Activation of phospholipase C γ by PI 3-kinase-induced PH domain-mediated membrane targeting

M.Falasca¹, S.K.Logan, V.P.Leh²,
G.Baccante³, M.A.Lemmon⁴ and
J.Schlessinger⁵

Department of Pharmacology, NYU Medical Center, 550 First Avenue, New York, NY 10016 and ⁴Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6089, USA, ¹Department of Cell Biology and Oncology, Istituto di Ricerche Farmacologiche 'Mario Negri', Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro, CH, Italy, ³Institute of Pathophysiology, School of Medicine, G.D'Annunzio University, 66013 Chieti, Italy and ²University of Oulu, Kajaanintie 52 D, SF-90220, Oulu, Finland

⁵Corresponding author

Signaling via growth factor receptors frequently results in the concomitant activation of phospholipase C γ (PLC γ) and phosphatidylinositol (PI) 3-kinase. While it is well established that tyrosine phosphorylation of PLC γ is necessary for its activation, we show here that PLC γ is regulated additionally by the lipid products of PI 3-kinase. We demonstrate that the pleckstrin homology (PH) domain of PLC γ binds to phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃], and is targeted to the membrane in response to growth factor stimulation, while a mutated version of this PH domain that does not bind PtdIns(3,4,5)P₃ is not membrane targeted. Consistent with these observations, activation of PI 3-kinase causes PLC γ PH domain-mediated membrane targeting and PLC γ activation. By contrast, either the inhibition of PI 3-kinase by overexpression of a dominant-negative mutant or the prevention of PLC γ membrane targeting by overexpression of the PLC γ PH domain prevents growth factor-induced PLC γ activation. These experiments reveal a novel mechanism for cross-talk and mutual regulation of activity between two enzymes that participate in the control of phosphoinositide metabolism.

Keywords: PH domains/phosphoinositides/phospholipase C γ /PI 3-kinase

Introduction

Phosphoinositide metabolism plays an important role in the control of diverse cellular processes such as cell proliferation and differentiation, cell survival, and control of cell shape and cell migration (Divecha and Irvine, 1995; Carpenter and Cantley, 1996). A large variety of extracellular signals stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] by activation of phospholipase C (PLC) isoforms (Cockcroft and Thomas, 1992; Lee and Rhee, 1995). It is now well established that the two products of this reaction, the intracellular second messengers inositol 1,4,5-trisphos-

phate [Ins(1,4,5)P₃] and diacylglycerol, mediate the release of Ca²⁺ from intracellular stores and activation of protein kinase C (PKC), respectively (Berridge, 1993; Nishizuka, 1995). The critical role of these pathways in mammalian growth and development is underscored by the recent finding that targeted deletion of PLC γ 1 causes embryonic lethality in mice (Ji *et al.*, 1997).

The PLC γ 1 and PLC γ 2 isoforms are activated by growth factor stimulation of both receptor and non-receptor protein tyrosine kinases (Cockcroft and Thomas, 1992; Rhee and Bae, 1997). Structurally, each PLC γ isoform has a putative pleckstrin homology (PH) domain at its amino-terminus, followed by the two conserved parts of the catalytic domain (themselves separated by two tandem SH2 domains), an SH3 domain and a putative split PH domain. Upon growth factor stimulation, the SH2 domains of PLC γ bind to tyrosine autophosphorylation sites in growth factor receptors, leading to tyrosine phosphorylation and stimulation of PLC γ enzymatic activity. It has been demonstrated that phosphorylation of Tyr783 of PLC γ is essential for stimulation of enzymatic activity in NIH-3T3 cells stimulated with platelet-derived growth factor (PDGF) (Kim *et al.*, 1991). Yet, in many cases, the tyrosine phosphorylation of PLC γ does not correlate with Ins(1,4,5)P₃ production; certain extracellular signals lead to weak tyrosine phosphorylation of PLC γ and extensive production of Ins(1,4,5)P₃, while other signals cause strong tyrosine phosphorylation of PLC γ but only low levels of Ins(1,4,5)P₃ production (Rhee and Bae, 1997). Therefore, additional mechanisms, independent of PLC γ tyrosine phosphorylation, are likely to play an important role in the control of PLC enzymatic activity in response to extracellular stimuli.

There is now good evidence that PH domains of signaling molecules are involved in their regulated targeting to cell membranes (Lemmon *et al.*, 1995, 1996). PH domains are protein modules of ~120 amino acids that have been identified in >100 different proteins (Haslam *et al.*, 1993; Mayer *et al.*, 1993; Musacchio *et al.*, 1993; Gibson *et al.*, 1994). These include protein kinases (Akt/PKB/Rac, Btk and the β -adrenergic receptor kinase, β ARK), guanine nucleotide exchange factors for small GTPases (e.g. Dbl and Sos), PLC isoforms (β , γ and δ) and GTPase-activating proteins (e.g. Ras-GAP). All PH domain-containing proteins appear to have a functional requirement for membrane association (Gibson *et al.*, 1994), and several recent studies indicate that PH domains play a role in directing this membrane targeting (Cifuentes *et al.*, 1993; Davis and Bennett, 1994; Lombardo *et al.*, 1994; Paterson *et al.*, 1995; Wang *et al.*, 1996; Zheng *et al.*, 1996; Chen *et al.*, 1997; Lemmon *et al.*, 1997). Efforts to identify the PH domain ligands responsible for membrane binding have led interest toward both protein-

