Phosphatidylinositol Phosphate 5-Kinase Iγ and Phosphoinositide 3-Kinase/Akt Signaling Couple to Promote Oncogenic Growth*

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Background: PIP$_2$ generated by PIPKI family members regulates many cellular functions, and PIP$_2$ is a PI3K substrate. Results: Loss of PIPKI$_{γ}$ or PIPKI$_{γ2}$ impaired Akt activation. PIPKI$_{γ2}$ and Src activate Akt and anchorage-independent growth. Conclusion: PI3K/Akt signaling is regulated by coupling with PIPKI$_{γ}$. Significance: The coupling of PIPKI$_{γ}$ and PI3K indicates a mechanism for Akt activation that enhances oncogenic growth.

The assembly of signaling complexes at the plasma membrane is required for the initiation and propagation of cellular signaling upon cell activation. The class I PI3K and the serine/threonine-specific protein kinase Akt signaling pathways (PI3K/Akt) are often activated in tumors. These pathways are initiated by the generation of phosphatidylinositol 3,4,5-triphosphate (PIP$_3$) by PI3K-mediated phosphorylation of phosphatidylinositol 4,5-biphosphate (PIP$_2$), synthesized by phosphatidylinositol 4-phosphate 5-kinase (PIPKI) enzymes. The mechanism of how tumor cells recruit and organize the PIP$_2$-synthesizing enzymes with PI3K in the plasma membrane for activation of PI3K/Akt signaling is not defined. Here, we demonstrated a role for the phosphatidylinositol 4-phosphate 5-kinase 1γ (PIPKI$_{γ}$) in PI3K/Akt signaling. PIPKI$_{γ}$ is overexpressed in triple-negative breast cancers. Loss of PIPKI$_{γ}$ or its focal adhesion-targeting variant, PIPKI$_{γ2}$, impaired PI3K/Akt activation upon stimulation with growth factors or extracellular matrix proteins in different tumor cells. PIPKI$_{γ2}$ assembles into a complex containing Src and PI3K; Src was required for the recruitment of PI3K enzyme into the complex. PIPKI$_{γ2}$ interaction with Src and its lipid kinase activity were required for promoting PI3K/Akt signaling. These results define a mechanism by which PIPKI$_{γ2}$ and PI3K are integrated into a complex regulated by Src, resulting in the spatial generation of PIP$_2$, which is the substrate PI3K required for PI3P$_3$ generation and subsequent Akt activation. This study elucidates the mechanism by which PIP$_2$-generating enzyme controls Akt activation upstream of a PI3K enzyme. This pathway may represent a signaling nexus required for the survival and growth of metastasizing and circulating tumor cells in vivo.

Tumor cells receive survival and proliferative signals from diverse stimuli, such as growth factors, cytokines, and extracellular matrix (ECM)$^2$ proteins (1). Among signaling pathways initiated by these stimuli, PI3K/Akt signaling is one of the most common pathways implicated in tumor cell growth and survival (2–4). The PI3K enzymes activated by these diverse stimuli convert PIP$_2$ into PIP$_3$, promoting the recruitment and activation of cytosolic proteins, PDK1 and Akt, to the plasma membrane and initiating PI3K/Akt signaling cascades (2–4). Cancer cells exploit various mechanisms to activate and sustain PI3K/Akt signaling (2–4). Most of them are associated with PTEN loss or the activation of the PI3K enzyme due to mutations in its catalytic subunit (2–4). However, the functional role of lipid kinases that synthesize PIP$_2$, precursor for PIP$_3$, remains poorly explored in the regulation of PI3K/Akt signaling. The localized synthesis of PIP$_2$ and PIP$_3$ suggest that lipid kinases generating PIP$_2$ and PIP$_3$ work together in a coordinated and regulated manner for Akt activation. In the context of highly divergent mechanisms for Akt activation in oncogenesis, the precise understanding of how PIP$_2$- and PIP$_3$-generating lipid kinases work together to regulate PI3K/Akt signaling paves the way for the development of a novel therapeutic approach in controlling the PI3K/Akt signaling axis in cancer.

In mammalian cells, PIP$_2$ is predominantly synthesized by type I PIPK enzymes (classified into PIPKI$_{α}$, PIPKI$_{β}$, and PIPKI$_{γ}$), which are targeted to distinct subcellular compartments via interactions with specific binding partners, which are often PIP$_2$ effector molecules (5–9). These interactions facilitate PIP$_2$ control of diverse cellular functions. PIPKI$_{α}$ is predominantly localized to the nucleus and controls the nuclear events, whereas PIPKI$_{β}$ is targeted to perinuclear regions/endosomes (9, 10). PIPKI$_{γ}$ is expressed as at least six splice variants (PIPKI$_{γ1}$, PIPKI$_{γ2}$, PIPKI$_{γ3}$, PIPKI$_{γ4}$, PIPKI$_{γ5}$, and PIPKI$_{γ6}$) in mammalian cells (11, 12); among them, PIPKI$_{γ2}$

