

Differential roles of Ras and Rap1 in growth factor-dependent activation of phospholipase C ϵ

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Phospholipase C ϵ is a phosphoinositide-specific phospholipase C that selectively associates with Ras and Rap small GTPases as a target. Here we explored the molecular basis of the Rap1- as well as Ras-mediated regulation of phospholipase C ϵ upon platelet-derived growth factor stimulation by using a receptor mutant deficient in its ability to phosphorylate and activate phospholipase C γ . Following platelet-derived growth factor treatment, this receptor induces persistent activation of ectopically expressed PLC ϵ through activation of Ras and Rap1. The rapid and initial phase of the activation is mediated by Ras, whereas Rap1 is responsible for the prolonged activation. We further demonstrate that the CDC25 homology domain, which exhibits guanine nucleotide exchange factor activity toward Rap1, but not Ras, is critical for the prolonged activation of phospholipase C ϵ . Platelet-derived growth factor prevented the hematopoietic BaF3 cells containing the mutant receptor from undergoing apoptosis, and enabled these cells to proliferate, only when phospholipase C ϵ was expressed. Therefore, the phospholipase C signal is suggested to be critical for survival and growth of BaF3 cells.

Oncogene (2002) 21, 8105–8113. doi:10.1038/sj.onc.1206003

Keywords: PLC ϵ ; Ras; Rap1

Introduction

Phosphoinositide-specific phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, yielding two intracellular second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃), which direct protein kinase C activity and calcium mobilization, respectively (Singer *et al.*, 1997). Thus, PLC plays a pivotal role in a diverse array of signal transduction pathways that

regulate cell growth, differentiation and development. In mammalian cells, four classes of PLCs (PLC β , PLC γ , PLC δ and PLC ϵ) have been identified, being regulated by distinct mechanisms. For instance, PLC β is activated upon direct binding of the α subunit (G α) or the $\beta\gamma$ subunits (G $\beta\gamma$) of heterotrimeric G proteins, whereas tyrosine phosphorylation is crucial for the activation of PLC γ . PLC ϵ is the most recently identified member of the PLC family. A *Caenorhabditis elegans* homolog of PLC ϵ , designated PLC210, was initially characterized as a novel Ras-binding protein through yeast two-hybrid screening (Shibatohge *et al.*, 1998), followed by the isolation of human (Song *et al.*, 2001), rat (Kelley *et al.*, 2001) and mouse (Wu *et al.*, unpublished data) cDNAs encoding PLC ϵ . Screening of human sequence databases by the use of amino acid sequences conserved in PLC isoforms also led to the identification of the same protein (Lopez *et al.*, 2001).

In addition to phospholipase catalytic (X and Y) and calcium-dependent lipid-binding (C2) domains, mammalian PLC ϵ contains two Ras/Rap1-associating (RA) domains (RA1 and RA2 domains) at the C terminus and a CDC25 homology domain near the N terminus, leading to the notion that Ras family GTPases may be intimately involved in the modulation of PLC ϵ activity. Although unidentified in initial studies, a pleckstrin homology domain and EF hands common to all PLC isozymes were recently reported to be found also in PLC ϵ (Wing *et al.*, 2001). However, their functions in the regulation of PLC ϵ remain to be clarified.

The RA2 domain of PLC ϵ associates with Ha-Ras and Rap1A (also called Krev-1) in a GTP-dependent manner, suggesting that PLC ϵ is regulated downstream of these small GTPases (Kelley *et al.*, 2001; Song *et al.*, 2001). In fact, PLC ϵ was translocated to the plasma membrane and the Golgi apparatus through the binding to GTP-bound forms of Ha-Ras and Rap1A, respectively (Song *et al.*, 2001). Ras-dependent subcellular translocation and activation of PLC ϵ were also reconstituted *in vitro* by the use of a liposome carrying recombinant Ras and phosphatidylinositol 4,5-bisphosphate (Song *et al.*, 2001). Additionally, PLC ϵ was significantly activated in cells when coexpressed with activated Ha-Ras (Kelley *et al.*, 2001). Although Ras-dependent activation of PLC ϵ is observed both *in vivo*

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Received 15 July 2002; revised 22 August 2002; accepted 29 August 2002

and *in vitro* as described above, it is unclear whether Rap1A as well can augment PLC ϵ activity upon the association with the RA2 domain. The RA1 domain, whose amino acid sequence is less conserved among mammalian PLC ϵ proteins, was reported to bind Ha-Ras with a much lower affinity than the RA2 domain in a GTP-independent manner (Kelley *et al.*, 2001). Therefore, the physiological role of the RA1 domain in the regulation of PLC ϵ remains elusive, and the RA2 domain will be designed simply the 'RA domain' in this study.

The CDC25 homology domain exhibits *in vitro* guanine nucleotide exchange factor (GEF) activity toward Rap1A, but not toward other Ras family GTPases tested including Ha-Ras (Jin *et al.*, 2001). Therefore, this domain may stimulate the formation of Rap1A·GTP at particular subcellular regions such as the Golgi apparatus. Indeed, the expression of PLC ϵ remarkably prolonged the time course of Rap1A·GTP formation upon epidermal growth factor (EGF) treatment (Jin *et al.*, 2001). However, the role of the CDC25 homology domain in the regulation of signaling mediated by Rap1A and PLC ϵ remains largely unknown.

