Phosphatidylinositol Phosphate 5-Kinase Iγi2 in Association with Src Controls Anchorage-independent Growth of Tumor Cells*

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Background: PIPKIγ isoforms play roles in cell migration, polarization, and membrane trafficking and are overexpressed in triple-negative breast cancers, indicating protumorigenic functions.

Results: PIPKIγi2 associates with the C terminus of Src, and this interaction interdependently controls their functioning.

Conclusion: PIPKIγi2 and Src synergistically control anchorage-independent tumor cell growth.

Significance: This study shows unexpected mechanisms for a phosphatidylinositol 4,5-biphosphate-generating enzyme that synergizes with the proto-oncogene Src to regulate oncogenic signaling.

A fundamental property of tumor cells is to defy anoikis, cell death caused by a lack of cell-matrix interaction, and grow in an anchorage-independent manner. How tumor cells organize signaling molecules at the plasma membrane to sustain oncogenic signals in the absence of cell-matrix interactions remains poorly understood. Here, we describe a role for phosphatidylinositol 4-phosphate 5-kinase (PIPKI) 1γi2 in controlling anchorage-independent growth of tumor cells in coordination with the proto-oncogene Src. PIPKIγi2 regulated Src activation downstream of growth factor receptors and integrins. PIPKIγi2 directly interacted with the C-terminal tail of Src and regulated its subcellular localization in concert with talin, a cytoskeletal protein targeted to focal adhesions. Co-expression of PIPKIγi2 and Src synergistically induced the anchorage-independent growth of nonmalignant cells. This study uncovers a novel mechanism where a phosphoinositide-synthesizing enzyme, PIPKIγi2, functions with the proto-oncogene Src, to regulate oncogenic signaling.

The ability of tumor cells to defy anoikis, cell death caused by lack of cell-matrix interaction, and grow in an anchorage-independent manner determines their capacity to survive in the vasculature and lymphatic circulation during tumor metastasis (1, 2). In adherent cells, focal adhesions are the contact points of cells to their underlying substrate and also serve as signaling hubs. Anchorage dependence of normal cells stems from the fact that they derive a large part of their proliferative and survival signals from their substrate via focal adhesions (3). Contradicting this, many focal adhesion proteins, including FAK, integrin-linked kinase, paxillin, Src, talin, and pCAS130, are actively involved in oncogenic processes that allow tumor cells to survive/grow in an anchorage-independent manner and promote tumorigenesis (4–7). However, the precise mechanism for how tumor cells assemble the signaling molecules at the plasma membrane following the disruption of cell-matrix interaction, thus bestowing anchorage independence for survival and growth, remains poorly understood (8–11).

Phosphatidylinositol 4,5-biphosphate represents the major phosphoinositide in the plasma membrane, where it functions as a pleiotropic lipid-signaling molecule regulating many cellular functions including cell survival, cell cycle progression, cell migration, vesicle trafficking, and actin cytoskeleton reorganization (12–19). The spatiotemporal generation of phosphatidylinositol 4,5-biphosphate regulates the targeting and/or functional activity of different molecules in specified subcellular compartments (12, 13, 20). Spatial signaling is achieved by an association of phosphatidylinositol 4,5-biphosphate-generating enzymes with molecules that are often phosphatidylinositol 4,5-biphosphate effectors (12, 20). For example, PIPKIγi2 is specifically targeted to focal adhesions via interaction with talin, where it generates phosphatidylinositol 4,5-biphosphate required for focal adhesion assembly and cell migration (21, 22). Phosphatidylinositol 4,5-biphosphate generation lies at a junction point in the phosphoinositide signaling, where it can function as a lipid messenger or be used as substrate for PI3K and PLC to generate the second messengers: phosphatidylinositol-3,4,5-triphosphate, inositol-1,4,5-triphosphate, and diacylglycerol (20).