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2 The abbreviations used are: ECM, extracellular matrix protein; PLC, phospholipase C; SH2, Src homology domain 2; SH3, Src homology domain 3; PIP$_2$, phosphatidylinositol 4,5-phosphate; PIP$_3$, phosphatidylinositol 3,4,5-triphosphate; PIPKI, phosphatidylinositol 4-phosphate 5-kinase; PTEN, phosphatase and tensin homolog.
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can be targeted to the cell-matrix interface via an interaction with talin and plays role in focal adhesion assembly (13–15), whereas PIPKIγ4 and PIPKIγ5 are localized to the nucleus and endosomes, respectively (11, 16). Src phosphorylation of PIPKIγ2 regulates its interaction with talin and its targeting to focal adhesion sites (17). Furthermore, Src and PIPKIγ2 are directly interacting partners, both required for oncogenic growth of tumor cells (18). Recently, PIPKIγ has been shown to modulate breast cancer metastasis, although the specific isoforms involved were not defined (19).

The class I PI3K enzymes are heterodimers of two distinct subunits, an adaptor (e.g. p85, p50, and p55) and a catalytic (e.g. p110α, p110β, and p110δ) (2–4). PI3K is rapidly recruited to activated growth factor receptors (SH2 domain of the adaptor subunit mediating the interaction to the phosphorylated YXXM motif of the receptor) or integrin-mediated adhesion complex (via interaction with focal adhesion kinase in the adhesion complex), promoting its activation and PIP₃ synthesis (2, 20). For PIPKI enzymes, the mechanism by which they are recruited to the proximity of the activated growth factor receptors or integrin-mediated adhesion complex to synthesize the spatial pool of PIP₃ is not understood. This may be a crucial mechanism for providing a discrete pool of PIP₃ required for PI3K synthesis and Akt activation. Therefore, we embarked on a systematic investigation to define the role of PIPKI enzymes in PI3K/Akt activation in response to stimulation with growth factors and ECM proteins.

Here we show that in a wide variety of cell lines, PIPKIγ was the major PIPKI enzyme contributing to PI3K/Akt signaling in response to activation of growth factor and adhesion receptors in suspension condition. The loss of PIPKIγ2, a focal adhesion-targeted variant of PIPKIγ, recapitulated the effect of PIPKIγ knockdown in PI3K/Akt activation. PIPKIγ2 integrated into a complex with Src and PI3K. Src mediated the incorporation of both PIPKIγ1 and PI3K into the complex via Src association with the p85 subunit of PI3K. The co-expression of PIPKIγ2 with Src resulted in sustained activation of PI3K/Akt. Furthermore, PIPKIγ2 and Src interaction, and its lipid kinase activity were required for PI3K/Akt activation. These results define a mechanism by which PIPKIγ2 functions with the proto-oncogene Src to activate and sustain the PI3K/Akt signaling required for the anchorage-independent or oncogenic growth of tumor cells.

**Experimental Procedures**

**Materials**—Antibodies used were as follows: pAkt (193H12), Akt (11E7), pErk1/2 (9101) and Erk1/2 (9102), Src (2108S), p110α (4255s), p110β (3011s), and p85 (4292) were purchased from Cell Signaling; antibody for HA (MMS-101R) was purchased from Covance. Antibodies for PIPKIα, PIPKIγ, and PIPKIγ2 were developed in the laboratory (13, 17, 21).

DNA Constructs and siRNA—DNA constructs used in the study were described previously (18, 22). siRNA oligonucleotides used were as follows: control siRNA, CCUGCAGACUGACGUAGUU; siPIPKIγ2, GAGCGACACAUAAAUUCUA; siPIPKIγ5, GGAUGGAGGUACUGAGAUU; siPIPKIγ, GCCACCTTCATTTCGAAGAA; siPIPKIα, GAAGUUGGACACUUCUUGG; siSrc, GCCUCCAGAUUGUCAACAA and GCCUCAACGUGAAAGCACUA.

**Cell Culture**—MDA-MB-231, Cal51, MCF-7, NIH3T3, HEK293, and HEK293FT cells were cultured in DMEM containing 10% FBS. T47D cells were cultured in RPMI-1640 containing 10% FBS. SUM159 and SUM153 cells were cultured in Ham’s F-12 supplemented with 5% FCS. All cells were grown at 37 °C in a 5% CO₂ incubator.

**Transfection or Lentiviral Infection**—For siRNA-mediated knockdown of gene expression, LipofectamineRNAiMAX (Invitrogen) was used following the protocol provided by manufacturer, and cells were used 48–72 h post-transfection. For transient transfection into HEK293 cells, Lipofectamine 2000 (Invitrogen) was used. Cells were harvested 24 h post-transfection. For the expression or co-expression of genes into MDA-MB-231 or NIH3T3, a lentiviral system was used as described previously (22). Cells were harvested 48 h postinfection (70–80% of infection efficiency was achieved for the experiments).

