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βPix-a Enhances the Activity of Phospholipase Cγ1 by Binding SH3 Domain in Breast Cancer

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Abstract Phospholipase C-γ1 (PLCγ1) plays a critical role in cell growth and proliferation by generating the second messengers, diacylglycerol and 1, 4, 5-inositol triphosphate. To investigate the roles of Src homology domain 2 and domain 3 of PLCγ1 in PLCγ1-mediated cell signaling, we characterized some proteins binding to these domains in the MCF7 and MDA-MB-231 breast cancer cell lines. Of the several proteins that bind to glutathione-S-transferase-SH2/SH2/SH3, we identified an 85 kDa protein that binds to the SH3 domain of PLCγ1 as the guanine nucleotide exchange factor, p21-activated protein kinase-interacting exchange factor-a (βPix-a). βPix-a co-immunoprecipitated with PLCγ1 in breast cancer tissues extracts and in MCF7 and MDA-MB-231 cell extracts. In addition, PDGF-stimulated PLCγ1 activity was elevated in βPix-a-overexpressing NIH3T3 cells. Our results suggest that βPix-a binds to the Src homology domain 3 of PLCγ1 and promotes tumor growth in breast cancer by enhancing the activity PLCγ1. J. Cell. Biochem. 94: 1010–1016, 2005.

Key words: PLCγ1; SH3 domain; breast cancer tissues; βPix-a

The hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), by a specific phospholipase C (PLC) is one of the earliest key events in the regulation of a number of cellular functions by a variety of extracellular signaling molecules [Cockcroft and Thomas, 1992; Rhee and Choi, 1992; Berridge, 1993; Noh et al., 1995]. This reaction produces two intracellular messengers, diacylglycerol (DAG) and 1, 4, 5-inositol triphosphate (IP3), which mediates the activations of protein kinase C (PKC) and intracellular Ca^{2+} release, respectively. Phospholipase C-γ isozymes, members of the 10 strong families of mammalian PLC isozymes identified to date, contain a long sequence of ~400 amino acids. Phospholipase C-γ1 (PLCγ1) isozymes are composed of Src homology domain 2 and domain 3 (SH2 and SH3) and the pleckstrin homology (PH) domain, which is split by the SH domains (Fig. 1A). The involvement of PLCγ1 in tumorigenecity was suggested in a study which found that malignant tumors were formed by injecting PLCγ1-overexpressing cells into nude mice [Dunk et al., 1997]. It has also been reported that breast cancer tissues exhibit elevated PLCγ1 expression [Noh et al., 1998]. Many biological processes require specific protein–protein interactions, and these interactions are often achieved by modular domains which mediate

Abbreviations used: PLCγ1, phospholipase C-γ1; βPix-a, p21-interacting exchange factor-a; SH3, Src homology 3 domain; DH, Db1 homology domain; PH, pleckstrin homology domain; PAK, p21 activated kinase; GEF, guanine nucleotide exchange factor.

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protein–protein associations. SH2 and SH3 are examples of such domains that appear to play critical roles in the formation of signaling complexes. The SH2 domain mediates protein–protein associations by binding to phosphorylated tyrosine residues, whereas the SH3 domain mediates protein–protein associations by binding to proline-rich sequences [Oh et al., 1997]. It is well established that the SH2 domain of PLCγ1 activates receptor tyrosine kinase, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors, as direct binding modules. When cells are stimulated with growth factors, PLCγ1 is recruited to the cellular membrane, where the PLCγ1 substrate is located, through the binding of the SH2 domain of PLCγ1 to an activated receptor [Pawson, 1995; Rameh et al., 1995]. However, the exact mechanism of the SH3 domain of PLCγ1, which plays an important role in PLCγ1-mediated cell signaling, has not been characterized.