In mammalian cells, phosphatidylinositol 4,5-biphosphate is largely generated by type 1 PIPK2 enzymes, which are classified into PIPK1α, PIPK1β, and PIPK1γ isoforms (12–14). PIPK1α is targeted to nucleus and controls nuclear events (14). PIPK1β is localized at vesicular structures in the perinuclear region (12, 13). Mammalian cells express at least five splice variants of PIPKIγ (e.g., PIPKIγ1, PIPKIγ2, PIPKIγ4, and PIPKIγ5); among them, PIPKIγ2 is targeted to both focal adhesion sites

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The abbreviations used are: PIPK, phosphatidylinositol 4-phosphate 5-kinase; PLCδ3, phospholipase Cδ3; Csk, C-terminal Src kinase; PH, pleckstrin homology; FAK, focal adhesion kinase; EGF, epidermal growth factor; IF, immunofluorescence microscopy.
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and endosomal membranes (12, 24). Studies are emerging that implicate PIPKιγ in tumor progression, where increased expression of PIPKιγ in breast cancer tissues correlated with poor patient survival (25). Increased PIPKιγ activity is observed in hepatocellular carcinoma (26), and PIPKιγ regulates the transcriptional activity of β-catenin downstream of growth factor signaling (27). Combined, these studies indicate a role for PIPKιγ in tumor progression.

Src, non-receptor tyrosine kinase and proto-oncogene, regulates the PIPKιγi2 interaction with talin (28). Src activation is a hallmark of many cancers, and several mechanisms are implicated in its activation of tumorigenic processes (29, 30). The plasma membrane recruitment and activation of Src is primarily mediated by myristoylation of glycine residue in its N terminus (31), although highly conserved basic residues in its C terminus (32), interacts with anionic lipid molecules (32). Here, we show that PIPKιγi2 and Src, both focal adhesion molecules, form a signaling complex following the disruption of cell-matrix interaction and support the anchorage-independence of tumor cells.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies for Src (antibody 2108S), ERK1/2 (antibody 9194), and pERK1/2 (antibody 4370) were purchased from Cell Signaling; antibodies for Src (antibodies 44660G and 44662G) and pFAK (antibody 44-624G) were purchased from Invitrogen; antibodies for talin (antibody 05-385), FAK (antibody 05-537), Src (antibody 05-184), and phosphotyrosine (antibody 4G10) were purchased from Millipore; antibodies for paxillin (antibody 61005) and Csk (antibody 610079) were from BD Biosciences. Other antibodies used were: HA (antibody HAA10; Covance), talin (antibody HPA004748; Sigma), cortactin (antibody 05-180; Upstate), and anti-GFP (antibody GF28R; Thermo Scientific). Antibodies for PIPKιγ, PIPKιγi2, and PIPKια were developed in the laboratory (28, 33, 34). Antibody specific for tyrosine-phosphorylated PIPKιγi2 was kind gift of Dr. Dianqing Wu (Yale University).

DNA Constructs, Mutagenesis, and siRNA—PIPKιγ isoforms or PIPKιγi2 mutants were subcloned into MluI and Sall sites in frame with HA tag in the N terminus of PWPT vector (Addgene) as described previously (22). Full-length chicken Src or Src mutants used in the study were cloned into BamHI and Sall sites of PWPT vector. All the mutations used in the study were created using a QuikChange site-directed mutagenesis kits (Stratagene) followed by DNA sequencing to confirm the integrity of the DNA sequence. For cloning the C terminus of Src, oligonucleotides used for annealing were: TCGAGGAGG-GCTCCCCGGGCTGGTACTGGGGCTCGGTGGACGTG-GGGAGAACCTCTAGG (sense) and TCGACCTAGAGGTACTACTTCACGTCCACCGAGCCCCAGTACCAGCCCG-TGCGACACAAUUUCUG (antisense). After annealing of these oligonucleotides, 5’-prime and 3’-prime ends harbor Xhol and Sall sites, respectively, for cloning into eGFP-C3 vector (Clontech) in frame with GFP in the N terminus. This construct was further subcloned into PWPT-GFP vector for retroviral infection.

siRNA—The following oligonucleotides were used: control siRNA, CCUUGGUGACUGUGAUUU; siPIPKιγi2, GAGGCACACAAUUUCUG; siPIPKιγi2 (second), CGCGCAGAGG-GGAGAACCTCTAGG (sense) and TCGACCTAGAGGTACTACTTCACGTCCACCGAGCCCCAGTACCAGCCCG-TGCGACACAAUUUCUG (antisense). For siRNA-mediated knockdown of genes, Lipofectamine RNAiMAX (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 hours post-transfection. For the expression or co-expression of genes into MDA-MB-231 or NIH3T3 or MCF-7 cells, the lentiviral system was used as described previously (22). Cells were harvested 24–48 h post-infection (70–80% infection efficiency were achieved for the experiments).