**Stimulation in Suspension Condition**—For the stimulation of cells in the suspension condition, cells were serum-starved overnight, followed by trypanosinization and resuspension of cells in serum-free DMEM containing 0.2% BSA. Cells were incubated for 1–2 h in a CO₂ incubator before stimulating with FBS (2.5–5% FBS) or EGF (1–10 ng/ml) or ECM protein (combination of fibronectin/collagen type I, 25 μg/ml each) for the indicated time periods. “Suspension condition” refers to the cells resuspended in their corresponding medium containing 0.2% BSA and 0.5% FBS after trypsinization/detachment. Cells were incubated at 37 °C in an incubator for 2–3 h except for in the time course study. For overnight culture in suspension condition, cells suspended in the medium were seeded into the culture plate coated with 0.3% agar to avoid cell attachment and incubated in a CO₂ incubator.

**Immunoprecipitation and Immunoblotting**—Cells were lysed using lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 10 mM NaF, 5 mM Na₃VO₄, and protease inhibitors). Clear supernatants were incubated with the indicated antibodies for 3–4 h to overnight at 4 °C, followed by isolation of immunocomplexes using protein G-Sepharose 4B beads (Amersham Biosciences). Beads were washed three times with lysis buffer before eluting the immunocomplexes with 2× sample buffer and then subjected to immunoblotting using specific antibodies.

**Anchorage-independent Growth**—For anchorage-independent growth, cells were suspended into the medium containing 0.3% agar and seeded into 24-well culture plates (18). To avoid cell attachment, culture plates were precoated with 0.5% agar before cell seeding. Cultures were fed with fresh medium in every 3–5 days and cultured for 1–3 weeks. For inhibition of Akt signaling, PI3K inhibitor (LY294002, 10 μM) was added into the medium. Colonies developed were fixed with 3.7% paraformaldehyde and stained with 0.1% crystal violet to facilitate the visualization and counting of colonies.

**Immunofluorescence Microscopy**—For the immunofluorescence study, colonies developed in the soft agar were fixed with 3.7% paraformaldehyde and stained with 0.1% crystal violet to facilitate the visualization and counting of colonies.

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**Immunofluorescence Microscopy**—For the immunofluorescence study, colonies developed in the soft agar were fixed with 3.7% paraformaldehyde, followed by cell permeabilization with 0.1% Triton-X and blocking with 3% BSA in PBS. Cells were incubated with primary antibody overnight at
4 °C, followed by incubation with Alexa555- and/or Alexa488-conjugated secondary antibodies (Molecular Probes) for 1 h at room temperature. Slides were mounted using Vectashield and visualized with a Nikon TE2000-U microscope. Images were acquired using MetaMorph and processed using Adobe Photoshop.

**Protein-Lipid Overlay Assay**—The effect of PIPKIγ knockdown or PIPKIγ2 overexpression on cellular level of PIP2 and PIP3 were examined by a protein-lipid overlay assay (23). Briefly, acidic lipids containing PIP2 and PIP3 were isolated from unstimulated or stimulated cells following the protocol provided by Echelon Biosciences. Equal numbers of cells were used for the lipid extraction (5 × 10^5 cells for PIP2 and 10 × 10^6 for PIP3). Isolated lipids were dissolved into MeOH/CHCl3/HCl and spotted onto nitrocellulose membrane, followed by blocking with 3% BSA in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T). Membranes were incubated with GST-PLCγ-PH (0.5 μg/ml; Echelon Biosciences) or GST-GRP1-PH (1 μg/ml; Echelon Biosciences) overnight at 4 °C. The bound proteins with the lipids in the membrane were detected using HRP-labeled anti-GST antibody (Sigma). The signals generated were measured by the ImageJ program (National Institutes of Health).

**Statistical Analysis**—The data are presented mean ± S.D. from at least three independent experiments. An unpaired t-test was conducted to determine the p value and the statistical significance between two groups (p < 0.05 was considered significant).

**Results**

**PIPKIγ Is Required for PI3K/Akt Activation**—The role of individual PIPKI enzymes was investigated in Akt activation in response to FBS or ECM protein stimulation. Cells were stimulated in suspension because this facilitated the segregation of PI3K/Akt signaling initiated in response to growth factors versus ECM proteins. These conditions are also relevant to metastasizing tumor cells in the vasculature or lymphatic circulation as well as circulating tumor cells found in cancer patients (24, 25).