Although evidence for the involvement of Ras and Rap small GTPases in the regulation of PLC ϵ has been accumulating, receptor-mediated signal transduction pathways that activate PLC ϵ in response to extracellular stimuli remain obscure. Schmidt and colleagues (Schmidt *et al.*, 2001b; Evellin *et al.*, 2002) recently documented that PLC ϵ was activated downstream of cAMP-dependent signaling triggered by stimulation of a subset of G protein-coupled serpentine receptors, such as β_2 -adrenergic, prostaglandin E $_1$ and M $_3$ muscarinic acetylcholine receptors. In these signaling pathways, Rap2B is reported to be specifically involved in the regulation of PLC ϵ although the molecular mechanisms underlying the activation of PLC ϵ by Rap2B remain to be solved (Schmidt *et al.*, 2001b; Evellin *et al.*, 2002).

Herein, we show that Rap1 as well as Ras plays an important role in the regulation of PLC ϵ following stimulation of the platelet-derived growth factor (PDGF) receptor. Ras mediates the rapid and transient signal for the activation of PLC ϵ , whereas Rap1 is responsible for the sustained activation. Furthermore, we describe that the CDC25 homology domain, which exhibits a Rap1-specific GEF activity, is critical for the prolongation of PLC ϵ activation in Rap1-dependent signaling.

Results

Activation of PLC ϵ by coexpression of Ras or Rap in COS-7 cells

PLC ϵ binds to Ras and Rap in a GTP-dependent manner through its C-terminal RA domain. Although the activation of PLC ϵ upon binding to the GTP-bound form of Ras has been demonstrated both *in vivo* (Kelley *et al.*, 2001) and *in vitro* (Song *et al.*, 2001), the

role of Rap in the regulation of PLC ϵ remains elusive. As an initial step to clarify this point, the activation of PLC ϵ by coexpression of constitutively active forms of Ras subfamily members was examined in COS-7 cells (Figure 1). As previously described (Kelley *et al.*,

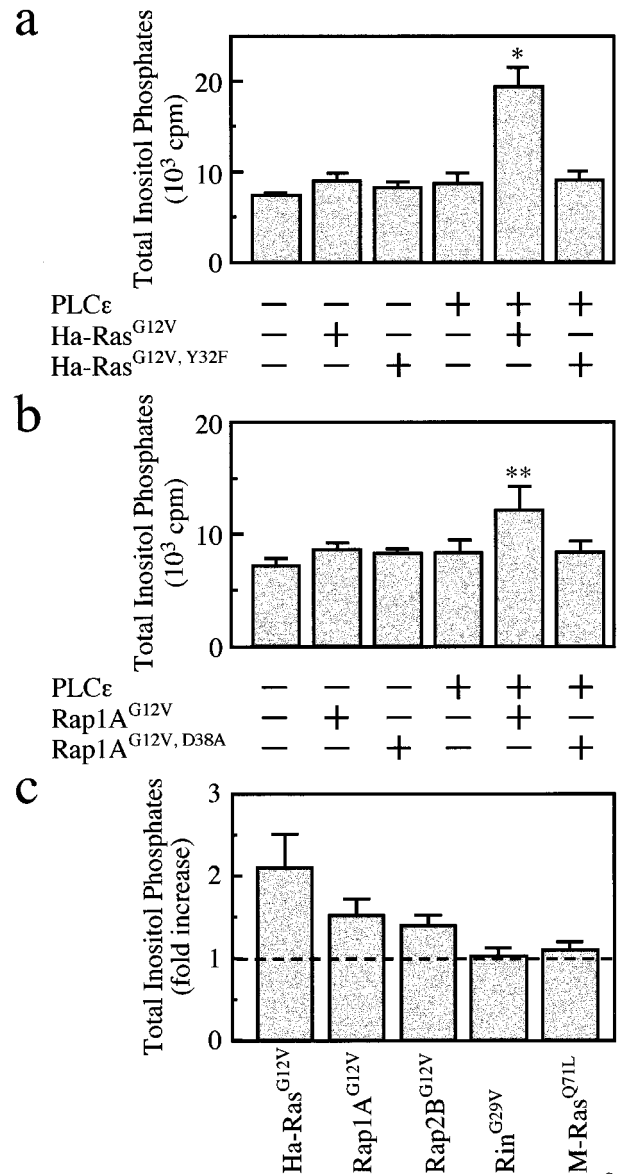


Figure 1 Activation of PLC ϵ by coexpression of Ras or Rap. (a) Activation of PLC ϵ by constitutively active Ha-Ras, but not its effector region mutant. Ha-Ras^{G12V} or its effector region mutant Ha-Ras^{G12V, Y32F} was expressed with or without PLC ϵ in COS-7 cells, and inositol phosphates were quantitated. Results are shown as the means \pm s.d. ($n=3$). * $P<0.01$ compared with mock-transfected cells. (b) Activation of PLC ϵ by constitutively active Rap1A, but not its effector region mutant. Rap1A^{G12V} or its effector region mutant Rap1A^{G12V, D38A} was expressed with or without PLC ϵ in COS-7 cells, and inositol phosphates were quantitated. Results are shown as the means \pm s.d. ($n=3$). ** $P<0.05$ compared with mock-transfected cells. (c) Activation of PLC ϵ by constitutively active forms of Ras family GTPases. Inositol phosphates in COS-7 cells ectopically expressing PLC ϵ and indicated small GTPases were quantitated. Fold increase in the level of inositol phosphates relative to that in cells expressing PLC ϵ alone is shown as the means \pm s.d. ($n=3$)