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 indicated antibodies for 3–4 h overnight at 4 °C followed by isolation of immune complexes using protein G-Sepharose 4B beads (Amersham Biosciences). The beads were washed three times with lysis buffer before eluting the immune complexes with 2× sample buffer and then subjected to immunoblotting using indicated antibodies.

GST Pulldown Assay—Different regions of chicken Src were PCR-amplified and cloned into pGEX-6P-1 (Novagen). Proteins were expressed into BL21 and purified using glutathione-Sepharose 4B beads (Amersham Biosciences). For in vitro binding study, purified GST fusion proteins immobilized on the Sepharose beads were incubated with His-tagged PIPKιγi2 purified from bacteria with or without cell lysates prepared from HEK293 cells transfected with HA-PIPKιγi2 at 4 °C for 1 h followed by elution of bound proteins with 2× sample buffer for immunoblotting.

Cell Proliferation and Anchorage-independent Growth—For cell proliferation assay, MDA-MB-231 cells were seeded into 12-well culture plate (1,000 cells/well) in DMEM containing 10% FBS. T47D and HCC1954 cells were cultured in RPMI 1640 containing 10% FBS. SUM1315 cells were culture in Ham’s F12 supplemented with 5% FCS. Suspended cells in the study refer to the cells resuspended in medium containing 0.1% BSA and 1% FBS after trypsinization/detachment and incubated at 37 °C in the incubator for 2–3 h except for time course study. For overnight culture in suspension condition, cells were seeded into the culture plate coated with 0.3% agar and incubated at 37 °C. For the stimulation of cells in adherent condition, cells were serum-starved overnight before stimulating the cells with 10% FBS or EGF (50 ng/ml) for the indicated time periods. For the stimulation of cells in suspension condition, cells were resuspended as described above and incubated for 2–3 h before stimulating with FBS (10% FBS) or EGF (50 ng/ml) or extracellular matrix protein (combination of fibronectin/collagen type I, 25 μg/ml each) for the indicated time periods.

Cell Culture—MDA-MB-231, NIH3T3, HEK293, and HEK293FT cells were cultured in DMEM containing 10% FBS. T47D and HCC1954 cells were cultured in RPMI 1640 containing 10% FBS. SUM1315 cells were culture in Ham’s F12 supplemented with 5% FCS. Suspended cells in the study refer to the cells resuspended in medium containing 0.1% BSA and 1% FBS after trypsinization/detachment and incubated at 37 °C in the incubator for 2–3 h except for time course study. For overnight culture in suspension condition, cells were seeded into the culture plate coated with 0.3% agar to avoid cell attachment. For the stimulation of cells in adherent condition, cells were serum-starved overnight before stimulating the cells with 10% FBS or EGF (50 ng/ml) for the indicated time periods. For the stimulation of cells in suspension condition, cells were resuspended as described above and incubated for 2–3 h before stimulating with FBS (10% FBS) or EGF (50 ng/ml) or extracellular matrix protein (combination of fibronectin/collagen type I, 25 μg/ml each) for the indicated time periods.
FBS. Cells were manually counted every second day for up to 8 days.

For anchorage-independent growth, cells were suspended in medium containing 0.3% agar and seeded into 24-well culture plates. 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 10–28 days depending upon the cells type used. Similarly, cell numbers used for seeding were also adjusted depending upon the efficiency of cells to form colonies. In some cases, Src inhibitor (PP1, 0.5 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.

