylation of H2B. **C:** p85-CT and p85-DN block Akt activation by Ras in the absence of PTEN. The PTEN null (Li et al., 1997) human glioblastoma cell line, U87MG, was transfected with the indicated constructs. Phosphorylation of Akt at Thr308 was measured in lysates of the transfected cells by immunoblotting.

The p85 inhibitory domain targets PI3K type IA and not PTEN

The p85 inhibitory domain may target molecules that regulate the enzymatic activity of p110. Alternatively, it may target molecules that function upstream of p110 to regulate its activation. To distinguish between these possibilities, we examined whether p85-CT inhibits the activity of p110-CAAX, a membrane bound, constitutively active p110 mutant (Klippel et al., 1996). To this end, p85-CT or p85-DN constructs were transfected into NIH3T3 cells in combination with constitutively active p110α and FLAG-Akt. In vitro kinase assays of Akt immunoprecipitated from the transfected cells 48 hr later showed that the p85 inhibitory domain blocks Akt activation by p110α-CAAX (Figure 5A). Therefore, the p85 inhibitory domain targets molecules that directly regulate the activity of p110. A similar experiment comparing the effects of p85-CT on membrane-targeted p110α and p110γ, two type I PI3K molecules of which only the former binds p85, showed that p85-CT inhibits only p110α (Figure 5B).

PtdIns-3,4,5-P_3 levels may be induced because of PI3K activation. Alternatively, they may be induced because of inactivation of PTEN, a tumor-suppressor gene that encodes a PtdIns-3,4,5-P_3 phosphatase (Cantley and Neel, 1999). The difference between p110α and p110γ suggests that the p85 inhibitory domain targets PI3K and not PTEN. To address this hypothesis, p85-CT or p85-DN constructs were transfected into the PTEN null cell line, U87MG (Li et al., 1997), in combination with constitutively active H-Ras G12V and FLAG-Akt. The results showed that p85-CT blocks activation of the PI3K/Akt pathway by Ras, even in the absence of PTEN (Figure 5C). We conclude that the p85 inhibitory domain targets PI3K and not PTEN.

Tyrosine kinase signals neutralize p85 inhibitory function

Rodriguez-Viciana et al. showed that forced expression of p85 fails to block Src-induced transformation of NIH3T3 cells (Rodriguez-Viciana et al., 1997). We, therefore, reasoned that p85 may not inhibit PI3K activation by Src. To test this hypothesis, NIH3T3 cells were transfected with expression constructs of p85-WT, H-Ras G12V, Src Y527F, and FLAG-Akt in the combination shown in Figure 6B. In vitro kinase assays of Akt carried out 48 hr later, after overnight serum starvation, confirmed that p85-WT blocks Akt activation by H-Ras but not by Src.

One possible explanation for the failure of p85 to block PI3K activation by Src is that Src may neutralize the p85 inhibitory domain. Therefore, when coexpressed with constitutively active Src, wild-type p85 may become functionally equivalent to p85Δonc. This model, if correct, would explain our previous finding that Src and H-Ras synergize to activate the PI3K/Akt pathway (Datta et al., 1996). To test this hypothesis, we wanted to examine whether constitutively active Ras enhances Src plus p85-induced PI3K/Akt activation in NIH3T3 cells. However, such an experiment would be difficult to interpret, because Src is known to activate Ras (Marais et al., 1995). To carry out this experiment, therefore, we wanted to use a Src mutant that retained the ability to neutralize the p85 inhibitory domain, but no longer activated Ras. A G2A mutation that inactivates the Src myristoylation signal (Figure 6A) renders Src defective in Ras activation (Marais et al., 1995) without affecting its tyrosine kinase activity (Kamps et al., 1986). Consistent with these data, a Src G2A mutant (NM-Src) retained full tyrosine kinase activity but did not activate the Ras/MAP kinase pathway in NIH3T3 cells (Figure 6C). In addition, this mutant did not activate the PI3K/Akt pathway (Figure 6C). This mutant, similar to wild-type Src, was able to phosphorylate both endogenous and exogenous p85 (data not shown).