Fig. 1. (a) Fluorescence micrographs of cells expressing GST-PLC γ_1 -PH (A–C) or GFP-PLC γ_1 -PH (D–F) fusion proteins. Confocal immunofluorescence microscopy of IMR33 cells microinjected with GST alone (A) or GST-PLC γ_1 -PH (B and C) and grown in the absence of serum. Cells were unstimulated (B) or stimulated with fetal bovine serum (C). A diffuse cytoplasmic staining can be seen in starved cells (B). In stimulated cells (C), a distinct accumulation of fluorescence can be seen in the lamellar region of the cell (arrows). Confocal fluorescence micrograph of L6 cells expressing GFP alone (D) or GFP-PLC γ_1 -PH fusion protein (E and F). In starved cells (E), a diffuse cytoplasmic fluorescence is seen. In serum-stimulated cells (F), a distinct accumulation of fluorescence is seen in the lamellar region of the cell (arrows). (b) Fluorescence micrographs of L6 cells expressing GFP-PLC γ_1 -PH at the indicated time points following PDGF stimulation. Bar, 10 μ m.

protein and protein–lipid interactions (Lemmon *et al.*, 1996).

Several possible PH domain targets have been proposed for different PH domains, including the $\beta\gamma$ subunits ($G_{\beta\gamma}$)

of heterotrimeric G proteins (Touhara *et al.*, 1994; Tsukuda *et al.*, 1994), PKC (Konishi *et al.*, 1994; Yao *et al.*, 1994) and phosphoinositides (Harlan *et al.*, 1994; Garcia *et al.*, 1995; Hyvönen *et al.*, 1995; Lemmon *et al.*, 1995; Pitcher

Table I. Conditions affecting cellular distribution of PLC γ -PH mutants or isoforms

PH domain	Treatment	Cell membrane	Intracellular
PLC γ_1	untreated	–	+
PLC γ_1	serum or PDGF	+	–
PLC γ_1 6/7	serum or PDGF	+	–
PLC γ_1 3/4	serum or PDGF	–	+
PLC δ_1	untreated, serum, PDGF	+	–
PLC γ_1	PDGF + Wortmannin	–	+
PLC γ_1	PDGF + DN PI 3-kinase	–	+
PLC γ_1	activated PI 3-kinase	+	–
PLC γ_1	inactive PI 3-kinase	–	+

The table summarizes the cellular distribution of various PH domain isoforms or mutants following a variety of cellular treatments. Experiments using serum, PDGF or Wortmannin (the top six lines of the table) were done in L6, COS and IMR33 cells. Experiments utilizing dominant-negative, constitutively active or kinase-inactive forms of PI 3-kinase were done in COS cells and HeLa cells.

et al., 1995). The most convincing cases for highly specific, physiologically relevant PH domain targets are seen with PLC δ_1 PH domain binding to PtdIns(4,5)P₂ (Garcia *et al.*, 1995; Lemmon *et al.*, 1995) and binding of the PH domain of the Ser/Thr kinase Akt/PKB/Rac to PtdIns(3,4)P₂ (Franke *et al.*, 1997; Frech *et al.*, 1997; Klippel *et al.*, 1997). The PH domain of PLC δ_1 is both necessary and sufficient for binding of the enzyme to the plasma membrane (Paterson *et al.*, 1995). It is connected to the rest of the enzyme by a flexible linker (Essen *et al.*, 1996), and tethers intact PLC δ_1 to membranes containing its substrate PtdIns(4,5)P₂, thus allowing processive PtdIns(4,5)P₂ hydrolysis (Cifuentes *et al.*, 1993; Lemmon *et al.*, 1996). By competing with PtdIns(4,5)P₂ for binding to the PH domain, the PLC δ_1 product Ins(1,4,5)P₃ removes this tether and reduces catalytic efficiency (Kanematsu *et al.*, 1992; Cifuentes *et al.*, 1994).

Although their precise roles and ligands are not well defined, it is clear that PH domains are important for the biological activity of proteins involved in intracellular signaling. PLC δ_1 and Akt/PKB/Rac provide two examples, and a third is provided by the Btk PH domain, in which mutations cause X-linked agammaglobulinemia that results from defects in B-cell signaling (Mattsson *et al.*, 1996). Here we describe studies of the role of the putative PLC γ_1 -PH present at the amino-terminus of the molecule (Parker *et al.*, 1994). PLC γ_1 is well known to be recruited to the plasma membrane by activated receptor tyrosine kinases (Margolis *et al.*, 1990). We find that the PH domain of PLC γ_1 is targeted to the plasma membrane, in response to serum and growth factor stimulation. More specifically, we find that PLC γ_1 -PH binds to PtdIns(3,4,5)P₃ and that phosphatidylinositol (PI) 3-kinase activity plays an important role in PLC γ activation and membrane localization in multiple cell types.

Results and discussion

In order to explore the possible role of the PH domain in signal-dependent localization of PLC γ_1 , a glutathione-S-transferase (GST) fusion protein containing the PLC γ_1 PH domain was microinjected into serum-starved IMR33 cells. The localization of the protein was analyzed using confocal