Immunofluorescence Microscopy (IF)—For examining the co-localization of PIPKIγ2 and Src at focal adhesions, cells were seeded into collagen type I- or fibronectin-coated coverslip and incubated for 30 min before fixing the cells with 3.7% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 before blocking with 3% BSA in PBS. The same procedures were used for IF study of the colonies developed in the soft agar. Cells were incubated with primary antibody overnight at 4 °C followed by incubation with Alexa 555- and/or Alexa 488-conjugated secondary antibody (Molecular Probes) for 1 h at room temperature. Slides were mounted using Vectashield and visualized with a Nikon TE2000-U microscope using 63× objective lenses. The images were acquired using MetaMorph and processed using adobe Photoshop.

For examining the phosphatidylinositol 4,5-biphosphate distribution in the PIPKIγ or PIPKIγ2 knockdown cells, MDA-MB-231 cells were transfected with siRNA as described above. After FIGURE 1. PIPKIγ/PIPKIγ2 is required for anchorage-independent growth of tumor cells. A, schematic diagram of PIPKIγ2 showing the domain organization and the specific amino acid sequence at the C terminus. Red letters indicate Src phosphorylation sites crucial for talin binding. B–D, 48 hours after siRNA transfection, cells were cultured in soft agar for 2–4 weeks. Immunoblotting shows the knockdown of PIPKIγ and PIPKIγ2. Representative images of colonies developed by T47D cells are shown. E, for cell proliferation assay, MDA-MB-231 cells after siRNA transfection were seeded into 12-well culture plates (1,000 cells/well). Cells were counted manually every second day. F, 24–36 h after siRNA transfection, cells were retransfected with plasmids for expression of GFP-PLCγ-PH or GFP-PLCγ-PH mutant. Cells were processed for IF study after overnight culture (scale bar, 10 μM). G, siRNA was used to knock down endogenous PIPKIγ2 from MDA-MB-231 cells or MDA-MB-231 cells expressing siRNA-resistant wild type PIPKIγ2 (PIPKIγ2WT) or its kinase dead mutant (PIPKIγ2KD). Cells were cultured in soft agar 48 hours after transfection. Immunoblotting shows the knockdown of endogenous PIPKIγ2 in the cells. The expression of siRNA-resistant PIPKIγ2WT or PIPKIγ2KD was confirmed by immunoblotting using antibodies specific for PIPKIγ2 or HA tag. All the values are means ± S.D. from three-independent experiments. The error bars represent S.D. (p values are indicated).
24–36 h, cells were retransfected with plasmids for the expression of GFP-PLC\_H9254-PH or GFP-PLC\_H9254-PH mutant. Cells were processed for IF study following the overnight culture.

Statistical Analysis - The data are presented as means ± S.D. from at least three-independent experiments. Unpaired \( t \) test was conducted to determine the \( p \) value, and the statistical significance between two groups (\( p \) value equal to or less than 0.05 were considered significant).