Specific siRNA was used to knock down individual PIPKI isoforms from MDA-MB-231 or other cell lines. The knockdown of PIPKIγ impaired Akt activation in response to both FBS or ECM protein stimulation. Cells were transfected with isoform-specific siRNA for PIPKI knockdown. 48 h post-transfection, cells were serum-starved overnight and resuspended into the serum-free medium. Cells were stimulated with FBS (A) or ECM protein (B) for 10 min in the suspension condition. Activated Akt was examined by immunoblotting using phospho-specific antibody for activated Akt. C, PIP2 level in the cells was examined by a protein-lipid overlay assay. Acidic lipids were isolated from an equal number of cells as described under "Experimental Procedures." The isolated lipids were spotted into the nitrocellulose membrane before incubating with purified GST-PLCγ-PH protein. HRP-labeled anti-GST antibody was used to detect the bound GST-PLCγ-PH. A.U., arbitrary units. D, siRNA was used to knock down PIPKIγ expression in MDA-MB-231 cells. As described above, cells were stimulated with FBS or ECM for different time periods before examining the activated Akt and Erk1/2 by immunoblotting. The same experiments were repeated using SUM159 cells (E). Results are represented as means ± S.D. (error bars) from three independent experiments.

**FIGURE 1. PIPKIγ knockdown blocked Akt activation upon FBS or ECM stimulation.** A and B, MDA-MB-231 cells were transfected with isoform-specific siRNA for PIPKI knockdown. 48 h post-transfection, cells were serum-starved overnight and resuspended into the serum-free medium. Cells were stimulated with FBS (A) or ECM protein (B) for 10 min in the suspension condition. Activated Akt was examined by immunoblotting using phospho-specific antibody for activated Akt. C, PIP2 level in the cells was examined by a protein-lipid overlay assay. Acidic lipids were isolated from an equal number of cells as described under "Experimental Procedures." The isolated lipids were spotted into the nitrocellulose membrane before incubating with purified GST-PLCγ-PH protein. HRP-labeled anti-GST antibody was used to detect the bound GST-PLCγ-PH. A.U., arbitrary units. D, siRNA was used to knock down PIPKIγ expression in MDA-MB-231 cells. As described above, cells were stimulated with FBS or ECM for different time periods before examining the activated Akt and Erk1/2 by immunoblotting. The same experiments were repeated using SUM159 cells (E). Results are represented as means ± S.D. (error bars) from three independent experiments.
FBS and ECM protein stimulation of the cells (Fig. 1, A and B). This is consistent with the PIPKIγ function in organizing the signaling complex at the plasma membrane and in PI3K synthesis, the PI3K substrate for PI3 generation and Akt activation. The knockdown of PIPKIα in comparison showed little impact on Akt activation, demonstrating specificity for PIPKIγ. The impaired activation level of PI3K/Akt was correlated with a significantly decreased PIP2 level in PIPKIγ knockdown cells upon stimulation with FBS and ECM protein in suspension condition (Fig. 1C). Because PIPKIγ is overexpressed in triple-negative breast cancer tissues and plays a role in anchorage-independent growth of tumor cells (18, 26), its role in PI3K/Akt signaling was further examined. The effect of PIPKIγ knockdown on the time course of Akt activation after FBS or ECM stimulation of MDA-MB-231 cells was examined (Fig. 1D).

**PIPKIγ Knockdown Impaired Persistent PI3K/Akt Signaling**—Persistent Akt activation is a key mechanism in oncogenic growth regulation (2, 4). The role of different PIPKI enzymes in protracted PI3K/Akt signaling was examined in different tumor cell lines, including T47D, Cal51, and MCF-7, that show constitutive and higher activation levels of PI3K/Akt signaling due to mutation in the p110α catalytic subunit of the PI3K enzyme (27, 28). As shown in Fig. 2, A–C, the activation level of Akt maintained in T47D, Cal51, and MCF-7 cells growing in normal growth medium was diminished upon PIPKIγ knockdown. Similarly, the knockdown of PIPKIγ in T47D cells significantly abrogated the strength and duration of Akt activation upon withdrawal of serum-containing medium (Fig. 2D).
the adherent condition, these cells maintained a higher level of activated Akt in the suspension condition, which plays a critical role in the oncogenic growth regulation of these tumor cells. As shown in Fig. 2, E and F, endogenous PIPKIγ2 was silenced using siRNA from MDA-MB-231 cells or MDA-MB-231 cells overexpressing siRNA-resistant PIPKIγ2 (WT) or its kinase dead mutant, PIPKIγ2 (KD). Cells were stimulated with FBS and ECM protein as described above before examining the activated Akt. G, siRNA was used to knockdown PIPKIγ2 in SUM159 cells before examining its effect on the activation level of Akt and Erk1/2 upon stimulation with FBS as described above. H and I, MDA-MB-231 cells transfected with siRNA specific for PIPKIγ5 were stimulated with FBS and ECM protein as described above, and the activation level of Akt was examined as described above.