In order to gain a further insight into the functions of the SH domains of PLCγ1, we attempted to identify the proteins that bind to the SH2 and SH3 domains of PLCγ1. By using the glutathione-S-transferase (GST)-SH2/SH2/SH3 fusion protein of PLCγ1 as an affinity ligand, we detected an 85 kDa PLCγ1 SH3 domain-binding protein and identified it as the p21-activated protein kinase (PAK)-interacting exchange factor (βPix-a), a recently identified guanine nucleotide exchange factor for Rac1/Cdc42. Interaction between the two proteins was confirmed in extracts of breast cancer tissues and in extracts of MCF7, MDA-MB-231 cells. Furthermore, PDGF-stimulated PLCγ1 activity was elevated in the βPix-a-overexpressing NIH3T3 cell line. Together with previous findings of elevated expressions of PLCγ1 and βPix-a in breast cancer tissues [Noh et al., 1998; Ahn et al., 2003], our results suggest that βPix-a contributes to tumor growth in breast cancer by interacting with PLCγ1 and enhancing its activity.

MATERIALS AND METHODS

Materials

Phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, Triton X-100, DTT, and HEPES, were obtained from Sigma-Aldrich (Seoul, Korea). The Enhanced Chemiluminescence Detection (ECL) system was from Amersham, Seoul, South Korea. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit from PIERCE, Seoul, South Korea and anti-βPix-a antibody was used as in our previous experiment [Ahn et al., 2003].

Collection of Surgical Specimens

Tissues were obtained from surgical specimens submitted to the Surgical Pathology Service at Seoul National University Hospital, Seoul, South Korea. Fresh specimens of normal and carcinomatous tissues were immediately frozen in liquid nitrogen and stored at −80°C until required for immunoprecipitation and Western blot analysis.

Construction of GST-Fusion Proteins

Fusion proteins comparing various combinations of the SH2 and SH3 domains of PLCγ1 were fused with glutathione-S-transferase (GST) (Fig. 1A). For each protein (SH22 (PLCγ1 residues 545–759), SH3 (residues 798-850), and SH223 (residues 545-850)), polymerase reaction chain products flanked by *Bgl*II and *Eco*RI linkers were inserted into the *Bam*HI and *Eco*RI sites of pGEX-2TK vector (Amersham Pharmacia). The subcloned GST fusion vectors were used to transform *Escherichia coli* DH5α competent cells.

Purification of Binding Proteins and Immunochemical Procedures

All procedures were carried out at 4°C unless otherwise indicated. After an incubation period of 2 h at 37°C, the expression of GST fusion protein in transformed *E. coli* was induced with 0.1 mM isopropyl-D-thiogalato-pyranoside (IPTG), and cells were harvested by centrifugation (5,000*g* for 15 min). Then the cells were sonicated in Buffer A (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EGTA, and 1.5 mM PMSF) and centrifuged at 100,000*g* for 1 h. The obtained supernatant was mixed with 50% slurry of glutathione Sepharose 4B for 2 h, and the glutathione Sepharose 4B pellet was collected by centrifugation (5,000*g* for 15 min) and washed four times with 10 bed volumes of buffer A. Sepharose beads were used to pull down the binding proteins in the breast tissue and cell extracts.

Tissue samples were thawed in 10 ml of Buffer B (20 mM HEPES, pH 7.2, 200 mM sodium chloride, 1% Triton X-100, 2% cholic