RESULTS

PIPKI\_\gamma i2 Regulate the Anchorage-independent Growth of Tumor Cells - PIPKI\_\gamma i2 is a phosphatidylinositol 4,5-bisphosphate-generating enzyme targeted to cell-matrix interaction sites via an interaction with talin (33, 35). Src phosphorylation of tyrosine residues at the C terminus of PIPKI\_\gamma i2 (red Tyr residues in Fig. 1A) regulates its interaction with talin (28). Increased expression of PIPKI\_\gamma in breast cancer tissues inversely correlates with patient survival, indicating its potential role in tumor progression (25). To define an oncogenic role for PIPKI\_\gamma, pan-PIPKI\_\gamma or PIPKI\_\gamma i2 was knocked down, and the effect on anchorage-independent growth of breast cancer cell lines in soft agar was examined. The knockdown of endogenous PIPKI\_\gamma or PIPKI\_\gamma i2 significantly impaired the ability of MDA-MB-231, SUM1315, and T47D cells to grow in an anchorage-independent manner (Fig. 1, B–D). However, cell proliferation was not affected by PIPKI\_\gamma i2 knockdown but was affected by pan-PIPKI\_\gamma knockdown in MDA-MB-231 cells in two-dimensional culture (Fig. 1E). Consistently, the loss of PIPKI\_\gamma i2 was not sufficient to affect the localization of GFP-PLC\_PH, a biosensor of phosphatidylinositol 4,5-bisphosphate, whereas pan-PIPKI\_\gamma knockdown resulted in impaired localization of GFP-PLC\_PH in the plasma membrane (Fig. 1F). These results indicate the collective function of different PIPKI\_\gamma variants in phosphatidylinositol 4,5-bisphosphate synthesis in the plasma membrane. Furthermore, the knockdown of endogenous PIPKI\_\gamma i2 in MDA-MB-231 cells expressing siRNA-resistant PIPKI\_\gamma i2 did not affect the anchorage-independent growth (Fig. 1G). Further, the kinase-dead mutant of PIPKI\_\gamma i2 poorly rescued anchorage-independent growth, signifying the importance of kinase activity. The expression level of ectopically expressed siRNA-resistant PIPKI\_\gamma i2 was severalfold higher than that of endogenous PIPKI\_\gamma i2 and resulted in induced anchorage-independent growth.
To investigate the role of each PIPKI splice variant, PIPKI variants were ectopically expressed into MDA-MB-231 cells, which express a low level of PIPKI/H9253 compared with other breast cancer cell lines examined (not shown). As shown in Fig. 2 (A–C), the expression of each variant significantly promoted anchorage-independent growth, although PIPKI/H9253 expression had substantially greater effect, which correlated with its expression level (Fig. 2D). The knockdown of ectopically expressed PIPKI/H9253 before examining the activation level of Src and other molecules in suspension condition showed significantly impaired Src activation (tyrosine phosphorylated Src in its activation site) upon PIPKI/H9253 or PIPKI/H9253i2 knockdown (Fig. 3, A and B, and data not shown). Corroborating this, the overexpression of PIPKI/H9253i2 increased the activation level of Src, without a noticeable change in FAK activation (assessed by autophosphorylation of FAK at Tyr397) (Fig. 3C). The knockdown of ectopically expressed PIPKI/H9253i2 abrogated activation level of Src (Fig. 3D), and this coincided with significantly reduced anchorage-independent growth (Fig. 2E). However, the impact of PIPKI/H9253 or PIPKI/H9253i2 knockdown or overexpression on Src activation was less obvious in the adherent condition (not shown).

In suspension culture, MDA-MB-231 cells overexpressing PIPKI/H9253 showed prolonged activation of endogenous Src (Fig.
Src is activated downstream of integrins and growth factor receptors (29, 36). Consistently, EGF- or FBS-stimulated Src activation in MDA-MB-231 cells was significantly impaired upon PIPKIγ2 knockdown (Fig. 3F). Also, in suspension condition, PIPKIγ2 knockdown affected Src activation in response to stimulation with FBS or extracellular matrix proteins (Fig. 3G). In corroboration with knockdown studies, the increased expression of PIPKIγ2 promoted FBS- or extracellular matrix protein-stimulated activation of Src, indicating PIPKIγ2 regulation of Src activation downstream of integrins and growth factor receptors (Fig. 3H).

**Src Is Required for PIPKIγ2-induced Anchorage-independent Growth and Vice Versa—**In support of the above results, the knockdown of endogenous Src or the use of a Src inhibitor blocked the PIPKIγ2-induced anchorage-independent growth (Fig. 4, A–D), indicating the role for Src in PIPKIγ2-regulation of oncogenic growth. These results are consistent with a key role for Src in mediating cell survival and growth in both cellular and in vivo systems (6, 36).