To determine whether NM-Src induces Ras-dependent PI3K activation, we examined whether, similar to its wild-type counterpart, NM-Src synergizes with Ras to activate the PI3K/Akt pathway. To this end, we coexpressed nonmyristoylated Src (NM-Src Y527F) or inactive Src (Src-K297M) with Ras-G12V in NIH3T3 cells. The results (Figure 6D) showed that NM-Src-Y527F and Ras-G12V indeed synergizes to activate Akt in NIH3T3 cells. This synergy strongly suggests that NM-Src neutralizes the p85 inhibitory domain. To test this hypothesis directly, we transfected H-Ras-G12V and p85-WT in combination with NM-Src and measured both the induction of PtdIns-3,4,5-P_3 and the phosphorylation of Akt at Thr308 in the transfected cells (Figure 6E). Both measurements confirmed that p85-WT does not inhibit PI3K activation by Ras signals in the presence of NM-Src. We conclude that Src transmits tyrosine-phosphorylation signals that neutralize the inhibitory domain of p85.

Many cytoplasmic tyrosine kinases and receptor tyrosine kinases are known to activate the PI3K/Akt pathway. We, therefore, tested some of these tyrosine kinases for their ability to neutralize the p85 inhibitory domain. Using synergy with Ras as the assay, we found that the Src catalytic domain (without its SH2/SH3 domains), other cytoplasmic tyrosine kinases (Syk and Abl but not $p125^{FAK}$), and receptor tyrosine kinases (EGF, PDGF, and IGF-1 receptors) all neutralize the inhibitory function of p85 (see Supplemental Figure S4 at http://www.cancercell.org/cgi/content/full/1/2/181/DC1). Using the same assay, we also showed that in addition to Ras and Rac1, small GTPases R-Ras and CDC42, but not RhoA, cooperate with tyrosine kinases to activate the PI3K/Akt pathway (see Supplemental Figure S5).