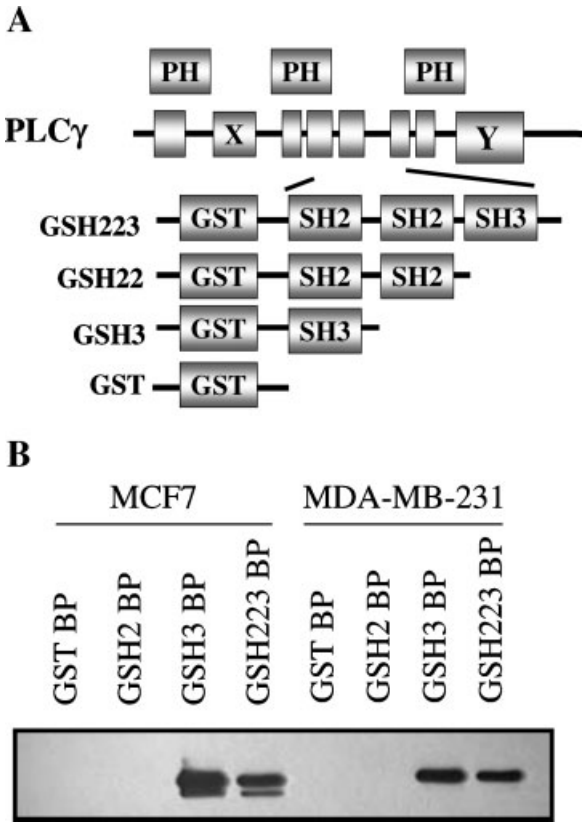

Fig. 1. **A**: Structures of PLCγ and GST fusion proteins. Schematic representation of fusion constructs containing the SH2 and SH3 domains of phospholipase C-γ1 (PLCγ1). GST-SH223 contains two SH2 domains and one SH3 domain. GST-SH22 contains two SH2 domains. GST-SH3 contains a SH3 domain. **B**: Binding of p21-interacting exchange factor-a (βPix-a) to different GST-fusion proteins containing the SH-domain of PLCγ1. Bacterially expressed GST-fusion proteins containing SH-domains of PLCγ1, as indicated, were immobilized on glutathione Sepharose, washed, and incubated with MCF7 and MDA-MB-231 extracts. The bead samples were then separated on 10% SDS–PAGE and analyzed by immunoblotting with anti-βPix-a antibodies.

acid, 1 mM EDTA, 1 mM EGTA, 0.1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF), homogenized three times in a Polytron homogenizer, and centrifuged at 100,000*g* for 1 h. For the affinity precipitation of the proteins which bind to the GST fusion proteins, the supernatants were incubated with fusion proteins for 3 h and then washed extensively with Buffer A.

Cell Culture and Transfection

NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. A day before transfection, NIH3T3 Cells were split onto 60 mm plates to allow them to reach 40%–80% confluence the next day and transfected with 5 μg of total plasmid DNA using Lipofectamine reagent (Life Technologies, Inc., Seoul, South Korea) according to the manufacturer's recommendations.

Immunoprecipitation and Immunoblot Analysis

For the immunoprecipitation of βPix-a and PLCγ1, breast tissues were homogenized in Buffer B. Protein content was determined using a BCA protein assay using bovine serum albumin as a standard. One milligram of the tissue homogenate was immunoprecipitated with βPix-a or PLCγ1 antibodies, and after incubation for 2 h in an ice bath, 40 μl of Protein G Sepharose was added. The immune complexes were recovered by centrifugation at 2,000*g* for 5 min. For the immunoblot analysis, immunoprecipitates were washed five times with 1 ml of buffer containing 50 mM Tris-HCl (pH 8.0), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), and 200 mM sodium chloride. Immune complexes were released by heating the sample at 95°C for 10 min with Laemmli sample buffer. After 8% SDS–polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose filters and incubated with βPix-a or PLCγ1 antibodies for 2 h. Immunoreactive bands were visualized using peroxidase conjugated goat anti-rabbit IgG antibody and detected using the ECL system.

Measurement of Phosphoinositide Hydrolysis by PLC

Cells were plated into 60 mm dishes at 5×10^5 cells per dish, grown for 1 day, and then labeled with *myo*-[2-^{3}H]inositol (1 μCi/ml) in inositol-free DMEM for 20 h. Subsequently, the labeled cells were washed and pretreated with 20 mM of LiCl for 15 min in DMEM containing 20 mM HEPES (pH 7.2) and 1 mg/ml bovine serum albumin. Stimulation was initiated by adding PDGF and terminated after 30 min by adding ice-cold 5% $HClO_4$. After incubation for a further 30 min in an ice bath, the extracts were centrifuged, diluted with distilled water, and applied to a Bio-Rad Dowex AG 1-X8 anion exchanger column. The column was washed with 10 ml of distilled water and then by 10 ml of 60 mM ammonium formate containing 5 mM of sodium tetraborate. The inositol phosphates

were then eluted using a solution containing 1M ammonium formate and 0.1M formic acid.