After demonstrating the Src function in PIPKIγ2-induced anchorage-independent growth, we inquired whether PIPKIγ/ PIPKIγ2 is required for Src function. A feature of Src-transformed cells is a disorganized cytoskeleton with multiple cell protrusions (37). Consistently, the knockdown of PIPKIγ2, but not PIPKIα, abrogated the disorganized actin cytoskeleton phenotype induced by Src expression in MDA-MB-231 cells (Fig. 4E, left panels). Ectopically expressed Src localized to cell protrusions, and this targeting was also significantly reduced by PIPKIγ or PIPKIγ2 knockdown (Fig. 4E, right panels). Similarly, tyrosine phosphorylation of FAK and cortactin, Src substrates, induced by Src expression was reduced upon PIPKIγ2 knockdown (Fig. 4F). Decreased anchorage-independent growth of Src-expressing cells upon PIPKIγ or PIPKIγ2 loss was also accompanied by reduced Src activa-

**FIGURE 4.** PIPKIγ2 and Src reciprocally regulate their oncogenic function. A–C, siRNA were used to knock down endogenous Src from MDA-MB-231 cells overexpressing PIPKIγ2. Cells were cultured in soft agar for 2–3 weeks before counting the colonies. ConsiRNA, control siRNA. D, PIPKIγ2-overexpressing cells were treated with a pharmacological inhibitor of Src (PP1 or control PP3, 0.5 μM) during suspension culture, and the colonies developed were counted after 2–3 weeks culture in soft agar. All the values are means ± S.D. from three-independent experiments. The error bars represent S.D. (p values are indicated). E, siRNA was used to knock down PIPKIγ2 or PIPKIα from MDA-MB-231 cells transfected with Src. Cells were fixed for IF study to examine the actin cytoskeleton (left panels) and Src localization (right panels). Scale bar, 20 μm. F, cortactin and FAK were immunoprecipitated from mock and Src-infected cells after siRNA transfection for PIPKIγ2 knockdown. Cells were harvested 48 hours post-transfection to immunoprecipitate the endogenous cortactin and FAK followed by immunoblotting using phosphotyrosine antibody. G, siRNA was used for PIPKIγ2 knockdown in MDA-MB-231 cells infected with lentivirus for Src overexpression. Cells were cultured in soft agar for 2 weeks before counting the colonies.
tion (Fig. 4G), indicating that PIPKIγi2 is required for Src activation and function.

PIPKIγi2 and Src Synergistically Induce Anchorage-independent Growth—After demonstrating the Src requirement for PIPKIγi2-induced anchorage-independent growth and vice versa, we examined the ability of PIPKIγi2 (and other PIPKIγ variants) to induce oncogenic growth of the nontransformed NIH3T3 or MCF10A cells. Independent expression of PIPKIγi2 or Src poorly induced the anchorage-independent growth of NIH3T3 cells (Fig. 5A). Strikingly, co-expression of PIPKIγi2 and Src dramatically increased the anchorage-independent growth. The synergistic effect of PIPKIγi2 and Src was further demonstrated in MDA-MB-231 cells (Fig. 5B). Among PIPKIγ variants, PIPKIγi2 showed the most potent effect, emphasizing the functional specificity of PIPKIγi2 and Src in anchorage-independent growth regulation (not shown). However, a kinase dead mutant of PIPKIγi2 poorly induced anchorage-independent growth in synergy with Src, indicating the role of kinase activity of PIPKIγi2 enzyme (Fig. 5C). Furthermore, an analysis of the expression of PIPKIγ/PIPKIγi2 demonstrated the link between PIPKIγ/PIPKIγi2 expression and Src activation in many breast cancer cell lines examined (data not shown). In all tumor cell lines examined, the loss of PIPKIγ/PIPKIγi2 and/or Src inhibited oncogenic growth on soft agar (Fig. 5, D and E).

PIPKIγi2, Src, and Talin Interdependently Regulate Their Subcellular Localization, and All Are Integrated into the Signaling Complex—Targeting of Src to focal adhesions is a key for its oncogenic activity (37–39). Immunofluorescence studies
revealed extensive co-localization of endogenous active Src with PIPKιγ2, predominantly at focal adhesions (Fig. 6A, upper panels). Phosphatidylinositol 4,5-biphosphate-generating enzymes regulate intracellular vesicle trafficking and targeting of signaling molecules to the plasma membrane at sites of adhesion (40, 41). PIPKιγ2 knockdown modestly affected active Src localization at focal adhesions (Fig. 6, A, lower panels, and B) However, the loss of talin resulted in a more profound defect on Src localization at focal adhesions (Fig. 6, A and B) and is consistent with the role of talin in focal adhesion assembly.