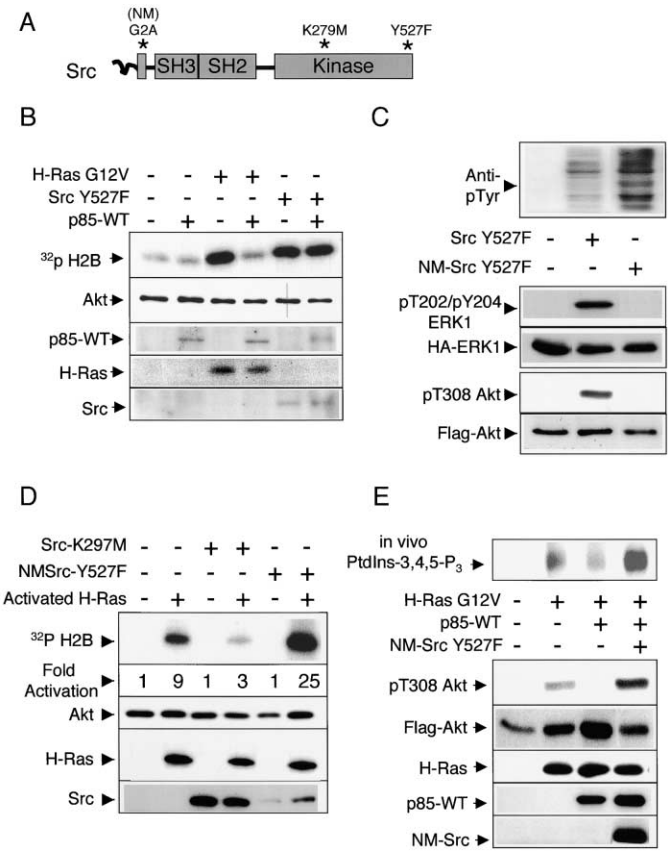

Figure 6. Ras-independent Src signals neutralize the p85 inhibitory domain

A: Schematic diagram of the chicken Src tyrosine kinase and its mutants used in this report. **B:** p85-WT inhibits Akt activation by Ras but not by Src. NIH3T3 cells were transfected with the indicated constructs. Akt activity was measured by in vitro phosphorylation of H2B. The expression of transfected proteins p85-WT, H-Ras, and Src-Y527F were monitored by probing immunoblots of total cell lysates with anti-HA antibody (H-Ras and p85-WT) or anti-Src antibody (Src). **C:** Mutation of the Src myristoylation signal does not affect Src tyrosine kinase activity, but abolishes Src-induced activation of the Ras/ERK pathway and the PI3K/Akt pathway. The indicated Src constructs were transfected into NIH3T3 cells in combination with either HA-ERK1 or FLAG-Akt. Src tyrosine kinase activity in vivo was determined by probing total cell extracts with the anti-phosphotyrosine antibody, 4G10. HA-ERK1 phosphorylation at Thr202/Tyr204 was measured by probing HA immunoprecipitates with an antibody that recognizes only the phosphorylated ERK1. Phosphorylation of Akt at Thr308 was also measured by immunoblotting. Expression of transfected constructs was determined by probing immunoblots of the cell lysates with the corresponding antibodies. **D:** The nonmyristoylated Src mutant synergizes with H-Ras to activate the PI3K/Akt pathway. The indicated constructs were transfected into NIH3T3 cells. Akt activity was measured by in vitro phosphorylation of H2B. Expression of transfected constructs was determined by probing immunoblots of total cell lysates with the corresponding antibodies. **E:** The nonmyristoylated Src abolishes the inhibition of PtdIns-3,4,5-P_3 production by p85. Constitutively active H-Ras-G12V was transfected in combination with p85-WT or nonmyristoylated Src Y527F into NIH3T3 cells. Sixteen hours after transfection, ^{32}P-labeled PtdIns-3,4,5-P_3 was measured as in Figure 1 (top gel). Akt phosphorylation at Thr308 was measured by immunoblotting in cell lysates from duplicate transfections. Expression of transfected constructs was determined by probing immunoblots with the corresponding antibodies.

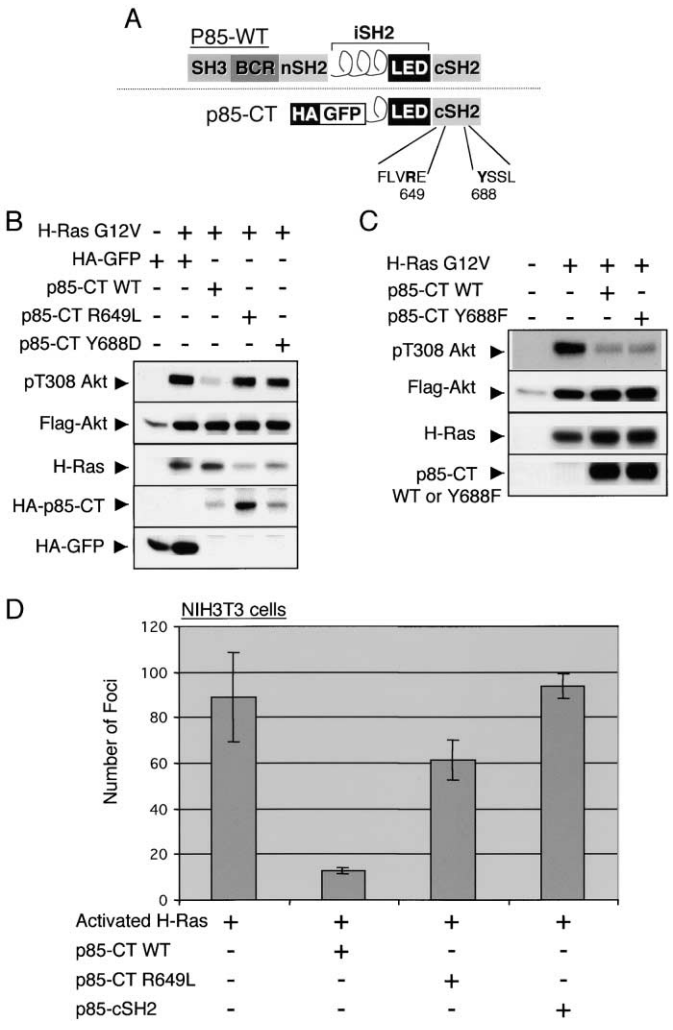

Figure 7. Mutations of the phosphotyrosine binding site and the Tyr688 phosphorylation site of the cSH2 motif neutralize the inhibitory domain