The Loss of PIPKIγ2 Mimics the Loss of PIPKIγ on PI3K/Akt Activation—Previously, we have shown that PIPKIγ2 and Src control anchorage-independent growth of tumor cells (18). The role of PIPKIγ2, a focal adhesion-targeting variant of PIPKIγ (for a schematic diagram of PIPKIγ variants, see Fig. 3A), was investigated with the hypothesis that it plays a major role in the spatial synthesis of PIP2 required for PIP3 generation in response to growth factor receptor and adhesion receptor activation, consistent with the compartmentalization of cellular signaling (6, 29). As shown in Fig. 3, B and C, the knockdown of PIPKIγ2 recapitulated the effect of PIPKIγ knockdown on PI3K/Akt activation. However, unlike the knockdown of pan-PIPKIγ, the knockdown of the PIPKIγ2 variant did not show any obvious decrease in the PIP2 level (Fig. 3D), indicating that the effect of PIPKIγ2 may result from localized changes in PIP2 and PIP3 levels in the vicinity of activated growth factor and adhesion receptors in the plasma membrane. Furthermore, kinase activity of PIPKIγ2 was required for rescuing the defect on Akt activation in PIPKIγ2 knockdown cells (Fig. 3, E and F).

The effect of PIPKIγ2 knockdown was more specific toward PI3K/Akt activation because the phosphorylation level of Erk1/2 was minimally affected in SUM159 and SUM1315 cells (Fig. 3G) (data not shown). Furthermore, the knockdown of PIPKIγ5, an endosomal targeting variant of PIPKIγ, showed no effect on Akt activation when stimulated with ECM protein and FBS in the suspension condition (Fig. 3, H and I). These results are also consistent with the role of PIPKIγ2 in PIP2 synthesis required for the assembly of the adhesion complex at the plasma membrane (13, 30).

PIPKIγ2 Overexpression Induces PI3K/Akt Activation and Oncogenic Growth—PIPKIγ is overexpressed in triple-negative breast cancers, but the expression of different splices variants remains undefined (26). The impact of overexpression of PIPKIγ variants on Akt activation was examined using MDA-MB-231 cells. In the adherent condition, the loss or overexpression of PIPKIγ2 or other variants showed minimal effect on
Akt activation in response to FBS stimulation (Fig. 4A). However, in the suspension condition, PIPKIγ2-overexpressing cells showed a modest increase in Akt activation in response to FBS and ECM protein stimulation of the cells (Fig. 4B) (data not shown). Analysis of phosphoinositide contents in the cells indicated a significantly increased PIP₃ level in PIPKIγ2-overexpressing cells, which was further increased upon cell activation (Fig. 4C). Consistent with the increased level of activated Akt, we observed a significantly increased level of PIP₂ level in PIPKIγ2-overexpressing cells. This indicates that PIP₂ generated by PIPKIγ2 is associated with PIP₃ synthesis, leading to PI3K/Akt activation in the plasma membrane. Furthermore, PIPKIγ2-overexpressing cells maintained the higher level of activated Akt following the disruption of the cell-matrix interaction and incubation in the suspension condition (Fig. 4D), which also corroborates with its anchorage-independent growth-promoting effect (18).

After demonstrating the role of PIPKIγ2 in PI3K/Akt signal transduction, the role of PI3K/Akt signaling in oncogenic growth regulation by PIPKIγ2 was investigated. The PI3K inhibitor, LY294002, was used to block PI3K/Akt signaling in MDA-MB-231 cells overexpressing PIPKIγ2. As expected, the LY294002 compound significantly impaired the anchorage-independent growth induced by the PIPKIγ2 expression (Fig. 4E). Anchorage-independent growth is one of the most commonly utilized in vitro methods to define cell transformation/oncogenic growth that directly correlate with in vivo tumor growth and metastasis (31, 32).

PIPKIγ2 and Src Cooperate to Regulate PI3K/Akt Signaling—PIPKIγ2 interacts with Src, and they collaboratively control anchorage-independent growth of tumor cells (18). Src is rapidly recruited to a wide spectrum of growth factor receptors and adhesion molecules and controls the oncogenic growth of tumor cells by regulating downstream signaling pathways, including PI3K/Akt signaling (33–35). Similarly, Src phosphorylation of PIPKIγ2 regulates its interaction with the cytoskeletal protein, talin, which mediates its recruitment to the integrin-mediated adhesion complex (17, 18). The direct association of PIPKIγ2 with talin and Src may facilitate its recruitment/assembly in the proximity of activated growth factor receptors and integrin-mediated adhesion complex in the plasma membrane to synthesize the spatial pool of PIP₂ for PIP₃ generation and Akt activation.