A

B

Fig. 2. Binding specificity of GST-PLC γ_1 -PH domain. GST fusion proteins attached to beads were incubated with a deacylated lipid extract from PDGF-stimulated [³H]inositol-labeled IMR33 fibroblasts (A). The beads were washed three times and the glycerophosphoinositols that remained associated were eluted with 0.1 M HCl and analyzed by HPLC as described (Logan *et al.*, 1997). The amount of radioactivity of each compound was determined as a percentage of the total radioactivity present in the extract, and then represented as a percentage of gPI(3,4,5)P₃ bound. Under the same conditions, no association of radioactivity was detected when GST alone was used. The data shown are means of quadruplicate experiments with error bars indicating standard deviation. The relative abundance of different inositol phospholipids present in the extract were approximately: PI, 88.8%; PI(3)P, 0.51%; PI(4)P, 5.84%; PI(3,4)P₂, 0.10%; PI(4,5)P₂, 3.82%; PI(3,4,5)P₃, 0.12%. (B) Ligand displacement of [³²P]gPI(3,4,5)P₃ binding to GST-PLC γ_1 -PH by Ins(1,3,4,5)P₄. [³²P]gPI(3,4,5)P₃ (100 000 c.p.m.) was incubated with GST-PLC γ_1 -PH and the indicated concentrations of Ins(1,3,4,5)P₄. The values are expressed as the percentage of radioactivity bound in the absence of competitor and are means of quadruplicate experiments. Error bars show standard deviation.

fluorescence microscopy in fixed cells incubated with polyclonal anti-GST antibodies followed by rhodamine-conjugated anti-goat antibodies. As shown in Figure 1aB, the fusion protein is homogeneously dispersed in the cell cytoplasm in the absence of serum stimulation. However, within 5 min of stimulation with either serum (Figure 1aC) or PDGF (Table I), the PH domain was recruited to the plasma membrane. No membrane recruitment was seen when GST alone was used (Figure 1aA). These data

Fig. 3. (A) Primary structures of PLC γ_1 -PH mutants. Mutations in the 3/4 and 6/7 loops are shaded. (B) Specificity of [³²P]gPI(3,4,5)P₃ binding to GST PLC γ_1 -PH compared with other GST-PH fusion proteins.

show that plasma membrane localization of PLC γ_1 -PH occurs only in stimulated cells.

The dynamics of PLC γ_1 -PH localization to the plasma membrane were investigated using the PLC γ_1 -PH fused to green fluorescent protein (GFP) and transiently expressed in IMR33 fibroblasts, L6 myoblasts, HeLa and COS-1 cells. Due to its intrinsic fluorescence, the subcellular localization and dynamics of the GFP fusion protein can be observed in living cells without the fixing and staining procedures required for indirect immunofluorescence of, for example, GST fusion proteins. Thus, alterations in GFP fusion protein localization resulting from different cellular treatments can be monitored directly, allowing analysis of the dynamic localization events occurring in living cells. Cells were transfected with a vector directing the expression of a GFP-PLC γ_1 -PH fusion protein. As with the microinjection studies described above, GFP-PLC γ_1 -PH was found to be present only in the cytoplasm when cells were starved of serum following transfection (Figure 1aE). However, when cells were stimulated with serum (Figure 1aF) or PDGF (Table I), the GFP-PH fusion protein was seen to associate with

the plasma membrane within 3–5 min. In control cells expressing free GFP, an intense cytoplasmic fluorescence was seen especially around the nucleus, but no membrane localization was observed in either starved or unstimulated cells (Figure 1aD).

Use of the GFP fusion protein allowed us to follow the time course of PLC γ_1 -PH membrane association in living cells. Membrane localization was first seen after 3–5 min, and became pronounced at 10 min, appearing as a distinct, rim-like fluorescence along the leading edge of the cell. During the subsequent 10–15 min, highly dynamic changes in this staining pattern were seen; it first became associated with ruffle-like structures, typically located at some distance from the growth substratum. Distinct fluorescent lines of the ruffles, probably representing upward-bent membrane veils, underwent rapid changes in their orientation such that 90° turns could be seen within 1 min (Figure 1b). Extensive membrane ruffling was apparent at 27 min, while from 30 min onwards there was a gradual disappearance of the distinct membrane-associated staining. Thus, PLC γ_1 -PH recruitment to the plasma membrane in these cells is transient, dependent upon growth factor

Fig. 4. Effect of PI 3-kinase inhibition or activation on membrane localization of GFP-PLC γ_1 -PH. Confocal fluorescence micrograph of serum-starved COS-1 cells expressing GFP-PLC γ_1 -PH fusion proteins, stimulated with 25 nM PDGF for 5 min (A), in the presence of co-transfected dominant-negative p85 (Kotani *et al.*, 1994) (B) or pre-treated with 100 nM Wortmannin for 10 min (C). (D) Confocal fluorescence micrographs of serum-starved COS-1 cells that had been co-transfected with vectors expressing GFP-PLC γ_1 -PH fusion protein and membrane-targeted activated p110 β (PI 3-kinase). All p110 variants have been described and characterized (Logan *et al.*, 1997). In this and all presented transfection experiments, the total amount of DNA in each transfection was held constant by the addition of empty vector. Bar, 10 μ m.

stimulation, and is seen especially in the highly dynamic membrane areas, correlating with the enzymatic activity of PLC γ after growth factor stimulation.

Specific binding of the PLC γ -PH to PtdIns(3,4,5)P $_3$

Previous studies have demonstrated specificity of phospholipid binding by PH domains. For example, the PH domain of PLC δ_1 binds with high affinity and specificity to PtdIns(4,5)P $_2$, (Ferguson *et al.*, 1995; Lemmon *et al.*, 1995) while the PH domain of GRP1 binds to PtdIns(3,4,5)P $_3$ and Akt/PKB binds to PtdIns(3,4)P $_2$ (Franke *et al.*, 1997; Frech *et al.*, 1997; Klarlund *et al.*, 1997). Binding of PtdIns(3,4,5)P $_3$ to PH domains has been reported for other proteins containing PH domains, such as Btk (Salim *et al.*, 1996; Kojima *et al.*, 1997) and Akt-1 (James *et al.*, 1996). To characterize the lipid-binding properties of the PLC γ_1 -PH, we incubated deacylated lipid extracts from PDGF-stimulated [3 H]inositol-labeled IMR33 fibroblasts with beads to which the GST-PLC γ_1 -PH was attached. The