A: Schematic diagram of p85-CT. The diagram shows the phosphotyrosine binding FLVR motif and the Y668 tyrosine-phosphorylation site. **B:** The R649L and Y688D mutants of p85-CT do not inhibit activation of the PI3K/Akt pathway by Ras. The indicated constructs were transfected into NIH3T3 cells. Akt phosphorylation at Thr308 was determined by immunoblotting. Expression of transfected constructs was determined by probing immunoblots of total cell lysates with the corresponding antibodies. **C:** The phosphorylation site mutant Y688F continues to inhibit PI3K/Akt activation by Ras. Expression constructs of p85-CT or p85-CT Y688F were transfected into NIH3T3 cells in combination with FLAG-Akt, as indicated. Akt phosphorylation at Thr308 and expression of the transfected constructs were measured by immunoblotting as in **B**. **D:** p85-CT blocks cell transformation by activated H-Ras. NIH3T3 ($\sim 1.5 \times 10^5$) cells, cultured in100 mm petri dishes, were transfected with expression constructs of constitutively active H-Ras and wild-type or mutant p85-CT in the indicated combinations. Three weeks after transfection, the cells were fixed with 70% methanol, and they were stained with Giemsa (Chan et al., 1994).

cSH2 mutations abolish p85 inhibitory function

The preceding data suggest that Src and other tyrosine kinases transmit signals that neutralize the p85 inhibitory domain. Since the inhibitory domain encompasses the p85 carboxy-terminal SH2 motif (cSH2; Figure 7A), we asked whether its inhibitory function depends on phosphotyrosine binding. Binding of all SH2 domains to phosphotyrosine can be abolished by mutating the arginine residue of the FLVR motif (Kuriyan and Cowburn, 1997). We, therefore, coexpressed the p85-CT R649L FLVR mutant, which no longer binds tyrosine-phosphorylated molecules (data not shown), in combination with Ras G12V into NIH3T3 cells, and we examined whether the mutant continues to inhibit PI3K/Akt activation by Ras. The result showed that the R649L mutation abolished the inhibition of p85-CT (Figure 7B). We conclude that phosphotyrosine binding is required for inhibition.

In addition to enabling phosphotyrosine binding, tyrosine kinases, such as Abl and Lck, directly phosphorylate p85 cSH2 at tyrosine residue 688 (von Willebrand et al., 1998). To determine whether phosphorylation of this residue is involved in the neutralization of the p85 inhibitory domain, we mutated it to a phosphomimic aspartic acid (p85-CT Y688D). Coexpression of p85-CT Y688D and Ras G12V in NIH3T3 cells showed that this mutation abolished the inhibitory effect of p85-CT (Figure 7B). On the other hand, p85-CT with a tyrosine to phenylalanine mutation at this site (Y688F) continued to block Akt activation by Ras (Figure 7C). Both p85-CT mutants were able to bind tyrosine-phosphorylated molecules (data not shown), suggesting that they fold properly. Consistent with these data, a Y688D mutant of p85 continued to bind p110 but failed to inhibit the basal activity of Akt in Cos7 cells (Cuevas et al., 2001). We, therefore, conclude that both phosphotyrosine binding and Y688 phosphorylation regulate p85 inhibitory function.

Since Rodriguez-Viciana et al. showed that full-length p85 blocks cell transformation by Ras (Rodriguez-Viciana et al., 1997), we tested whether the inhibitory domain alone also blocks transformation. To this end, we transfected p85-CT together with activated Ras into NIH3T3 cells. Scoring for foci of transformation two weeks later revealed that the p85 inhibitory domain is sufficient to block Ras-induced transformation (Figure 7D). This blockage is specific because both the p85-CT (R649L) and p85-cSH2 constructs failed to block. Blockage of transformation, therefore, correlates perfectly with the blockage of PI3K/Akt activation (Figures 4D and 7B).