A number of studies demonstrate Src regulation of PI3K/Akt signaling via diverse mechanisms (36–42). Ectopic expression of Src promoted PI3K/Akt activation in MDA-MB-231 cells (Fig. 5A). Consistently, in the suspension condition, Src-expressing cells rapidly induced Akt activation in response to FBS, ECM protein, and EGF stimulation (Fig. 5, B–D). These cells also showed persistent Akt activation in a low FBS-containing medium (not shown). Furthermore, the inhibition of PI3K by LY294002 significantly impaired the anchorage-independent growth of Src-expressing cells (Fig. 5E), indicating the role of PI3K/Akt signaling in the oncogenic growth regulation of Src.

The role of Src and PIPKIγ2 in Akt activation in PIPKIγ2-overexpressing cells was investigated. Src interacts with PIPKIγ2,
and Src knockdown severely impaired the anchorage-independent growth of PIPKIγ2-overexpressing cells (18). As shown in Fig. 5, F and G, the knockdown of Src abrogated Akt activation in PIPKIγ2-overexpressing MDA-MB-231 cells. These results indicate that PIPKIγ/PIPKIγ2 functions together with Src in the regulation of PI3K/Akt signaling, which supports their role in the organization of signaling complexes and their co-targeting to plasma membrane/adhesion complexes along with the PI3K enzyme.

After demonstrating the role of Src in PI3K/Akt signaling, siRNA was used to knockdown PIPKIγ2 from Src-transfected cells, with the aim of determining whether Src recruits and/or collaborates with PIPKIγ2 in the regulation of PI3K/Akt signaling. As shown in Fig. 5, H and I, PIPKIγ2 knockdown significantly impaired Akt activation in Src-expressing cells stimulated with ECM protein and growth factor (e.g. EGF). These results are also consistent with previous findings that PIPKIγ2 regulated Src activation downstream of the growth factor receptor and integrins (18) and indicate the cooperative role of PIPKIγ2 and Src in the regulation of both signaling and function.

PIPKIγ2 Forms a Signaling Complex with Src and PI3K—To define a mechanism for PIPKIγ2 regulation of PI3K/Akt signaling, we investigated whether the PIPKIγ2 and PI3K enzymes are integrated into a complex upon cell stimulation. PIPKI enzymes often assemble into complexes, where the PIP₂ generated modulates an effector molecule (5, 6, 9). In this case, the proximity of PIPKIγ2 and PI3K may facilitate the generation of the spatial pool of PIP₃ that is used by PI3K for generation of the PIP₃ that then activates Akt. PI3K utilizes SH2
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FIGURE 6. Src promotes the integration of PIPKIγ2 and PI3K into a complex. A, PIPKIγ2 or Src was immunoprecipitated from MDA-MB-231 cells coexpressing PIPKIγ2 and Src. The coimmunoprecipitation of PI3K enzyme into the immunocomplex was examined by immunoblotting using the antibody specific for PI3K subunits (e.g. p85 and p110α). B, MDA-MB-231 cells expressing PIPKIγ2 were incubated in the suspension condition or stimulated with EGF and ECM for the indicated time periods. PIPKIγ2 was immunoprecipitated, and coimmunoprecipitation of Src and PI3K into the immunocomplex was examined by immunoblotting. C, MDA-MB-231 cells expressing PIPKIγ1 or PIPKIγ2 were stimulated with EGF and ECM protein in the suspension condition as described above. Then PIPKIγ1 or PIPKIγ2 was immunoprecipitated to examine the coimmunoprecipitation of Src and PI3K into the immunocomplex. D, PIPKIγ2 was immunoprecipitated from MDA-MB-231 cells transfected with siRNA for the knockdown of Src. Cells were stimulated as described above, and the coimmunoprecipitation of PI3K with PIPKIγ2 in the immunocomplex was examined by immunoblotting. E, endogenous Src was immunoprecipitated from HEK293 cells transfected with siRNA for PIPKIγ or PIPKIγ2 knockdown. The coimmunoprecipitation of PI3K with Src was examined by immunoblotting. F, HEK293 cells were co-transfected with Src and p85. Src was immunoprecipitated, and coimmunoprecipitation of p85 was examined by immunoblotting. Reciprocally, coimmunoprecipitation of Src with p85 was examined.

domains of the p85 adaptor subunit for its recruitment to tyrosine-phosphorylated motifs of activated receptors (2). Similarly, PIPKIγ2 may utilize its direct interacting partners, Src and talin, in its recruitment to the proximity of activated growth factor receptors or adhesion complexes.

The incorporation of PIPKIγ2, Src, and PI3K enzymes into a complex was examined in MDA-MB-231 cells ectopically expressing PIPKIγ2 and Src (Fig. 6A). The assembly of these complexes was induced by activated growth factors and adhesion receptors, as demonstrated by the increased association of PIPKIγ2 with Src and PI3K in the cells stimulated with EGF and ECM protein (fibronectin/collagen type I) (Fig. 6B). The interaction of PIPKIγ2 with PI3K was specific because PIPKIγ1, which is deficient in focal adhesion targeting and talin binding, did not integrate into the complex (Fig. 6C). Most significantly, Src knockdown severely impaired PIPKIγ2 association with PI3K (Fig. 6D). The loss of PIPKIγ1 or PIPKIγ2 did not affect the Src association with PI3K (Fig. 6E). The co-expression and coimmunoprecipitation study demonstrated Src association with PI3K (Fig. 6F) and is consistent with reported studies (38, 42). All of these results indicate that Src functions as a bridging molecule in incorporating both PIPKIγ2 and PI3K into a complex. This provides the mechanism for the spatial generation of PIP2 and PIP3 for Akt activation upon growth factor and adhesion receptor activation.