2001), coexpression of a constitutively active form of Ha-Ras, Ha-Ras^{G12V}, resulted in an increase in the level of inositol phosphates in PLC ϵ -expressing COS-7 cells, reflecting the activation of PLC ϵ (Figure 1a). An effector region mutant of Ha-Ras, Ha-Ras^{G12V,Y32F}, which did not bind to PLC ϵ (Song *et al.*, 2001), exerted no effect on inositol phosphates production, supporting a notion that the observed activation of PLC ϵ is mediated through direct binding (Figure 1a). Similarly, an activated form of Rap1A (Rap1A^{G12V}), but not its effector region mutant (Rap1A^{G12V,D38A}) that did not bind to PLC ϵ (data not shown), increased the level of inositol phosphates (Figure 1b). The effect of Rap1A was significant but modest compared to that of Ha-Ras, which may be ascribed to difference in the size of phosphatidylinositol 4,5-biphosphate pools in different subcellular regions where Ras or Rap1A exists. Like Rap1A, both Rap2A and Rap2B associated with PLC ϵ in a GTP-dependent manner as revealed by an *in vitro* binding assay (Figure 2a), whereas other Ras family members Rin and M-Ras did not bind to PLC ϵ (data not shown). Consistent with the binding activity to PLC ϵ , Rap2B, but not Rin and M-Ras, in their active forms, caused modest PLC ϵ activation in COS-7 cells (Figure 1c).

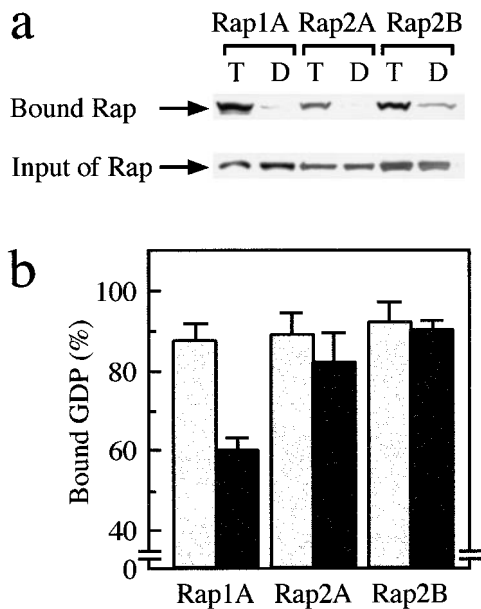


Figure 2 Specificity of the RA and CDC25 homology domains. (a) *In vitro* binding of the RA domain to Rap GTPases. GST-tagged Rap GTPases were preloaded with GTP γ S (T) or GDP (D) and incubated with MBP-PLC ϵ RA (2094–2302) immobilized on amylose resin. GTPases bound to MBP-PLC ϵ RA(2094–2302) (Bound Rap) and one-tenth aliquots of GTPases in the reaction mixture (Input of Rap) were detected by immunoblotting using an anti-GST antibody. Representative results of three independent experiments are shown. (b) GEF activity of the CDC25 homology domain. Rap GTPases preloaded with [³H]GDP were incubated at 30°C for 20 min with excess amounts of unlabeled GTP in the presence (black bars) or absence (gray bars) of PLC ϵ . [³H]GDP remaining bound to the protein was quantitated and expressed as the percentage of the values at the zero time point. Results are shown as the means \pm s.d. ($n = 3$)

Specificity of the RA and CDC25 homology domains of PLC ϵ

We recently reported that the RA domain of PLC ϵ associates with both Ha-Ras and Rap1A in a GTP-dependent manner (Song *et al.*, 2001). On the other hand, the CDC25 homology domain of PLC ϵ exhibits GEF activity toward Rap1A, but not Ha-Ras (Jin *et al.*, 2001). On the basis of these biochemical properties, we assumed that the CDC25 homology domain might be important for amplification and prolongation of Rap1A-mediated signaling (Jin *et al.*, 2001). However, it remains unclear whether the Rap2 signal as well is modulated by the CDC25 homology domain, and therefore we further examined the interaction of PLC ϵ with Rap2A and Rap2B. Like Rap1A, GTP-bound forms, but not GDP-bound forms, of both Rap2A and Rap2B associate with the PLC ϵ RA domain (Figure 2a). In contrast, GDP release from Rap1A was enhanced by PLC ϵ , whereas that from Rap2A or Rap2B remained unaffected (Figure 2b).

Isolation of cell lines containing PLC ϵ and a PDGF receptor mutant that is incapable of activating PLC γ

Previously, we addressed translocation of PLC ϵ in response to EGF stimulation to the plasma membrane and the Golgi apparatus through binding to Ras and Rap1, respectively (Song *et al.*, 2001). However, we failed to detect a significant increase in the catalytic activity of PLC ϵ in response to EGF treatment because EGF also stimulated another subtype of PLC, PLC γ , through tyrosine phosphorylation. In an attempt to elucidate the role of Ras and Rap in the regulation of PLC ϵ in response to extracellular stimuli, we isolated cell lines harboring PLC ϵ and a PDGF receptor mutant that is incapable of stimulating PLC γ . Phosphorylation of two tyrosine residues (Y977 and Y989) at the C-terminal intracellular portion of the PDGF receptor is essential for PDGF-dependent PLC γ activation, and therefore, phenylalanine substitution of these tyrosines renders the PDGF receptor incapable of stimulating PLC γ without affecting Ras activation (Fantl *et al.*, 1993; Satoh *et al.*, 1993). The cDNA encoding full-length PLC ϵ or PLC ϵ lacking the N-terminal portion including the CDC25 homology domain (PLC ϵ Δ N) was introduced into a hematopoietic BaF3 cell line-derived clone carrying the PDGF receptor mutant PDGFR(Y977F/Y989F) (designated BaF3-PDGFR(Y977F/Y989F)) (Satoh *et al.*, 1993). The parental BaF3 cell line does not have a detectable amount of endogenous PLC ϵ . Resulting stable transfectant clones (designated BaF3-PDGFR(Y977F/Y989F)/PLC ϵ and BaF3-PDGFR(Y977F/Y989F)/PLC ϵ Δ N, respectively) were subjected to further analyses.