Role of complimentary tyrosine kinase and small GTPase signals on PI3K activation in integrin or growth factor-stimulated cells

Earlier studies by us and others (Clark et al., 1998; Djouder et al., 2001; Franke et al., 1995; Genot et al., 2000; Klinghoffer et al., 1996; Rodriguez-Viciana et al., 1994) had shown that small GTPase signals are required for PI3K/Akt activation by growth factors or integrin stimulation. To determine whether tyrosine kinase signals are also required, we examined whether a kinase-inactive, dominant-negative mutant of Src inhibits PI3K/Akt activation induced by integrin stimulation. To address this question, we took advantage of observations showing that in serum-starved adherent cells, tyrosine kinase and small GTPase signals are induced by matrix adhesion and that the small GTPase signals are required for PI3K/Akt activation (Khwaja et al., 1997; King et al., 1997). We, therefore, transfected mouse 3Y1 fibroblasts with wild-type FLAG-Akt alone or in combination with kinase-inactive Src K279M. Following serum starvation, the cells were trypsinized and replated onto fibronectin-coated plates or control polylysine-coated plates for 30 min. The results showed that Akt activation in the fibronectin-coated plates is

inhibited by Src K279M (Figure 8A). These results support the hypothesis that Src signals are required, in combination with small GTPase signals, for PI3K/Akt activation by integrin stimulation.

To further explore the physiological role of complimentary signals transduced by tyrosine kinases and small GTPases, we employed suspension cultures of immortalized keratinocytes (HaCaT cells; Frisch and Francis, 1994). Cell culture under conditions of forced suspension precludes integrin engagement and signaling and can be performed in defined media free of exogenous growth factors. In the absence of integrin-generated signals, these cells fail to activate the PI3K/Akt pathway and undergo apoptosis (anoikis). Previous studies, using suspension cultures of MDCK cells, suggested that activated Ras alone activates the PI3K/Akt pathway and rescues the cells from anoikis (Khwaja et al., 1997). These findings suggested that, in this experimental setting, Ras alone was sufficient to activate PI3K/Akt and seemingly argued against an obligatory role of tyrosine kinases in this process. However, all these experiments were performed in serum-containing media which could generate tyrosine kinase signals that synergize with Ras. To avoid this problem, our experiments were carried out in serum-free defined media. The results (Figure 8B) showed that constitutively active H-Ras G12V activates the PI3K/Akt pathway and rescues cells from anoikis only in suspension cultures supplemented with epidermal growth factor (EGF). Ras G12V-alone in the absence of EGF and EGF-alone in the absence of H-Ras G12V were inefficient in PI3K/Akt activation and failed to rescue the cells from anoikis. These results support the hypothesis that PI3K/Akt activation and inhibition of anoikis depend on the combination of tyrosine kinase and small GTPase signals.