In three-dimensional suspension culture, PIPKιγ2 knockdown impaired both Src and talin localization at plasma membrane (Fig. 6C). Src extensively co-localized with PIPKιγ2 at cell-cell contact sites at the plasma membrane (Fig. 6D), whereas other PIPKιγ variants deficient in focal adhesion targeting (PIPKιγ1) were poorly localized with Src at plasma membrane. A PIPKιγ2 mutant deficient in Src phosphorylation and talin binding, thus defective in focal adhesion localization were also poorly co-localized with Src at plasma membrane in suspension culture (not shown). In suspension condition, PIPKιγ2 forms a stable complex with talin that is promoted by Src expression and PIPKιγ2 phosphorylation (Fig. 6E). All of these results indicate that PIPKιγ2 in coordination with talin regulates Src localization at focal adhesions and the plasma membrane in three-dimensional culture. Furthermore, impaired anchorage-independent growth in PIPKιγ2-overexpressing cells after talin knockdown (Fig. 6F) supports
the coordinated roles of the focal adhesion molecules, PIPKIγ2, Src, and talin, in oncogenic signaling.

**An Interaction between PIPKIγ2 and Src Is Required for Their Oncogenic Growth Control**

PIPKIs often associate with proteins they regulate (12, 20, 22) as such an interaction between PIPKIγ2 and Src was explored. The interaction between PIPKIγ2 variants and Src was observed in their endogenous levels and after co-expression and co-immunoprecipitation (Fig. 7A). The full-length Src bound with His-tagged PIPKIγ2 or HA-PIPKIγ2, indicating that either the kinase domain or the C terminus mediates the Src interaction with PIPKIγ2. Co-expression and co-immunoprecipitation studies demonstrated that the Src C-terminal deletion mutant was defective in PIPKIγ2 binding (Fig. 7C), indicating that the C terminus is necessary for this interaction. The constitutively active Src (Y527F mutation) showed a modest increase in PIPKIγ2 interaction (Fig. 7C). Csk kinase that phosphorylates Src in Tyr527, promoting an autoinhibitory intramolecular interaction and Src inactivation, binds to C terminus of Src (29). However, PIPKIγ2 expression did not affect the Src association with Csk nor Tyr527 phosphorylation of Src (not shown).

Deletion of the Src C terminus leads to constitutive activation of Src (42). Remarkably, the co-expression of the C-terminal deletion mutant of Src with PIPKIγ2 failed to induce anchorage-independent growth in synergy with Src (Fig. 7D), indicating the importance of the PIPKIγ2 interaction with Src. Further, the expression of the GFP fusion protein with C-terminal tail of Src (GFP-C-tail) inhibited the localization of active Src at focal adhesions (not shown) and PIPKIγ2 interaction with Src (Fig. 7E). It also inhibited the anchorage-independent growth induced by co-expression of PIPKIγ2 and Src (Fig. 7F).
The highly conserved basic residues in the N terminus of Src play an important role in Src function and its recruitment to plasma membrane via electrostatic interaction with anionic phospholipids, including phosphatidylinositol 4,5-biphosphate and others (32). However, the precise role of phosphatidylinositol 4,5-biphosphate in Src function is not defined. As shown in Fig. 8A, mutations of all of these basic residues to neutral amino acids impaired Src association with PIPKIγi2 (Fig. 8B) and the ability of Src to induce oncogenic growth in synergy with PIPKIγi2 (Fig. 8C). Furthermore, kinase dead PIPKIγi2 showed impairment in inducing anchorage-independent growth in synergy with Src (Fig. 5C). Taken together, these results indicate a coordinated role of PIPKIγi2 and Src and phosphatidylinositol 4,5-biphosphate generation in oncogenic signaling and anchorage-independent growth (illustrated in schematic diagram in Fig. 8D).