associated glycerophosphoinositides (gPIs) were eluted and analyzed by HPLC. This is a rigorous test of lipid binding, since the ratio of lipids provided in the extract is physiological. The experiment presented in Figure 2A shows that the PH domain of PLC γ_1 binds strongly to PtdIns(3,4,5)P $_3$. Only small quantities of PtdIns(3,4)P $_2$ and PtdIns(3)P were bound. The binding of deacylated PtdIns(3,4,5)P $_3$ was displaced by the water-soluble head group Ins(1,3,4,5)P $_4$ at a half-maximal concentration of 1–2 μ M (Figure 2B). The binding affinity appears to be similar to that of the GRP1 PH domain, where the concentration of Ins(1,3,4,5)P $_4$ that inhibited binding of PtdIns(3,4,5)P $_3$ half maximally was 3–8 μ M (Klarlund *et al.*, 1997).

Binding of wild-type and mutants PLC γ -PH to plasma membrane and to PtdIns(3,4,5)P $_3$

To analyze further the specificity of the PLC γ_1 -PH membrane localization and binding to PtdIns(3,4,5)P $_3$, and in

Fig. 5. (A) Effect of dominant-negative p85, Wortmannin and GFP-PLC γ_1 -PH on enzyme activity of PLC γ_1 in COS-1 cells. Cells were transiently transfected with GFP-PLC γ_1 -PH or dominant-negative p85. The effect of Wortmannin was evaluated by pre-treating serum-starved COS-1 cells for 10 min with 100 nM Wortmannin. Cells were stimulated with 25 nM PDGF for 3 min. The levels of [³H]inositol-labeled Ins(1,4,5)P₃ were determined as described (Falasca *et al.*, 1997). PDGF increased the basal levels of Ins(1,4,5)P₃ 2- to 3-fold. The data presented are the mean $\pm$ SE of four separate experiments performed in duplicate. (B) Top: a phosphotyrosine immunoblot of lysates from cells which were not stimulated (lane 1) or stimulated with PDGF (lane 2), in the absence or presence of Wortmannin (lane 3). Cell lysates were immunoprecipitated with anti-PLC γ antibodies, separated by gel electrophoresis, transferred to nitrocellulose and probed with anti-phosphotyrosine antibodies. Bottom: the same blot was stripped and reprobed with anti-PLC γ antibodies.

order to determine which portion of the PH domain is important for ligand and membrane binding, we analyzed the binding properties of several PH domain mutants. The mutations are reported schematically in Figure 3A. From structural analysis of PLC δ_1 PH domain binding to Ins(1,4,5)P₃ and PtdIns(4,5)P₂, it has been shown that three loops separating β -strands in PH domains (the β_1/β_2 , β_3/β_4 and β_6/β_7 loops) are particularly variable

in both length and sequence, and are critical for ligand binding. Ins(1,4,5)P₃ contacts only these loops in its high-affinity complex with the PLC δ_1 PH domain (Ferguson *et al.*, 1995; Lemmon *et al.*, 1995). We individually replaced two of the variable loops of the PLC γ_1 -PH with the equivalent loops of the dynamin-1 PH domain, which does not localize to the membrane in our studies (data not shown). Figure 3B compares PtdIns(3,4,5)P₃ binding to mutated PLC γ_1 -PH with that to the wild-type domain. Mutation of the β_6/β_7 loop had no effect upon ligand binding of PLC γ_1 -PH, while replacement of the β_3/β_4 loop with the aligned dynamin-1 PH domain sequences completely abolished ligand binding. In agreement with this result, mutation of the β_6/β_7 loop had no effect on membrane localization, while mutation of the β_3/β_4 loop abolished membrane localization of the PLC γ_1 PH domain (Table I). These results indicate that PLC γ_1 -PH binding to PtdIns(3,4,5)P₃ and to the plasma membrane is sequence specific, and further implicate the β_3/β_4 loop in key interactions, as seen for Ins(1,4,5)P₃ binding to PLC δ_1 .

Effect of Wortmannin and dominant-negative PI-3 kinase on PLC γ -PH plasma membrane localization

The experiments presented above indicate that PLC γ interacts via its PH domain with PtdIns(3,4,5)P₃, the major lipid product of activated PI 3-kinase (Traynor-Kaplan *et al.*, 1988; Auger *et al.*, 1989; Stephens *et al.*, 1991). Therefore, several approaches were taken to investigate the role of PI 3-kinase in the translocation and activation of PLC γ . We first tested the effect of PI 3-kinase inhibition on the cellular distribution of the PLC γ_1 -PH using a dominant-interfering mutant of p85 (the regulatory subunit of PI 3-kinase) and the PI 3-kinase inhibitor Wortmannin. The experiments presented in Figure 4 and Table I show that either expression of dominant-interfering p85 or treatment with Wortmannin blocked serum- and PDGF-induced membrane localization of the PLC γ_1 -PH, further indicating that the lipid products of PI 3-kinase serve as targets of the PLC γ_1 -PH in living cells.