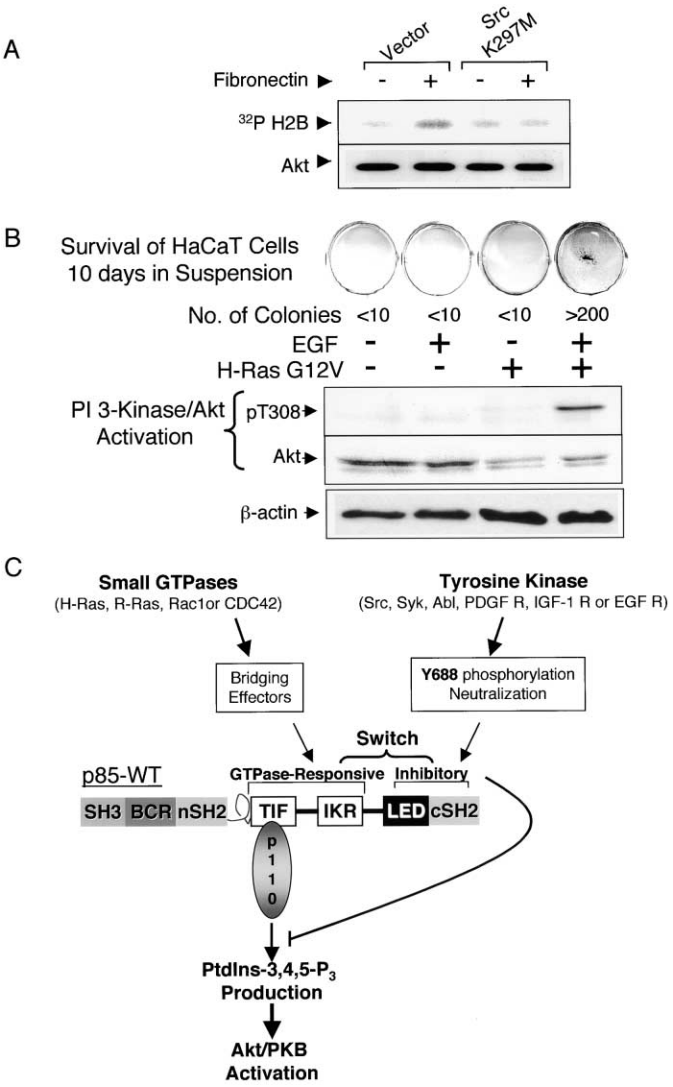

Figure 8. Complimentary roles of tyrosine kinase and small GTPase signals induced by integrin or growth factor stimulation, in PI3K/Akt activation and cell survival

A: Inhibition of fibronectin-stimulated Akt activity by kinase-inactive Src. Serum-starved Mouse 3Y1 fibroblasts, cotransfected with FLAG-Akt and kinase-inactive Src-K297M or vector, were trypsinized and replated in fibronectin or polylysine-coated plates as indicated. Akt kinase activity in cell lysates harvested 30 min later was measured by in vitro phosphorylation of H2B. **B:** Activated Ras and EGF cooperate to support long-term cell survival (10 days) and PI3K/Akt activation in HaCaT keratinocytes in suspension cultures. HaCaT cells or HaCaT cells stably expressing activated H-Ras G12V were cultured on 0.9% agarose in MCDB base medium. EGF was used at 2 nM concentration in the indicated cultures. Cell aliquots were removed after 6 hr in suspension for immunoblot analysis. Cell viability was examined after 10 days in suspension as described (Jost et al., 2001). **C:** A model of PI3K activation by tyrosine kinase signals and small GTPase signals. According to this model, p85 binds, via its cSH2 motif, tyrosine-phosphorylated proteins and undergoes phosphorylation at Y688. Phosphorylation at this site functionally inactivates the p85 inhibitory domain. Neutralization of the inhibitory domain renders p85 responsive to PI3K activation signals that are transduced by small GTPases.

Discussion

Based on the preceding data, we suggest a regulatory mechanism to explain how cytoplasmic tyrosine kinases and small GTPases regulate PtdIns-3,4,5-P_3 production by type IA PI3K. This model also explains how the carboxy-terminally truncated p85 functions as an oncogene. According to this model (Figure 8C), type IA PI3K activity is under the dual control of a p85 inhibitory domain, which is regulated by binding to tyrosine-phosphorylated molecules and by tyrosine phosphorylation, and a GTPase-responsive domain (GRD). Because of the inhibitory domain, PI3K either does not respond or responds poorly to signals from small GTPases such as Ras or Rac1. Removal of the inhibitory domain, as in the case of the p85 oncogene, or neutralization of its inhibitory function by tyrosine phosphorylation allows small GTPases to activate PI3K through the GRD. The inhibitory domain and the GRD together form a molecular switch that regulates the enzyme.

These data raise the question of the molecular mechanism through which tyrosine kinases and small GTPases regulate the switch. The inhibitory domain, which consists of the cSH2 and the adjacent LED motifs, was inactivated by a mutation (R649L) in the FLVR region of cSH2. Since this mutation eliminates binding to phosphotyrosine, we conclude that the inhibitory function of this domain depends on its binding to tyrosine-phosphorylated molecules. One potential explanation for this result is that the overexpressed p85 inhibitory domain competes with endogenous p85 for binding to tyrosine-phosphorylated molecules. However, this hypothesis cannot fully explain our data because the cSH2 domain alone, which should also bind these sites, has no inhibitory activity. Therefore, the LED motif is an indispensable part of the inhibitory domain.