Activation of Ras and Rap1 following PDGF stimulation in BaF3-derived transfectants

GTP-bound forms of Ras and Rap1 in BaF3-derived transfectants were detected by the pull-down assays

(Figure 3). In BaF3-PDGFR(Y977F/Y989F)/PLC ϵ cells, PDGF caused a rapid and transient increase in Ha-Ras-GTP with a peak level at 1 to 5 min (Figure 3a). In contrast, the Rap1-GTP level increased more slowly and remained elevated for at least 20 min (Figure 3b). In BaF3-PDGFR(Y977F/Y989F)/PLC ϵ Δ N cells, PDGF-dependent Rap1-GTP formation became transient as shown in Figure 3c, suggesting that the CDC25 homology domain is required for the sustained Rap1 activation observed in the cells expressing full-length PLC ϵ . The time course of Rap1 activation in BaF3-PDGFR(Y977F/Y989F) cells was similar to that of BaF3-PDGFR(Y977F/Y989F)/PLC ϵ Δ N cells (data not shown).

IP₃ production following PDGF stimulation in BaF3-derived transfectants

Intracellular IP₃ levels were quantitated following PDGF treatment of BaF3-derived cells (Figure 4). We also stimulated these cells with interleukin-3 (IL-3) as a control and detected an increase in the level of intracellular IP₃. PDGF did not induce elevation of the IP₃ level in the absence of PLC ϵ because the PDGF receptor mutant could not activate PLC γ (Figure 4a). In marked contrast, PDGF caused a remarkable increase in the IP₃ level when full-length PLC ϵ was ectopically expressed (Figure 4b). The increase in the IP₃ level is presumably ascribed to the activation of PLC ϵ mediated by Ras and Rap1 because both small

GTPases are activated in response to PDGF as described (Figure 3). To further clarify the involvement of Ras and Rap in the PLC ϵ -dependent accumulation of IP₃, a dominant-negative mutant of Ras (Ha-Ras^{S17N}) or a Rap GTPase-activating protein (SPA-1) was expressed in BaF3-PDGFR(Y977F/Y989F)/PLC ϵ

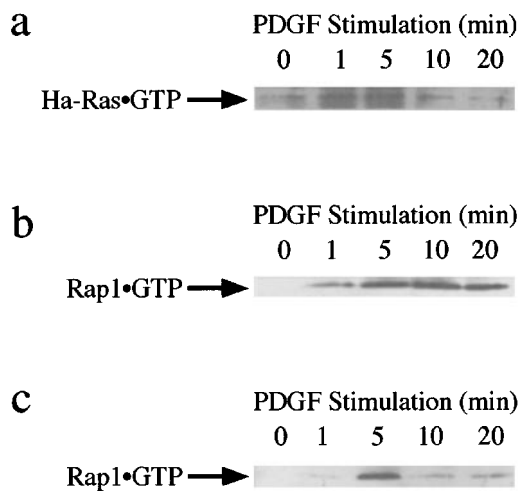


Figure 3 Activation of Ras and Rap following PDGF stimulation. (a) BaF3-PDGFR(Y977F/Y989F)/PLC ϵ cells were transiently transfected with the HA-tagged Ha-Ras cDNA. The GTP-bound form of HA-Ha-Ras in these cells was detected by the pull-down assay following PDGF stimulation for indicated times. Representative results of three independent experiments are shown. (b) The GTP-bound form of endogenous Rap1 in BaF3-PDGFR(Y977F/Y989F)/PLC ϵ cells was detected by the pull-down assay following PDGF stimulation for indicated times. Representative results of three independent experiments are shown. (c) The GTP-bound form of endogenous Rap1 in BaF3-PDGFR(Y977F/Y989F)/PLC ϵ Δ N cells was detected by the pull-down assay following PDGF stimulation for indicated times. Representative results of three independent experiments are shown