Activated PI 3-kinase-induced PLC γ -PH plasma membrane localization

Next we examined the effect of expression of a constitutively activated PI 3-kinase on the cellular localization of the GFP-PLC γ_1 -PH. Expression of membrane-targeted p110 β leads to accumulation of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ (Logan *et al.*, 1997). In cells that overexpress this constitutively active PI 3-kinase mutant, the PH domain of PLC γ_1 is targeted to the plasma membrane even in serum-starved cells (Figure 4D and Table I). By contrast, membrane localization of the PLC γ PH domain is not seen in cells that express a membrane-targeted, kinase-inactivated form of PI 3-kinase (Table I), providing additional evidence that accumulation of 3' phosphorylated phosphoinositides in the cell membrane generates binding sites for the PH domain of PLC γ . Protein expression of the membrane-targeted active p110 β , the kinase-inactivated membrane-targeted p110 β and the dominant-interfering p85 was verified by immunoblot analysis in each case. Taken together, the experiments described above demonstrate that PtdIns(3,4,5)P₃ functions as a specific ligand for the PH domain of PLC γ_1 at the cell membrane.

Fig. 6. Cross-talk between PLC γ_1 and PI 3-kinase. Both PI 3-kinase and PLC γ_1 are recruited to activated growth factor (GF) receptors following stimulation. PI 3-kinase and PLC γ_1 bind to specific phosphotyrosine residues in activated growth factor receptors via their SH2 domains. In addition, PI 3-kinase generates PI(3,4,5)P₃ which binds to the PH domain of PLC γ_1 leading to translocation of PLC γ_1 to the plasma membrane concomitant with tyrosine phosphorylation and activation.

Effect of the PH domain on PLC γ enzyme activity

To determine the effect of membrane localization on PLC γ_1 enzymatic activity, Ins(1,4,5)P₃ production was examined in PDGF-stimulated COS-1 cells and IMR33 cells in the presence of Wortmannin, and also in COS-1 cells that overexpress the PLC γ_1 PH domain or a dominant-interfering mutant of p85. The experiment presented in Figure 5A shows that PDGF-induced Ins(1,4,5)P₃ production is reduced by ~65% in cells that overexpress the PH domain or are treated with 100 nM Wortmannin. Approximately 90% inhibition of PDGF-induced Ins(1,4,5)P₃ production was recorded in cells overexpressing the dominant-interfering mutant of PI 3-kinase p85. By contrast, overexpression of the PH domain mutant that does not localize to the plasma membrane or bind to PtdIns(3,4,5)P₃ (3/4 mutant) did not affect PDGF-induced stimulation of Ins(1,4,5)P₃ production (data not shown), demonstrating that the inhibition induced by the wild-type PH domain is specific. Interestingly, Wortmannin treatment did not influence the ability of PDGF to induce tyrosine phosphorylation of PLC γ_1 (Figure 5B), indicating that PI 3-kinase does not regulate the activity of PLC γ at the level of tyrosine phosphorylation.

The experiments presented here demonstrate that growth factor-induced generation of PtdIns(3,4,5)P₃ leads to translocation of PLC γ_1 to the plasma membrane, a step that increases the local concentration of the enzyme in the vicinity of its substrate PtdIns(4,5)P₂, probably leading to

more efficient substrate hydrolysis. Activation of PLC γ_1 in response to extracellular signals involves several regulatory steps. Following growth factor stimulation, receptor tyrosine kinases undergo autophosphorylation on tyrosine residues, which function as docking sites for the SH2 domains of PLC γ_1 , thus providing one mechanism for targeting the enzyme to the cell surface. Membrane association is stabilized further by binding of the PH domain of PLC γ to PtdIns(3,4,5)P₃ molecules in the membrane that are generated by PI 3-kinase in response to growth factor stimulation (Figure 6). Recent experiments by Bae *et al.* (1988) indicate that the SH2 domains of PLC γ_1 may also contribute to its binding to PtdIns(3,4,5)P₃, thus providing an additional anchor in the plasma membrane. Thus, several targeting signals appear to act together to generate a stable anchor for PLC γ in the plasma membrane where its substrate is found. An additional step in the activation of PLC γ involves tyrosine phosphorylation of PLC γ_1 mediated by either receptor or non-receptor protein tyrosine kinases, which leads to activation of PLC γ_1 by a poorly understood allosteric mechanism.

A similar molecular scenario was described recently for growth factor-induced activation of the Ser/Thr kinase Akt/PKB. It has been shown that this kinase is targeted to the plasma membrane in response to growth factor stimulation by means of the Akt/PKB PH domain binding to PtdIns(3,4)P₂. This brings Akt/PKB in proximity to a membrane-bound protein kinase which phosphorylates and

activates Akt (Alessi *et al.*, 1997; Stokoe *et al.*, 1997). In this case, Akt/PKB is activated by the concerted action of PI 3-kinase and a Ser/Thr kinase. Here we demonstrate that PLC γ_1 is activated by PI 3-kinase acting in concert with a receptor or non-receptor protein tyrosine kinase. In the case of PLC γ , the activity of an enzyme that plays a crucial role in phosphoinositide metabolism is regulated by another enzyme that plays a key role in the same metabolic process (PI 3-kinase), thus revealing a previously unappreciated connection within the intricate circuitry that takes place in the control of this family of second messengers.

Materials and methods

Construction of expression plasmids

The cDNA fragments corresponding to the PH domains from rat PLC- γ_1 (residues 14–150) and rat PLC- δ_1 (residues 11–140) were amplified using PCR. PCR products were digested with *Bam*HI alone (for PLC γ_1 -PH) or *Bam*HI plus *Eco*RI (for PLC δ_1 -PH), depending on the sites present in the oligonucleotides used for PCR, and were ligated into the appropriately digested pGEX-2T bacterial expression vector to direct expression of GST-PH domain fusion proteins. Mutated PH domains were generated by four-primer PCR mutagenesis, as previously described (Artalejo *et al.*, 1997). The DNA sequence of each PH domain insert was verified by dideoxynucleotide sequencing.