RESULTS

Binding Site of PLCγ1 to βPix-a

We previously reported the overexpression of PLCγ1 and βPix-a in human breast cancer tissues [Noh et al., 1998; Ahn et al., 2003] and we noticed that two proteins were associated with binding each other. To investigate the interaction between these two proteins further, we examined the binding of βPix-a to the SH domains of PLCγ1. Affinity matrices were prepared by immobilizing purified GST-PLCγ1 SH domain fusion proteins on glutathione-Sepharose beads (Fig. 1A), and this was used to pull-down βPix-a from the extracts of the MCF7 and MDA-MB-231 cell lines. After incubation and washing, the bead-bound proteins were analyzed by SDS–PAGE and by immunoblotting with anti-βPix-a antibodies. As shown in Figure 1B, βPix-a bound to GSH3 and GSH223 fusion proteins, whereas no binding to GSH22 fusion protein was detected in either MCF7 or MDA-MB-231 extract. This result indicates that βPix-a can interact with PLCγ1 via the SH3 domain of PLCγ1.

Expression Levels of PLCγ1 and βPix-a in Breast Cancer Tissue and Association Between Two Proteins In Vivo

To investigate whether PLCγ1 and βPix-a are associated with each other in vivo, we co-immunoprecipitated the two proteins from human breast cancer tissues. As previously reported, enhanced expressions of PLCγ1 and βPix-a were observed (Fig. 2A). Compared to normal breast tissue obtained from the same patients, the cancer tissue overexpressed these two proteins. To demonstrate the association between βPix-a and PLCγ1, βPix-a or PLCγ1 was immunoprecipitated from human breast cancer tissue lysates using their respective antibodies, and the presence of PLCγ1 or βPix-a in immunoprecipitates was determined by immunoblotting using specific antibodies. As shown in Figure 2B, PLCγ1 was detected in immunoprecipitates of βPix-a, and βPix-a was detected in immunoprecipitates of PLCγ1. This association between the two proteins was consistently found in all human breast cancer tissues used in the present experiments.

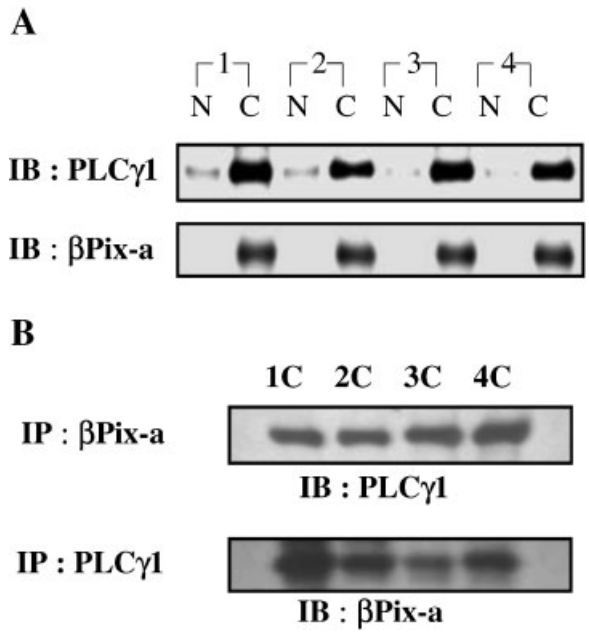

Fig. 2. **A**: Immunoblot analysis (IB) of βPix-a and PLCγ1 in normal breast tissue and breast cancer tissue. Normal and cancer tissue samples were homogenized with ice-cold homogenizing buffer. Ten milligrams of extracted proteins were separated on 10% SDS–PAGE, and transferred to a nitrocellulose membrane. Proteins were identified by immunoblot using anti-βPix-a and PLCγ1 antibody, as described in ''Materials and Methods.'' **Lanes**: N, normal tissue; C, cancerous tissue. **B**: The association between βPix-a and PLCγ1 in breast cancer tissue. Lysates of breast cancer tissues were immunoprecipitated (IP) with the indicated antibodies, as described in ''Materials and Methods.'' Immunoprecipitates were separated by 10% SDS–PAGE and blotted with the indicated antibodies. Results shown represent one of three independent experiments.