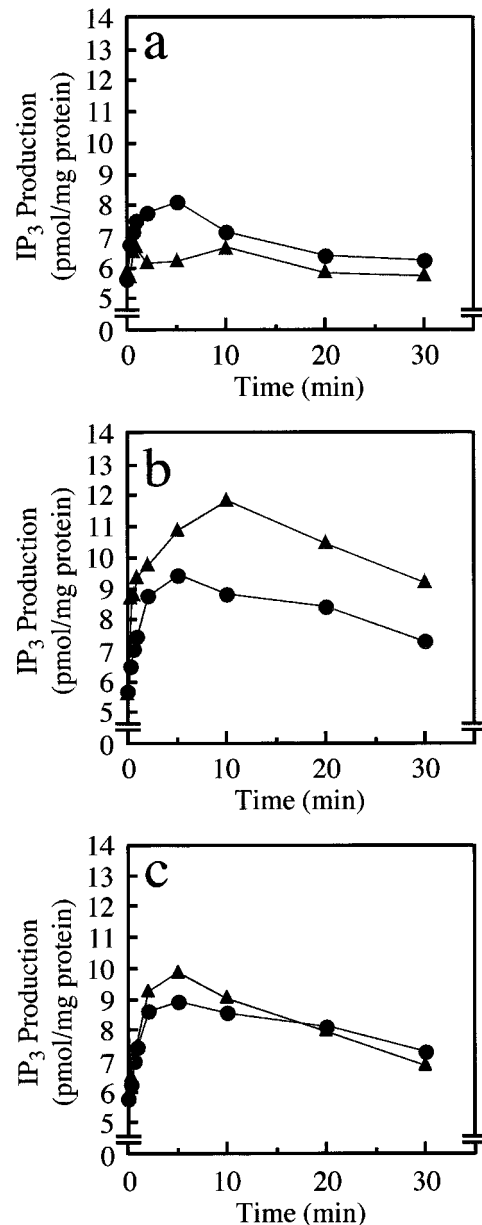


Figure 4 Time courses of IP₃ production following IL-3 or PDGF stimulation. (a) BaF3-PDGFR(Y977F/Y989F) cells were stimulated with IL-3 (circles) or PDGF (triangles), and intracellular IP₃ levels were quantitated. Representative results of three independent experiments performed in duplicate are shown. (b) BaF3-PDGFR(Y977F/Y989F)/PLC ϵ cells were stimulated with IL-3 (circles) or PDGF (triangles), and intracellular IP₃ levels were quantitated. Representative results of three independent experiments performed in duplicate are shown. (c) BaF3-PDGFR(Y977F/Y989F)/PLC ϵ Δ N cells were stimulated with IL-3 (circles) or PDGF (triangles), and intracellular IP₃ levels were quantitated. Representative results of three independent experiments performed in duplicate are shown

cells by the use of a retroviral expression system (Figure 5). Ha-Ras^{S17N} potently inhibited the PDGF-triggered IP₃ production, but a slight increase in the IP₃ level was observed with delayed time kinetics at 10 to 20 min. In contrast, in SPA-1-expressing cells, the rapid response remained unaffected whereas the prolonged increase in the IP₃ level was significantly abrogated. Therefore, it is feasible that the PLC ϵ -dependent IP₃ production, particularly at the initial phase, is mediated mainly by Ras, while Rap may be responsible for the sustained activation of PLC ϵ . The activity of PLC ϵ may also be modulated by the CDC25 homology domain because this domain exhibits Rap1-specific GEF activity (Figure 2), and is responsible for the prolongation of the Rap1 signal (Figure 3). Indeed, prolonged IP₃ production was impaired in BaF3-PDGFR(Y977F/Y989F)/PLC ϵ Δ N cells, and consequently the time course resembled that observed in the SPA-1-expressing cells (Figures 4c and 5). Considering that the CDC25 homology domain is specific to Rap1, but not Rap2, Rap1 rather than Rap2 is likely to be implicated in the regulation of PLC ϵ .

Proliferation of PLC ϵ -expressing BaF3 cells

Lastly, we examined whether the PLC ϵ pathway stimulates survival and growth of BaF3 cells, which undergo apoptosis upon IL-3 deprivation (Johnson, 1998). Cell number increase of two BaF3-PDGFR(Y977F/Y989F)/PLC ϵ cell clones (#8 and #10) in the presence or absence of PDGF is illustrated in Figure 6. PDGF did not stimulate growth of BaF3-PDGFR(Y977F/Y989F) and BaF3-PDGFR(Y977F/Y989F)/vector cells. In contrast, both PLC ϵ -expressing clones proliferated in the presence of PDGF. Additionally, growth rates of the two clones in the presence of IL-3 were higher compared to that of BaF3-PDGFR(Y977F/Y989F) cells. Apoptosis of the two

clones was also prevented when stimulated by PDGF although BaF3-PDGFR(Y977F/Y989F) cells underwent apoptosis in the presence of PDGF. Therefore,

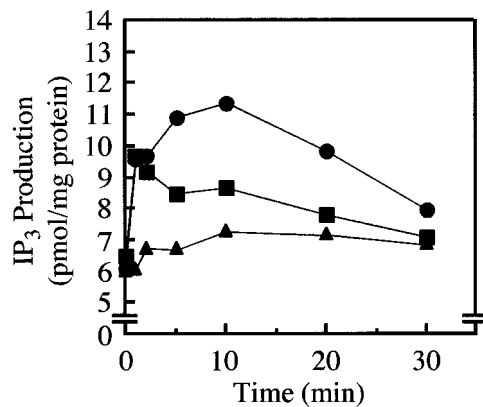


Figure 5 Effect of dominant-negative Ras and the Rap GTPase-activating protein SPA-1 on PDGF-induced production of IP₃ in BaF3-PDGFR(Y977F/Y989F)/PLC ϵ cells. BaF3-PDGFR(Y977F/Y989F)/PLC ϵ cells were mock-infected (circles) or infected with a retrovirus expressing Ha-Ras^{S17N} (triangles) or SPA-1 (squares). Following stimulation with PDGF, intracellular IP₃ levels were quantitated. Representative results of three independent experiments performed in duplicate are shown