GFP expression vector

cDNA encoding the different PH domains was subcloned (in-frame with GFP) into the GFP fusion protein expression vector pEGFP-C1 (Clontech), using the *Bgl*II and *Bam*HI sites for PLC γ_1 -PH and the *Bgl*II and *Eco*RI sites for PLC δ_1 -PH. The sequence of each insert was verified by dideoxynucleotide sequencing.

Expression and purification of PH domains

The pGEX-2T plasmid containing the wild-type or mutated PH domains was transformed into *Escherichia coli* DH5 α . Cells were grown to an OD₆₀₀ of 0.3–0.4, and induced with 1 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG) (Boehringer Mannheim) for 3 h at 37°C (for PLC δ_1 -PH), or for 4–7 h at 25°C (for PLC γ_1 -PH and mutants). After pelleting, cells were lysed by sonication in 50 mM Tris, pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol (DTT), containing protease inhibitors [1 mM phenylmethylsulfonylfluoride (PMSF), 10 μ g/ml leupeptin and 10 μ g/ml aprotinin]. Triton X-100 was added to 1% in the lysate and particulates were removed by centrifugation for 20 min at 16 000 r.p.m. in an SS34 (Sorvall) rotor. The clarified lysate was incubated with glutathione-agarose beads (Sigma) for 1 h at 4°C, washed three times with ice-cold phosphate-buffered saline (PBS) containing 1 mM DTT plus protease inhibitors, and protein was stored at 4°C bound to the glutathione-agarose beads. For microinjection studies, protein was eluted from the beads by incubation with 15 mM reduced glutathione in PBS, and the eluted protein was buffer-exchanged into microinjection buffer (PBS) using a Centricon-10 (Amicon).

Cell culture and transfection

IMR33, L6, HeLa and COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were plated onto gridded glass coverslips. Cells were starved in 0.5% FBS for 16–24 h in DMEM. GST fusion proteins (0.5–2 μ g/ml) in PBS were then microinjected into the cytoplasm of cells. At 2–3 h after microinjection, cells were serum stimulated with 20% FBS in DMEM for 5–10 min, or PDGF stimulated with 25 ng/ml for 5–10 min. The transfection of GFP fusion protein vectors was done with cells grown on glass coverslips in 2.5 cm dishes using 2 μ g of plasmid DNA and 5 μ l of Lipofectamine (Gibco/BRL) according to the manufacturer's instructions. Following transfection, cells were serum starved in 0.5% FBS overnight.

Microinjection

Cells were injected in HEPES-buffered culture medium at room temperature using a Zeiss (Oberkochen, Germany) Axiovert 35 microscope, an Eppendorf (Hamburg, Germany) micromanipulator 5170, a microinjector 5242 and Sutter capillaries (Novato, CA). The holding pressure was

30 hPa, the injection pressure was 80 hPa and the duration of the injection was 200 ms. The concentration of microinjected proteins was 0.5–2 mg/ml in PBS. After microinjection, cells were incubated in DMEM at 37°C.

Indirect immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and were then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. The coverslips were incubated for 1 h at room temperature with the primary antibody (goat anti-GST antibody), followed by a 30 min incubation with biotin-SP-conjugated donkey anti-goat IgG and Cys 3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), 30 min each. The stained cells were analyzed by both conventional fluorescence microscopy under a Zeiss microscope and confocal microscopy. For confocal microscopy, cells were viewed using an upright SARAstro 2000™ CLSM (Molecular Dynamics).

Observations on living cells transfected with GFP fusion constructs were done by using a Multiprobe 2001™ [CLSM Microscope (Molecular Dynamics)] equipped with an inverted microscope. For that purpose, 25 mm coverslips were placed, cell-side up, in an attofluor cell chamber (Molecular Probes). After adding serum or PDGF, the cells were scanned at a fixed focal level at 1 min intervals.

Analysis of [3 H]inositol phospholipids

For inositol metabolites evaluation, cells grown in 6-well plates were labeled for 24 h in medium 199 containing *myo*-[3 H]inositol (6 μ Ci/ml). Following the labeling with *myo*-[3 H]inositol, all cells were washed once with PBS and pre-incubated for 10 min in 2 ml of PBS containing 10 mM LiCl (pH 7.4) at 37°C prior to the addition of the agonist. The cells were then extracted with methanol/1 M HCl/chloroform 1:1:1 and analyzed by high-performance liquid chromatography (HPLC) as described (Falasca *et al.*, 1997).

Acknowledgements

M.F. is supported by the Italian Association for Cancer Research (AIRC, Milan, Italy). M.A.L. is a Damon Runyon Scholar (DRS-05).