Co-expression of PIPKIγ2 and Src Induces PI3K/Akt Activation and Anchorage-independent Growth—Previously, the synergistic role for PIPKIγ2 and Src in anchorage-independent growth regulation was demonstrated (18). Corroborating these results, co-expression of PIPKIγ2 and Src induced a dramatic and synergistic increase in Akt activation in suspension culture (Fig. 7A). This was further validated by demonstrating the significantly increased PIP3 level in the cells co-expressing PIPKIγ2 and Src (Fig. 7A, bottom). Furthermore, the colonies developed by PIPKIγ2- and Src-expressing cells in the soft agar also showed increased immunostaining for phosphorylated Akt (Fig. 7B). The PI3K/Akt signaling was required for increased anchorage-independent growth in PIPKIγ2- and Src-expressing cells because the PI3K inhibitor LY2940022 significantly abrogated colonies developed by these cells in soft agar (Fig. 7C), establishing the role for PI3K/Akt signaling in PIPKIγ2 and Src regulation of oncogenic growth. Corroborating this, the knockdown of Src and PIPKIγ2 impaired PI3K/Akt signaling in T47D and HCC1954 cells in suspension culture (data not shown). Furthermore, a kinase-dead mutant of PIPKIγ2 (D253A,D316A) was severely impaired in inducing PI3K/Akt activation in concert with Src upon stimulation of cells with growth factor and ECM protein (Fig. 7, D and E). This indicates that kinase activity of PIPKIγ2 and PIP3 generation is required for PI3K/Akt signaling. The interaction between PIPKIγ2 and Src was also required for induced Akt activation and oncogenic growth because the Src mutant deficient in PI3K binding was significantly impaired in inducing Akt activation (Fig. 7, F and G) and anchorage-independent growth in synergy with PIPKIγ2, as indicated previously (18). This was further substantiated by the disruption of the PIPKIγ2 and Src interaction by the C terminus of Src, which inhibited Akt activation induced by the co-expression of PIPKIγ2 and Src (data not shown).

Src contains highly conserved basic residues at the N terminus that are potential anionic phospholipid binding sites, and these residues play a crucial role in Src recruitment and its activation in the plasma membrane (18, 43, 44). The Src mutant, Src (3K/3T) (lysine residues mutated to threonine) poorly induced Akt activation in response to FBS stimulation in the suspension condition (Fig. 8A), although it was competent in the adherent condition (Fig. 8B). Consistently, this mutant showed impaired Akt activation in synergy with PIPKIγ2 in the suspension culture (Fig. 8C). This is consistent with the Src mutants’ impaired ability to induce anchorage-independent growth in collaboration with PIPKIγ2 (18). This indicates that Src association with the PIPKIγ2 and PIP3 generated also regulates Src function and is also consistent with our previous results that PIPKIγ2 knockdown affects Src activation downstream of EGF receptors (18). With these results, we conclude that PIPKIγ2 regulates PI3K/Akt signaling and oncogenic growth in coordination with the proto-oncogene Src.
PI3K/Akt signaling is one of the most commonly deregulated signaling pathways in cancer and plays a key role in oncogenic growth signaling (2, 3). Herein, we illustrated the mechanism by which cancer cells can activate and sustain the PI3K/Akt signaling downstream of growth factor and adhesion receptors in the suspension condition. PIPKIγ and its focal adhesion targeting variant, PIPKIγi2, appeared to provide PIP2 spatially for PIP3 generation and the activation of PI3K/Akt signaling downstream of activated growth factor and adhesion receptors. This indicates that cancer cells maintain their vital cellular signaling, such as PI3K/Akt, irrespective of adherent or suspension condition. This may provide the mechanism by which metastasizing and circulating tumor cells can sustain cellular signaling for their survival and growth.