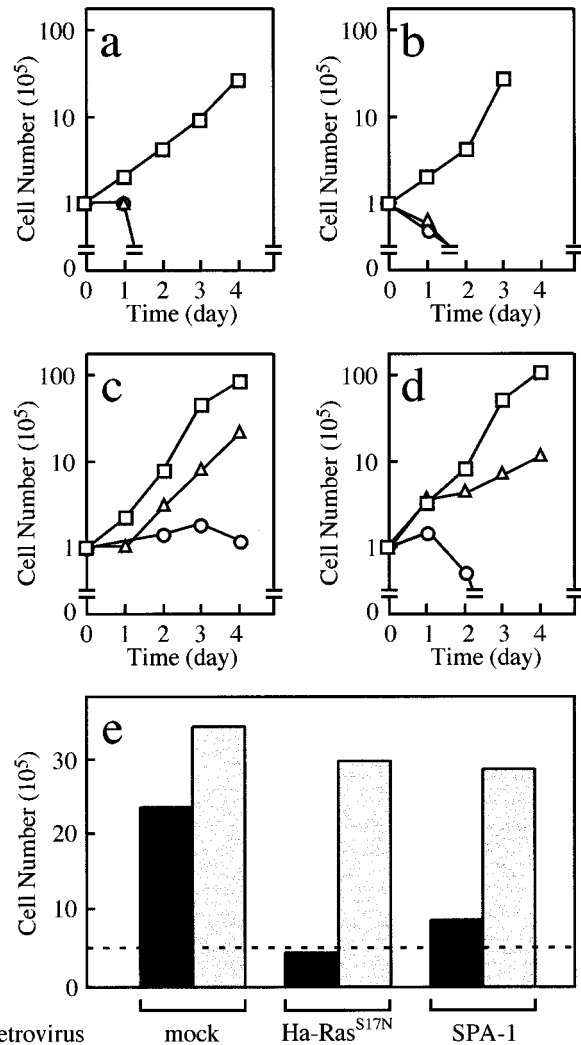


Figure 6 Effect of PLC ϵ on proliferation of BaF3-derived cells. (a) Proliferation of BaF3-PDGFR(Y977F/Y989F) cells. BaF3-PDGFR(Y977F/Y989F) cells (1.0×10^5 cells) were cultivated in the presence of FBS (circles), FBS plus PDGF (triangles) or FBS plus IL-3 (squares), and cell numbers were counted. Mean values of three or four independent experiments performed in duplicate are shown. (b) Proliferation of BaF3-PDGFR(Y977F/Y989F)/vector cells. BaF3-PDGFR(Y977F/Y989F)/vector cells were cultivated as in (a). Mean values of three or four independent experiments performed in duplicate are shown. (c) Proliferation of BaF3-PDGFR(Y977F/Y989F)/PLC ϵ (clone #8) cells. BaF3-PDGFR(Y977F/Y989F)/PLC ϵ (clone #8) cells were cultivated as in (a). Mean values of three or four independent experiments performed in duplicate are shown. (d) Proliferation of BaF3-PDGFR(Y977F/Y989F)/PLC ϵ (clone #10) cells. BaF3-PDGFR(Y977F/Y989F)/PLC ϵ (clone #10) cells were cultivated as in (a). Mean values of three or four independent experiments performed in duplicate are shown. (e) Effect of Ha-Ras^{S17N} and SPA-1. BaF3-PDGFR(Y977F/Y989F)/PLC ϵ (clone #8) cells were infected with retroviruses expressing indicated proteins. Infected cells (5.0×10^5 cells) were cultivated in the presence of FBS plus PDGF (black bars) or FBS plus IL-3 (gray bars) for 3 days, and cell numbers were counted. Mean values of three or four independent experiments performed in duplicate are shown

it is likely that PLC ϵ , when activated by PDGF, suppresses apoptotic cell death, and stimulates cell proliferation. BaF3-PDGFR(Y977F/Y989F)/PLC ϵ (clone #8) cells did not undergo apoptosis under growth factor-deprived conditions (Figure 6b), which may be due to basal unstimulated activity of PLC ϵ at a high expression level. Inhibition of Ras- and Rap-mediated activation of PLC ϵ by retroviral expression of Ha-Ras^{S17N} and SPA-1, respectively, almost completely abolished cell growth, suggesting that PLC ϵ activities in distinct subcellular regions, such as the plasma membrane and the Golgi apparatus, are indispensable for cell growth (Figure 6d).

Discussion

Both Ha-Ras and Rap1A in their GTP-bound forms bind to the RA domain of PLC ϵ , suggesting that PLC ϵ may function as a direct target of not only Ha-Ras but also Rap1A (Song *et al.*, 2001). This was further supported by our observation that PLC ϵ colocalized with Ha-Ras and Rap1A in the plasma membrane and the Golgi apparatus, respectively, after treatment with EGF (Song *et al.*, 2001). However, it remained unclear whether phosphoinositide-hydrolyzing activity of PLC ϵ is regulated by Rap1A, although Ha-Ras-dependent activation of PLC ϵ was shown both *in vivo* and *in vitro* (Kelley *et al.*, 2001; Song *et al.*, 2001). In this study, we further explored a role for Rap1A in the regulation of PLC ϵ activity. When coexpressed in COS-7 cells, activated Rap1A, like Ha-Ras, significantly stimulated PLC ϵ activity presumably through direct interaction (Figure 1). Therefore, it is feasible that Rap1A directly binds to, and thereby stimulates PLC ϵ in the Golgi apparatus in response to extracellular stimuli such as EGF treatment. Considering that protein kinase C exists in the Golgi apparatus (de Matteis *et al.*, 1993), and can act as a downstream target of DAG yielded by PLC ϵ , the Rap1A/PLC ϵ signaling pathway may provide a link between growth factor receptors and Golgi-localized protein kinase C.