References

- Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R.J., Reese, C.B. and Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B α . *Curr. Biol.*, **7**, 261–269.
- Artalejo, C.J., Lemmon, M.A., Schlessinger, J. and Palfrey, H.C. (1997) A specific role for the PH domain of dynamin-1 in the regulation of rapid endocytosis. *EMBO J.*, **16**, 1565–1574.
- Auger, K.R., Serunian, L.A., Soltoff, S.P., Libby, P. and Cantley, L.C. (1989) PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell*, **57**, 167–175.
- Bae, Y.S., Cantley, L.C., Chen, C.-S., Seung-Ryul, K., Kwon, K.-S. and Rhee, S.G. (1998) Activation of phospholipase C- γ by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.*, in press.
- Berridge, M.J. (1993) Inositol triphosphate and calcium signaling. *Nature*, **361**, 315–325.
- Carpenter, C.L. and Cantley, L.C. (1996) Phosphoinositide 3-kinase and the regulation of cell growth. *Biochim. Biophys. Acta*, **1288**, M11–M16.
- Chen, R.-H., Corbalan-Garcia, S. and Bar-Sagi, D. (1997) The role of PH domain in the signal-dependent membrane targeting of Sos. *EMBO J.*, **16**, 1351–1359.
- Cifuentes, M.E., Delaney, T. and Rebecchi, M.J. (1994) D-*myo*-inositol 1,4,5-trisphosphate inhibits binding of phospholipase C- δ_1 to bilayer membranes. *J. Biol. Chem.*, **269**, 1945–1994.
- Cockcroft, S. and Thomas, G.M. (1992) Inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. *Biochem. J.*, **288**, 1–14.
- Davis, L.H. and Bennett, V. (1994) Identification of two regions of β_G spectrin that bind to distinct sites in brain membranes. *J. Biol. Chem.*, **269**, 4409–4416.
- Divecha, N. and Irvine, R.F. (1995) Phospholipid signaling. *Cell*, **80**, 269–278.
- Essen, L.-O., Perisic, O., Cheung, R., Katan, M. and Williams, R. (1996) Crystal structure of a mammalian phosphoinositide-specific phospholipase C δ . *Nature*, **380**, 595–602.