The role of the LED motif is not clear to date. One potential

clue regarding its function is that the inhibitory domain is modular and functions in *trans*, despite the fact that it does not bind p110. This suggests that the LED motif may regulate the conformation of p85, perhaps through electrostatic and/or hydrophobic interactions with the IKR motif. Changes in the conformation of p85 may affect the activity of p110. One possible mechanism by which conformational changes of p85 regulate the activity of the catalytic subunit is that such changes may directly affect the conformation of the p85 bound p110. Alternatively, conformational changes of p85 may affect the ability of p110 to dimerize. This hypothesis is supported by earlier findings showing that PI3K dimerizes in vitro and that dimerization is promoted by binding to tyrosine-phosphorylated peptides (Layton et al., 1998). The interaction between the IKR and LED motifs was suggested by the abundance of conserved hydrophobic and positively charged amino acids in the IKR motif and hydrophobic and negatively charged amino acids in the LED motif. Supporting the hypothesis that such interactions indeed take place and that they are functionally important are recent findings showing that mutations designed to disrupt the interaction between the IKR and LED motifs abolish the function of the p85 inhibitory domain (data not shown). The inhibitory function of the LED motif could be affected by conformational changes of p85 induced by tyrosine phosphorylation. Cuevas et al. (2001), indeed, proposed that phosphorylation of Y688 promotes intramolecular interactions between nSH2 and cSH2. Such interactions may induce conformational changes that affect the inhibitory activity of the LED motif.

The preceding data suggest a model according to which PI3K is inactive in quiescent cells, in part because the p85 inhibitory domains binds tyrosine-phosphorylated molecules with inhibitory properties. Following signals that activate tyrosine kinases, p85 undergoes phosphorylation in a number of sites, including tyrosine 668. Since a Y668F mutation did not affect the function of the inhibitory domain while a phosphomimetic Y668D mutation completely abolished its activity, we conclude that phosphorylation of this site is sufficient to neutralize this domain. Neutralization of p85 by phosphorylation may be mediated by one of several tyrosine kinases, including Src, Lck, Abl, EGF-R, PDGF-R, and IGF-R, which are known to phosphorylate this molecule (von Willebrand et al., 1998; Wymann and Pirola, 1998) and to cooperate with small GTPases in PI3K activation (see Supplemental Figure S5 at http://www.cancercell.org/cgi/content/full/1/2/181/DC1). This raises the question of the mechanism through which phosphorylation of tyrosine 668 neutralizes the inhibitory domain. One possibility is that phosphorylation alters the conformation of the protein and makes the GRD domain accessible to small GTPase signals. This conformational change may be the direct outcome of phosphorylation. Alternatively, phosphorylation of tyrosine 668 may selectively alter the spectrum of proteins that bind the cSH2 motif. Previous studies by von Willebrand et al. (1998) indeed showed that phosphorylation at this site in pervanadate-treated Jurkat cells selectively alters the spectrum of p85 binding proteins. Please note that selective shifts in the spectrum of proteins binding the PI3K complex could potentially also occur because of oligomerization induced by binding of p85 to tyrosine-phosphorylated proteins (Layton et al., 1998). The conformational change that neutralizes the inhibitory domains may therefore be caused by the dissociation of inhibitory molecules from the PI3K complex and/or by the association of the complex with activating molecules.

The mechanism of PI3K activation via the synergistic action of two different classes of signaling molecules defines a signaling paradigm for PtdIns-3,4,5-P_3 regulation. According to this paradigm, a given molecule may be the target of two simultaneously activated, converging pathways, one of which molecularly modifies the target so that it can be recognized by the other. Synergism between a variety of tyrosine kinases and small GTPases suggests that external signals may use different combinations of these two classes of molecules to activate PI3K. In agreement with this, a kinase-inactive mutant of Src (Figure 8A) and a dominant-negative mutant of CDC42 (Clark et al., 1998) inhibit PI3K activation by integrin stimulation, and a dominant-negative mutant of H-Ras partially inhibits PI3K activation by PDGF and NGF (Franke et al., 1995; Klinghoffer et al., 1996; Rodriguez-Viciana et al., 1994). Moreover, in hematopoietic cells, PtdIns-3,4,5-P_3 induction by the IgE receptor, FcεRI, or by the T cell receptor requires Rac1 signals (Djouder et al., 2001; Genot et al., 2000). Data presented in this report showed that the p85 inhibitory domain blocks Ras-induced transformation of NIH3T3 cells. In addition, they showed that both constitutively active Ras and EGF are required for Akt activation and protection from apoptosis. These results strongly support the physiological relevance of the model of PI3K activation described in this report.