It is important to examine whether the activity of PLC ϵ is actually stimulated in response to extracellular signals in a Ras- or Rap1-dependent manner. However, we were unable to detect EGF-dependent increase in PLC ϵ activity specifically because other subtypes of PLCs were also activated following EGF treatment. To accomplish this, we isolated cell lines harboring PLC ϵ and a PDGF receptor mutant that activates Ras and Rap1, but not PLC γ . Indeed, the activity of PLC ϵ , as determined by intracellular IP₃ production, was stimulated upon PDGF treatment, and remained elevated at least for 30 min after PDGF was challenged (Figure 4).

Disruption of the Rap pathway by infection of a SPA-1-expressing virus resulted in a transient increase in the intracellular IP₃ level after PDGF treatment (Figure 5), suggesting that Rap-mediated signaling is required for the sustained activation of PLC ϵ . In contrast, Ras mediates the rapid and transient

activation of PLC ϵ as demonstrated by the effect of Ha-Ras^{S17N} (Figure 5). Although biological functions of PLC ϵ downstream of a variety of cell surface receptors remain obscure, Ras-mediated transient and Rap-mediated sustained activation of PLC ϵ may have different roles in cell response. In this respect, it is noteworthy that, in PC12 pheochromocytoma cells, Rap1 is involved in nerve growth factor-dependent sustained activation of extracellular signal-regulated kinases, which is prerequisite for neuronal differentiation, whereas Ras is responsible for the rapid activation (York *et al.*, 1998). Moreover, Ras- and Rap-dependent PLC ϵ activation may possibly occur in distinct subcellular compartments not only in COS-7 cells (Song *et al.*, 2001), but also in other cell types. Therefore, distinct sets of protein kinase C isozymes and calcium channels may selectively function in Ras/PLC ϵ and Rap/PLC ϵ signaling pathways, exerting different biological functions. Notably, PDGF-dependent growth of PLC ϵ -expressing BaF3-derived cells was totally sensitive to the inhibitory action of Ha-Ras^{S17N} or SPA-1 (Figure 6). Thus, both Ras- and Rap1-dependent functions of PLC ϵ in the plasma membrane and the Golgi apparatus, respectively, are indispensable for proliferation of this cell line.

The CDC25 homology domain of PLC ϵ exhibits GEF activity toward Rap1A *in vitro*, leading to the hypothesis that this domain may be required for prolonged activation of PLC ϵ through a positive feedback mechanism (Jin *et al.*, 2001). In support of this, the CDC25 homology domain was essential for prolonged formation of Rap1-GTP accompanied by prolonged translocation of PLC ϵ to the Golgi apparatus following EGF stimulation (Jin *et al.*, 2001). As illustrated in Figure 4, the increase in the intracellular level of IP₃ upon PDGF treatment in BaF3-derived cells that express a PLC ϵ mutant lacking the CDC25 homology domain was transient in contrast to the sustained increase in full-length PLC ϵ -expressing cells. Collectively, it is conceivable that the CDC25 homology domain functions as a module responsible for prolongation of Rap1/PLC ϵ signaling.

It remains unclear which subtype of the Rap family is involved in the activation of PLC ϵ in BaF3-derived transfectants because both Rap1 and Rap2 bind to the RA domain in a GTP-dependent manner, thereby stimulating the activity of PLC ϵ (Figures 1 and 2). We also cannot distinguish roles of Rap1 and Rap2 based on the inhibitory action of SPA-1 because SPA-1 serves as a GTPase-activating protein for both Rap1A and Rap2A (Kurachi *et al.*, 1997). Intriguingly, the CDC25 homology domain of PLC ϵ shows a GEF activity toward Rap1A, but neither Rap2A nor Rap2B, *in vitro* (Figure 2) and is needed for the PDGF-triggered Rap-dependent sustained activation of PLC ϵ (Figure 4). Hence, Rap1A rather than Rap2A and Rap2B is likely to be responsible for PLC ϵ signaling. On the other hand, Rap2 was reported to be specifically involved in the regulation of PLC ϵ following stimulation of Gs-coupled receptors (Schmidt *et al.*, 2001b; Evellin *et al.*, 2002), where the cAMP-dependent Rap GEF Epacl

serves as a regulator. However, PDGF stimulation of PLC ϵ presumably does not involve cAMP production. Additionally, more than 50% of Rap2A exists in the GTP-bound form in cells, leading to the notion that regulatory mechanisms for Rap2A are different from those for Rap1A (Ohba *et al.*, 2000). Thus, a GEF specific to Rap1 may play a crucial role for triggering Rap1 activation in our system.

In addition to signaling pathways involving Ras or Rap, PLC ϵ has been implicated in pathways immediately downstream of heterotrimeric G proteins. A constitutively activated form of G α 12, but not other G α subunits, such as G α s and G α i, stimulated PLC ϵ activity although the mechanism whereby the interaction between G α 12 and PLC ϵ occurs remains totally unknown (Lopez *et al.*, 2001). Moreover, G β γ enhanced PLC ϵ activity when coexpressed in COS-7 cells presumably through the association of G β γ with a pleckstrin homology domain newly identified in PLC ϵ (Wing *et al.*, 2001). The effect of G β γ seems to be independent of the Ras pathways, implying that multiple regulatory signals converge at PLC ϵ . Future studies will further reveal the molecular mechanisms underlying the complicated regulation of PLC ϵ .