- Falasca, M., Carvelli, A., Iurisci, C., Qiu, R.-G., Symons, M.H. and Corda, D. (1997) Fast receptor-induced formation of glycerophosphoinositol-4-phosphate, a putative novel intracellular messenger in the Ras pathway. *Mol. Biol. Cell*, **8**, 443–453.
- Ferguson, K.M., Lemmon, M.A., Schlessinger, J. and Sigler, P.B. (1995) Structure of a high affinity complex between inositol-1,4,5-trisphosphate and a phospholipase C pleckstrin homology domain. *Cell*, **83**, 1037–1046.
- Franke, T.F., Kaplan, D.R., Cantley, L.C. and Toker, A. (1997) Direct regulation of the *Akt* proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science*, **275**, 665–668.
- Frech, M., Andjelkovic, M., Reddy, K.K., Falck, J.R. and Hemmings, B.A. (1997) High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of Rac/protein kinase B and their influence on the kinase activity. *J. Biol. Chem.*, **272**, 8474.
- Garcia, P., Gupta, R., Shah, S., Morris, A.J., Rudge, S.A., Scarlata, S., Petrova, V., McLaughlin, S. and Rebecchi, M.J. (1995) The pleckstrin homology domain of phospholipase C- δ_1 binds with high affinity to phosphatidylinositol 4,5-bisphosphate in bilayer membranes. *Biochemistry*, **34**, 16228–16234.
- Gibson, T.J., Hyvönen, M., Musacchio, A., Saraste, M. and Birney, E. (1994) PH domain: the first anniversary. *Trends Biochem. Sci.*, **19**, 349–353.
- Harlan, J.E., Hajduk, P.J., Yoon, H.S. and Fesik, S.W. (1994) Pleckstrin homology domains bind to phosphatidylinositol 4,5-bisphosphate. *Nature*, **371**, 168–170.
- Haslam, R.J., Koide, H.B. and Hemmings, B.A. (1993) Pleckstrin domain homology. *Nature*, **363**, 309–310.
- Hyvönen, M., Macias, M.J., Nilges, M., Oschkinat, H., Saraste, M. and Wilmanns, M. (1995) Structure of the binding site for inositol phosphates in a PH domain. *EMBO J.*, **14**, 4676–4685.
- James, S.R., Downes, C.P., Gigg, R., Grove, S., Grove, J.A., Holmes, A.B. and Alessi, D.R. (1996) Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. *Biochem. J.*, **315**, 709–713.
- Ji, Q.-S., Winnier, G.E., Niswender, K.D., Horstman, D., Wisdom, R., Magnuson, M.A. and Carpenter, G. (1997) Essential role of the tyrosine kinase substrate phospholipase C- γ_1 in mammalian growth and development. *Proc. Natl Acad. Sci. USA*, **94**, 2999–3003.
- Kanematsu, T., Takeya, H., Watanabe, Y., Ozaki, S., Yoshida, M., Koga, T., Iwanaga, S. and Hirata, M. (1992) Putative inositol 1,4,5-trisphosphate binding proteins in rat brain cytosol. *J. Biol. Chem.*, **267**, 6518–6525.
- Kim, H.K., Kim, J.W., Zilberstein, A., Margolis, B., Kim, C.K., Schlessinger, J. and Rhee, S.G. (1991) Stimulation of inositol phospholipid hydrolysis requires PLC- γ phosphorylation on residues 783 and 1254. *Cell*, **65**, 435–441.
- Klarlund, J.K., Guilherme, A., Holik, J.J., Virbasius, J.V., Chawla, A. and Czech, M.P. (1997) Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing pleckstrin and Sec7 homology domains. *Science*, **275**, 1927–1930.
- Klippel, A., Kavanaugh, W.M., Pot, D. and Williams, L.T. (1997) A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Mol. Cell Biol.*, **17**, 338–344.
- Kojima, T., Fukuda, M., Watanabe, Y., Hamazato, F. and Mikoshiba, K. (1997) Characterization of the pleckstrin homology domain of Btk as an inositol polyphosphate and phosphoinositide binding domain. *Biochem. Biophys. Res. Commun.*, **236**, 333–339.
- Konishi, H., Kuroda, S. and Kikkawa, U. (1994) The pleckstrin homology domain of Rac protein kinase associates with the regulatory domain of protein kinase C ζ . *Biochem. Biophys. Res. Commun.*, **205**, 1770–1775.
- Kotani, K. et al. (1994) Involvement of phosphoinositide 3-kinase in insulin or IGF-1-induced membrane ruffling. *EMBO J.*, **13**, 2313–2321.
- Lee, S.B. and Rhee, S.G. (1995) Significance of PIP₂ hydrolysis and regulation of phospholipase C isozymes. *Curr. Opin. Cell Biol.*, **7**, 183–189.
- Lemmon, M.A., Ferguson, K.M., O'Brien, R., Sigler, P.B. and Schlessinger, J. (1995) Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain. *Proc. Natl Acad. Sci. USA*, **92**, 10472–10476.
- Lemmon, M.A., Ferguson, K.M. and Schlessinger, J. (1996) PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell*, **85**, 621–624.
- Lemmon, M.A., Falasca, M., Ferguson, K.M. and Schlessinger, J. (1997) Regulatory recruitment of signaling molecules to the cell membrane by pleckstrin-homology domains. *Trends Cell Biol.*, **7**, 237–242.
- Logan, S.K., Falasca, M., Hu, P. and Schlessinger, J. (1997) Phosphatidylinositol 3-kinase mediates epidermal growth factor-induced activation of the c-jun N-terminal kinase signaling pathway. *Mol. Cell Biol.*, **17**, 5784–5790.
- Lombardo, C.R., Weed, S.A., Kennedy, S.P., Forget, B.G. and Morrow, J.S. (1994) β II-spectrin (fodrin) and β 122-spectrin (muscle) contain NH₂- and COOH-terminal membrane association domains (MAD1 and MAD2). *J. Biol. Chem.*, **269**, 29212–29219.
- Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D.R., Zilberstein, A., Ullrich, A., Pawson, T. and Schlessinger, J. (1990) The tyrosine phosphorylated carboxy terminus of the EGF receptor is a binding site for GAP and PLC- γ . *EMBO J.*, **9**, 4375–4380.
- Mattsson, P.T., Vihinen, M. and Smith, C.I.E. (1996) X-linked agammaglobulinemia (XLA): a genetic tyrosine kinase (Btk) disease. *BioEssays*, **18**, 825–834.
- Mayer, B.J., Ren, R., Clark, K.L. and Baltimore, D. (1993) A putative modular domain present in diverse signaling molecules. *Cell*, **73**, 629–630.
- Musacchio, A., Gibson, T., Rice, P., Thompson, J. and Saraste, M. (1993) The PH domain: a common piece in a patchwork of signalling proteins. *Trends Biochem. Sci.*, **18**, 343–348.
- Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.*, **9**, 484–496.
- Parker, P.J., Hemmings, B.A. and Gierschik, P. (1994) PH domains and phospholipases—a meaningful relationship? *Trends Biochem. Sci.*, **19**, 54–55.
- Paterson, H.F., Savopoulos, J.W., Perisic, O., Cheung, R., Ellis, M.V., Williams, R.L. and Katan, M. (1995) Phospholipase C δ_1 requires a pleckstrin homology domain for interaction with the plasma membrane. *Biochem. J.*, **312**, 661–666.
- Pitcher, J.A., Touhara, K., Payne, E.S. and Lefkowitz, R.J. (1995) Pleckstrin homology domain-mediated membrane association and activation of the β -adrenergic receptor kinase requires coordinate interaction with G $\beta\gamma$ subunits and lipid. *J. Biol. Chem.*, **270**, 11707–11710.
- Rhee, S.G. and Bae, Y.S. (1997) Regulation of phosphoinositide-specific phospholipase C isozymes. *J. Biol. Chem.*, **272**, 15045–15048.
- Salim, K.O. et al. (1996) Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J.*, **15**, 6241–6250.
- Stephens, L.R., Hughes, K.T. and Irvine, R.F. (1991) Pathway of phosphatidylinositol (3,4,5)-trisphosphate synthesis in activated neutrophils. *Nature*, **351**, 33–39.
- Stokoe, D., Stephens, L.R., Copeland, T., Gaffney, P.R.J., Reese, C.B., Painter, G., Holmes, A., McCormick, F. and Hawkins, P.T. (1997) Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science*, **277**, 567–570.
- Touhara, K., Inglese, J., Pitcher, J.A., Shaw, G. and Lefkowitz, R.J. (1994) Binding of G protein $\beta\gamma$ -subunits to pleckstrin homology domains. *J. Biol. Chem.*, **269**, 10217–10220.
- Traynor-Kaplan, A.E., Harris, A., Thompson, B., Taylor, P. and Sklar, L.A. (1988) An inositol tetrakisphosphate-containing phospholipid in activated neutrophils. *Nature*, **334**, 353–356.
- Tsukuda, S., Simon, M.I., Witte, O.N. and Katz, A. (1994) Binding of $\beta\gamma$ subunits of heterotrimeric G proteins to the PH domain of Bruton's tyrosine kinase. *Proc. Natl Acad. Sci. USA*, **91**, 11256–11260.
- Wang, D.-S., Miller, R., Shaw, R. and Shaw, G. (1996) The pleckstrin homology domain of human β 122 spectrin is targeted to the plasma membrane *in vivo*. *Biochem. Biophys. Res. Commun.*, **225**, 420–426.
- Yao, L., Kawakami, Y. and Kawakami, T. (1994) The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C. *Proc. Natl Acad. Sci. USA*, **91**, 9175–9179.
- Zheng, Y., Zangrilli, D., Cerione, R.A. and Eva, A. (1996) The pleckstrin homology domain mediates transformation by oncogenic Dbl through specific intracellular targeting. *J. Biol. Chem.*, **271**, 19017–19020.

Received September 23, 1997; revised and accepted November 11, 1997