Enzymatic Activity of PLCγ1 in Overexpression of βPix-a

To examine the functional significance of the association between βPix-a and PLCγ1, we investigated the effect of βPix-a overexpression on the growth factor-mediated [inositol-^{3}H] PI(4, 5)P_2-hydrolyzing activity of PLC. Flag-tagged βPix-a transfected stable NIH3T3 cell lines were established as described above and this cell lines expressed more than twofold increased level of βPix-a than the control cells (Fig. 3A). The expression levels of endogenous βPix-a and PLCγ1 were affected by the exogenous expression of Flag-tagged βPix-a. As shown in Figure 3B, the basal levels of PLC activity were similar in both control and Flag-tagged βPix-a-expressing cell lines. However, the growth factor-dependent stimulation of PLC activity was enhanced in the Flag-tagged βPix-

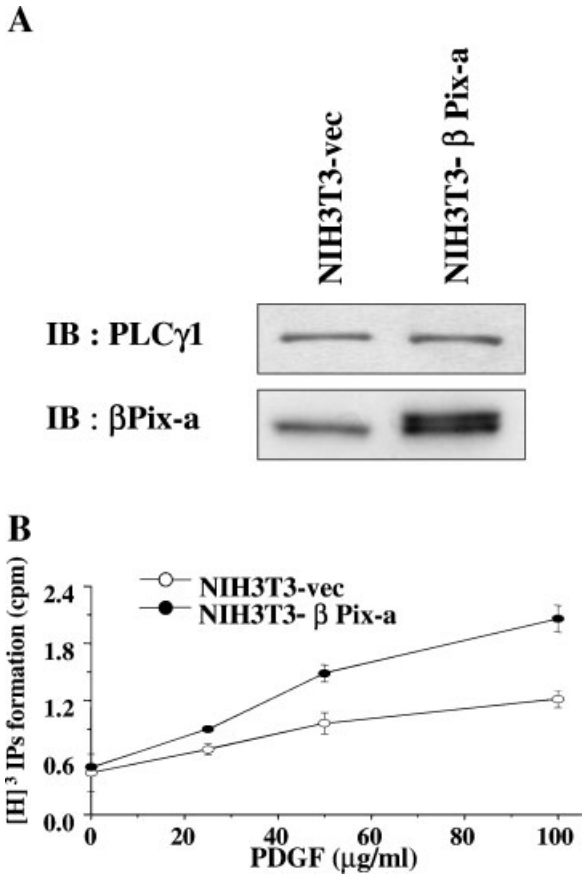

Fig. 3. **A**: Immunoblot analyses of βPix-a and PLCγ1 in Flag-tagged βPix-a transfected stable NIH3T3 cells and in control NIH3T3 cells. The expression levels of endogenous βPix-a and PLCγ1 were affected by the exogenous expression of Flag-tagged βPix-a. **B**: Effect of βPix-a on the activity of PLCγ1. Cells were cultured in 60 mm dishes at 5×10^5 cells per dish. The cells were then labeled with myo-[2-^{3}H]inositol (1 μCi/ml) in inositol-free DMEM for 20 h. Subsequently, the labeled cells were washed and pretreated with 20 mM LiCl for 15 min. Stimulation was initiated by adding PDGF for 30 min and terminated by adding ice-cold 5% $HClO_4$. Results shown represent one of three independent experiments.

a-expressing cell lines. Moreover, in response to stimulation with PDGF (100 μg/ml), the enzymatic activity of PLC increased twofold in control cell lines and fourfold in βPix-a-expressing cell lines (Fig. 3B). These results indicate that growth factor-dependent PLC activation can be stimulated by enhanced βPix-a expression. The significance of this observation is that binding between βPix-a and the SH3 domain of PLCγ1 might regulate the activity of PLCγ1, and that this binding between βPix-a and PLCγ1 may be involved in the regulatory signaling pathway.

DISCUSSION

The SH2 and SH3 domains of PLCγ1 are believed to play important regulatory and effector functions in intracellular signal transduction pathway by interacting with other proteins. Moreover, it is well established that the SH2 domains of PLCγ1 are acting as binding modules for activated receptor tyrosine kinases, like epidermal growth factor and platelet-derived growth factor receptor [Anderson et al., 1990; Kim et al., 1991; Bae et al., 1997]. In addition to receptor tyrosine kinases, several adaptor proteins, such as Grb2, Sos, and AP180 have been found to be associated with the SH2 domains of PLCγ1 [Seedorf et al., 1994; Khurana et al., 1997; Pei et al., 1997]. However, the identities of proteins binding to the SH3 domain of PLCγ1 are not well established. Here, we show that βPix-a binds to the SH3 domain of PLCγ1 in vitro, and confirm the association between these two proteins in human breast cancer tissues.