**DISCUSSION**

The ability to grow in an anchorage-independent manner is one of the fundamental properties of tumor cells and is a key for metastasis, although the underlying mechanisms are poorly understood. Here, we show that the focal adhesion-targeted, phosphatidylinositol 4,5-biphosphate-synthesizing enzyme PIPKIγi2 coordinates with the pro-oncogenic molecule, Src, and the cytoskeletal adaptor molecule, talin, to regulate oncogenic growth of tumor cells. This is consistent with results showing that PIPKIγ expression correlates with poor breast cancer patient survival (25) and supports a potential role for PIPKIγ and PIPKIγi2 in tumor progression.

The activation of Src is a hallmark of many tumors, and several mechanisms are reported for Src activation (29, 30). Inactive Src largely remains in the perinuclear region, whereas
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Active Src is targeted to the plasma membrane/cell adhesion sites in an actin-dependent manner (30). Myristoylation is required for Src recruitment to the plasma membrane and its activation (31). In addition, highly conserved basic residues at the N terminus of Src play an important role in Src function and its recruitment to the plasma membrane via electrostatic interaction with anionic phospholipids (32). Phosphoinositides, including phosphatidylinositol 4,5-bisphosphate, constitute the pivotal lipid molecules in the plasma membrane that play key roles in the recruitment of signaling molecules possessing phosphoinositide-binding motifs and/or domains (43). The inability of Src mutants (substitution of basic residues to neutral) to interact with and function in synergy with PIPKIγ2 to induce anchorage-independent growth strongly suggests the phosphoinositide regulation of Src function and functional integration of Src into phosphoinositide signaling pathways. Binding data indicate that PIPKIγ2 interacts with the C terminus of Src. This interaction may relieve the intramolecular interaction between the C-tail and Src homology 2 domain, facilitating Src activation. However, PIPKIγ2 interaction with Src C terminus did not abrogate the Src association with Csk nor Csk phosphorylation of Src. In the absence of PIPKIγ2/PIPKIγ2 expression or in cells expressing low levels of PIPKIγ, Src may remain in an inactive state. PIPKIγ2/PIPKIγ2 might be playing a role in Src activation as well as its targeting to plasma membrane/focal adhesion sites in coordination with talin. This is consistent with the results that show a requirement for PIPKIγ2 and talin in localization of Src at focal adhesions in adherent conditions and at cell-cell contact sites in three-dimensional culture.

Talin, Src, and PIPKIγ2, are all targeted to focal adhesions in adherent cells. In the absence of cell-matrix interactions in suspension culture, all of them are assembled into a complex, presumably at the vicinity of integrins and growth factor receptors that sustain oncogenic signaling necessary for anchorage-independent growth. In three-dimensional culture, their mutual interaction may promote their organization at cell-cell contact sites in the plasma membrane, although their major fractions remain in cytosol (22, 44, 45). Furthermore, Src is required for PIPKIγ2 association with talin in different tumor cells (not shown), and Src expression significantly promoted PIPKIγ2 association with talin. Conversely, talin interaction with actin is regulated by phosphatidylinositol 4,5-bisphosphate (46), suggesting their integrative and collaborative function. As talin is emerging as a potential regulator of oncogenesis, Src regulation of PIPKIγ2 interaction with talin is fully consistent with a collaborative role in anchorage-independent growth (35, 47, 48).

In the plasma membrane, PIPKIγ2 and activated Src may induce oncogenic signaling that contributes to anchorage-independent growth. Talin, a cytoskeletal protein and phosphatidylinositol 4,5-bisphosphate effector protein, is an important component of this signaling nexus because it may selectively promote the PIPKIγ2 targeting to the plasma membrane in suspension culture because of its ability to interact with actin cytoskeleton. With these results, we have uncovered the mechanism of how focal adhesion molecules PIPKIγ2, Src, and talin converge into a signaling complex to support oncogenic growth of tumor cells. This could be an oncogenic axis required for in vivo tumor growth and metastasis, including that of triple negative breast cancers, where PIPKIγ and Src are predominantly overexpressed (23, 49). Furthermore, the elucidation of oncogenic signaling molecules downstream of PIPKIγ2 and Src and their functional relevance in vivo are future directions for study.

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