In summary, data in this report show that p85 contains a molecular switch that regulates PI3K in response to signals transduced by tyrosine kinases and small GTPases. Given the significance of the PI3K pathway in oncogenesis, this molecular switch defines an important target for future chemotherapeutic drugs.

Experimental procedures

Cell culture, transfection, and plasmids

NIH3T3 fibroblasts were cultured in Dulbecco's Modified Eagle's Essential Medium (DMEM) supplemented with 10% calf serum and antibiotics. Immortalized, nontumorigenic human keratinocytes (HaCaT cells) (Boukamp et al., 1988) and their tumorigenic variant expressing the activated H-Ras G12V (Boukamp et al., 1990) were obtained from N. Fusenig. Cells were transfected using LipofectAMINE (GIBCO-BRL) or Fugene-6 (Roche) according to the manufacturer's protocols. All constructs, except where noted, were generated in the mammalian expression vector, CMV5. The Src myristoylation signal (MGSSKSKPK), an extended Hemagglutinin epitope (HA) (MASSYPYDVPDYASLGGPSRST), and a FLAG-epitope tag (MDYKDDDDK) were fused at the amino terminus of the indicated constructs. Green fluorescence protein (GFP) in pFred143(KH1035) was a gift from G. Pavlakis, p110-α-CAAX in pMT2 was a gift from K. Kotani, and p110-γ-CAAX in pCDNA3 was a gift from M. Wymann. Additional details are available in the Supplemental Data at http://www.cancercell.org/cgi/content/full/1/2/181/DC1.

In vivo labeling of PtdIns-3,4,5-P_3

PtdIns-3,4,5-P_3 was measured in lipid extracts of ^{32}P-labeled NIH3T3 cells by thin-layer chromatography (Maehama and Dixon, 1998). Approximately 2×10^5 NIH3T3 cells plated into 35 mm Petri dishes were transfected with constitutively active H-Ras G12V, p85-WT, and p85Δonc constructs in CMV5 using Fugene 6 (Roche). Sixteen hours later, the transfected cells were serum-starved for 5 hr and following this, they were labeled for 1.5 hr with 100 uci/ml ^{32}P-orthophosphate in a phosphate-free buffer (10 mM Hepes [pH 7.5], 136 mM NaCl, 4.9 mM KCl, and 5.5 mM glucose). For wortmannin treatment, 200 nM wortmannin was added to cells 45 min prior to cell extraction. Further details are provided in the Supplemental Data at http://www.cancercell.org/cgi/content/full/1/2/181/DC1.

Akt kinase assay

Akt/PKB kinase activation was determined either by measuring the phosphorylation of Akt at Thr308 using an antibody from Cell Signaling Technol-

ogy or by measuring the kinase activity of immunoprecipitated FLAG-tagged wild-type Akt (Bellacosa et al., 1998; Franke et al., 1995). Detailed protocols for the Akt kinase assay are available in the Supplemental Data at http://www.cancercell.org/cgi/content/full/1/2/181/DC1. Specific Akt kinase activity was determined by Phosphorimager quantitation of the ^{32}P-phosphorylated Akt substrate Histone 2B and by quantification of Akt in anti-Akt immunoprecipitates using a laser densitometry scanner (Molecular Dynamics).

Immunoblotting

Total cell lysates and protein immunoprecipitates were subjected to SDS-PAGE. The SDS-PAGE-resolved proteins were transferred onto PVDF membranes (Immobilon P, Millipore) and the resulting immunoblots were probed with the appropriate antibodies. Binding of antibodies to membrane-immobilized proteins was visualized by enhanced chemiluminescence (ECL, Amersham) following the manufacturer's protocol.

The origin of the antibodies we used is described in the Supplemental Data at http://www.cancercell.org/cgi/content/full/1/2/181/DC1.

Transformation assay and suspension survival assay

Transformation assays in NIH3T3 cells were performed as previously described (Chan et al., 1994; Wigler et al., 1977). Suspension survival assays were performed as previously described (Jost et al., 2001). Brief description of these assays is provided in the Supplemental Data at http://www.cancercell.org/cgi/content/full/1/2/181/DC1.

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