βPix-a is a recently identified guanine nucleotide exchange factor (GEF) for the small G proteins, Rac1 and Cdc42, which are critical regulators of actin cytoskeleton reorganization [Westwick et al., 1997; Daniels, 1999]. βPix-a can interact with several proteins like p21-activated protein kinase (PAK), and Git1 (G protein-coupled receptor kinase-interacting target 1) [Bagrodia et al., 1998; Manser et al., 1998]. It has a proline-rich region in its C-terminus that might serve as a binding site for SH3 domain-containing proteins. Thus, the binding of βPix-a to the SH3 domain of PLCγ1 suggests that PLCγ1 may interact with the proline-rich region of the βPix-a C-terminus.

The elevated expressions of both βPix-a and PLCγ1 in breast cancer tissues suggest that both proteins may have some role in tumorigenesis or in the maintenance of the tumor phenotype in breast tissues. The finding of overexpressed PLCγ1 in colorectal cancer [Riggins et al., 1995; Demtroder et al., 2002] and in breast cancer tissues supports this idea [Noh et al., 1998]. The activation of PLCγ1 by growth factors that stimulate cell proliferation suggests that the uncontrolled activation of PLCγ1 may contribute to the formation of malignant tumor phenotypes. Moreover, the role of βPix-a in malignant tumors has not been reported. However, the activations of Rac1 or Rac3, key downstream effectors of βPix-a, have been reported to

be required for Ras transformation and for the proliferation of breast cancer cells [Khosravi et al., 1995; He et al., 1998; Knaus et al., 1998; Cerk et al., 2001]. Therefore, the enhanced level of βPix-a in breast cancer tissues may contribute to cancer cell proliferation via the activation of its downstream effectors Rac1 or Rac3.

We found that the overexpression of βPix-a in NIH3T3 cells enhances the growth factor-dependent activation of PLC. These results suggest that the enhanced level of βPix-a expression in breast cancer tissues contributes to the proliferation of malignant cells by stimulating the enzymatic activity of PLCγ1, which is also overexpressed in breast cancer tissues. Although the exact mechanism of PLC activity stimulation through interaction with βPix-a is unknown, two mechanisms may be valid. First, βPix-a, which was reported to translocate to the cellular membrane upon PDGF stimulation, may recruit PLCγ1 to the cellular membrane where the enzyme's substrates are located. Second, the binding of βPix-a to the SH3 domain of PLCγ1 may convert PLCγ1, which has been proposed to be in an inactive conformation in the absence of stimulation by the intramolecular interaction, into its active conformation and thus contribute to the regulation of the cell growth and proliferation. However, these two mechanisms may not be mutually exclusive. It is possible that βPix-a binding converts PLCγ1 into an active conformation and that the active PLCγ1-βPix-a complex is then translocated to the membrane in the presence of PDGF. This model is consistent with the findings that the two proteins are associated with each other in the absence of PDGF and that PLC activity is stimulated in the presence of PDGF.

In this study, we suggest that βPix-a has a role in the proliferation of breast cancer cells through the stimulation of PLCγ1 activity via physical interaction. However, it is also possible that interaction between βPix-a and PLCγ1 may stimulate the GEF activity of βPix-a and activate downstream effectors such as Rac and Pak. Recently, it has been reported that PLCγ1 induces Rac activation and Iba1-dependent membrane ruffling; Iba1 is a calcium-binding protein that is involved in Rac-dependent membrane ruffling and phagocytosis [Fanger et al., 1997; Shin et al., 2002]. The involvement of PLCγ1 in cell migration and tumor cell invasion has also been reported [Kassis et al., 1999; Khoshyomn et al., 1999; Price et al., 1999]. Moreover, it is well documented that Rac activation is involved in both cell migration and cancer cell invasion [Obermeier et al., 1998; Zhou et al., 1998]. Therefore, it is possible that the recruitment of βPix-a to the cellular membrane via its association with PLCγ1 in a growth factor-dependent manner may play a role in PDGF-induced membrane ruffling and cell migration. Future experiments will clarify the role of the PLCγ1–βPix-a interaction in these contexts.

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