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PI3K/i2 is utilized by PI3K and PLC enzymes to generate the second messengers: PIP3, inositol triphosphate, and diacylglycerol, in response to growth factor and adhesion receptor activation (45–47). Despite these key functions, the content of PIP2 is maintained relatively constant (5, 6, 9). This indicates that PIP2 synthesis may be channeled for use as a messenger or a precursor for messenger generation (5, 6, 46). Here we show that the PIP2-synthesizing enzyme PIPKIγi2 regulated by Src is integrated into a PI3K complex that is required for the activation of PI3K/Akt signaling. The PI3K-mediated conversion of PIP2 into PIP3 is required for the activation of Akt. Our results support a model where PIPKIγi2-generated PIP2 is utilized by PI3K to synthesize PIP3 and activate Akt. The PI3K/Akt signaling nexuses have roles in oncogenesis (2), and the PIPKIγi2 is required for this pathway in the suspension condition and for anchorage-independent growth of the tumor cells (18). Among different isoforms of PIPKI enzymes, the knockdown of PIPKIγ, which is overexpressed in breast cancer and correlates with poor patient prognosis (26), showed specificity for PI3K/Akt signaling in the suspension condition. The focal adhesion targeting variant, PIPKIγi2, recapitulated the outcome of PIPKIγ knockdown on Akt activation.

The assembly of the signaling complex is the primary event in the initiation and transduction of intracellular signals (48, 49). Many signaling proteins are targeted to the activated growth factor receptors and integrins in the plasma membrane via their
SH2 or phosphotyrosine binding domain or similar domains (48, 49). The PI3K enzyme is rapidly recruited to the plasma membrane by utilizing the SH2 domain-mediated interaction of its adaptor subunit (e.g., p85) with phosphotyrosine residues of activated growth factor receptors (2, 3). Upon cell stimulation, PIPKIγ2 specifically associated with Src and the PI3K enzyme; Src facilitated the assembly of the complex of PIPKIγ2 and PI3K as Src knockdown impaired PI3K/Akt activation in PIPKIγ2-expressing cells as well as PIPKIγ2 assembly into the complex. Further, PIPKIγ2 (of the six different PIPKIγ variants) is targeted to the cell-ECM interface via interaction with the cytoskeletal protein, talin (13, 30), positioning PIPKIγ2 as a key enzyme synthesizing PIP3 in response to cell stimulation with ECM proteins through integrin activation. These concepts are supported by (i) impaired Akt activation by PIPKIγ2 knockdown, (ii) increased PIP2 and PIP3 levels in PIPKIγ2-overexpressing cells, and (iii) specific incorporation of PIPKIγ2 with the PI3K enzyme upon cell stimulation with growth factors and ECM protein. This supports a model in which the PIPKIγ2-generated pool of PIP2 is utilized by the PI3K to generate PIP3 for Akt activation (Fig. 8, D and E). Phosphorylation of tyrosine residues in the C terminus of PIPKIγ2 by Src (17) could also regulate interaction between PIPKIγ2 and PI3K enzyme, thus promoting the co-targeting of both PIP2- and PIP3-generating enzymes to the plasma membrane to facilitate PI3K/Akt signaling. These interactions may define the fundamental basis of PI3K/Akt regulation by the PIP2-synthesizing enzyme in response to some cell stimuli.

Src, a cytosolic tyrosine kinase, is activated downstream of many growth factor receptors, cytokine receptors, and adhe-
Regulation of PI3K/Akt Signaling by PIPKιγ

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tion receptors (34, 50, 51). By virtue of its SH2 and SH3 domains, Src interacts with different signaling molecules and modulates their functionality (34, 51). Along with its own defined sets of downstream signaling cascades, Src regulates PI3K/Akt signaling by different mechanisms. Some of these include phosphorylation of adaptor subunits of PI3K (e.g. Tyr-688 on p85) and alleviation of inhibitory constraints to its catalytic subunit, p110α (39); co-targeting with PI3K to activated growth factor receptors (38); and activation of Ras and inactivation of PTEN (37), a negative regulator of PI3K/Akt signaling.

Here, we show that Src facilitates the assembly of the PIP2-synthesizing enzyme, PIPKιγ2, in the proximity of activated receptors and adhesion complex to support PI3K/Akt activation required for the oncogenic growth of tumor cells. This is consistent with many studies indicating that PI3K/Akt activation is the cumulative outcome of several pathways (2).

With these results, we conclude that PIPKιγ2 coordinates with Src and possibly talin for spatial assembly with PI3K in the proximity of activated growth factor receptors and adhesion complexes for PIP2 and PIP3 synthesis for Akt activation even in the suspension condition (Fig. 8, D and E). This could represent a signaling nexus required for the survival and growth of metastasizing and circulating tumor cells in vivo and is also consistent with reported studies that have demonstrated the role of PIPKιγ/PIPKιγ2 in oncogenic growth and tumor metastasis (18, 19, 26). Because generation of PIP2 by PI3K and Akt activation has significant therapeutic implications for cancers, targeting PIPKιγ/PIPKιγ2 could pave the way for controlling the PI3K/Akt signaling nexus in metastasizing and circulating tumor cells in vivo.

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Regulation of PI3K/Akt Signaling by PIPKIγ


Phosphatidylinositol Phosphate 5-Kinase Iγ and Phosphoinositide 3-Kinase/Akt Signaling Couple to Promote Oncogenic Growth
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