It is important to clarify the physiological function of PLC ϵ activated downstream of Rap1. To date, Rap1 has been implicated in an array of intracellular signaling pathways, which regulate proliferation, differentiation, lymphocyte aggregation, T-cell anergy or platelet activation (Bos, 1998; Bos *et al.*, 2001). In addition, Rap1 may exert its function in a subcellular region-specific manner because Rap1 is present both in the Golgi apparatus and in the plasma membrane (Beranger *et al.*, 1991; Wienecke *et al.*, 1996; Matsubara *et al.*, 1999; Ohba *et al.*, 2000; York *et al.*, 2000; Gao *et al.*, 2001). Several lines of evidence have supported the notion that Rap1 plays an important role in integrin-mediated leukocyte adhesion. An active form of Rap1 potently induces the activation of integrins and subsequent cell aggregation (Katagiri *et al.*, 2000; Reedquist *et al.*, 2000), whereas a dominant-negative form of Rap1 and GTPase-activating proteins for Rap1 including SPA-1 inhibited cell adhesion triggered by ligation of the T-cell receptor or CD31 (Tsukamoto *et al.*, 1999; Katagiri *et al.*, 2000; Reedquist *et al.*, 2000). Likewise, lipopolysaccharide-induced activation of integrins in macrophages requires Rap1 (Schmidt *et al.*, 2001a). Currently, however, the involvement of PLC ϵ in these Rap1-dependent signaling pathways is totally unknown. Recently, DAG has been implicated in the recruitment of protein kinase D to the Golgi apparatus, which is important for protein transport to the cell surface (Baron and Malhotra, 2002). PLC ϵ may contribute to the generation of DAG in the Golgi apparatus and may be involved in protein kinase D regulation although other lipid metabolic pathways play a major role in controlling the DAG level. Further studies will be required for the elucidation of biological functions of the Rap1/PLC ϵ pathway particularly in the Golgi apparatus.

Materials and methods

Plasmids

cDNAs for full-length human PLC ϵ and its deletion mutant PLC ϵ Δ N (amino acids 1088–2302) lacking the CDC25 homology domain were subcloned into the mammalian expression vector pFLAG-CMV2 (Sigma), generating pFLAG-CMV2-PLC ϵ and pFLAG-CMV2-PLC ϵ Δ N, respectively (Jin *et al.*, 2001). Hemagglutinin (HA)-tagged small GTPases (Ha-Ras, Ha-Ras^{G12V}, Ha-Ras^{G12V,Y32F}, Rap1A^{G12V}, Rap1A^{G12V,D38A}, Rap2B^{G12V}, Rin^{G29V} and M-Ras^{Q71L}) were subcloned into the mammalian expression vector pEF-BOS (Mizushima and Nagata, 1990). The Ha-Ras^{S17N} cDNA was subcloned into the retroviral vector pLPCX containing the puromycin-resistant gene (Clontech), generating pLPCX-Ha-Ras^{S17N}. pLXSN-FLAG-SPA-1 (for retroviral expression of FLAG-tagged SPA-1), containing the neomycin-resistant gene, was kindly provided by Dr Nagahiro Minato (Kyoto University, Kyoto, Japan).

Cell lines

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). BaF3-PDGFR(Y977F/Y989F) cells (Sato *et al.*, 1993) were cultured in RPMI 1640 supplemented with 10% FBS, IL-3 (approximately 1 nM) and G418 (0.3 mg/ml). pFLAG-CMV2-PLC ϵ , pFLAG-CMV2-PLC ϵ Δ N or pFLAG-CMV2 (vector) was introduced into BaF3-PDGFR(Y977F/Y989F) cells with a hygromycin B-resistant marker plasmid by electroporation essentially as described (Sato *et al.*, 1993). Stable transfectants (designated BaF3-PDGFR(Y977F/Y989F)/PLC ϵ , BaF3-PDGFR(Y977F/Y989F)/PLC ϵ Δ N and BaF3-PDGFR(Y977F/Y989F)/vector, respectively) were selected and cultured in RPMI 1640 supplemented with 10% FBS, IL-3 (approximately 1 nM), G418 (0.3 mg/ml) and hygromycin B (0.5 mg/ml). Viable cells were counted after Trypan blue staining.

Retroviral gene transfer

The packaging cell line Phoenix Eco (American Type Culture Collection No; SD3444) was transfected with pLPCX-Ha-Ras^{S17N} and pLXSN-FLAG-SPA-1 and cultured in the presence of puromycin (1 μ g/ml, for pLPCX-Ha-Ras^{S17N}) or G418 (0.3 mg/ml, for pLXSN-FLAG-SPA-1) for 2 weeks. Recombinant retroviruses were obtained according to the instructions by Dr Garry Nolan (Stanford University, CA, USA). BaF3-PDGFR(Y977F/Y989F)/PLC ϵ cells infected with the Ha-Ras^{S17N} virus were selected by puromycin (1 μ g/ml). Expression of Ha-Ras^{S17N} and SPA-1 was confirmed by immunoblotting.

Quantitation of total inositol phosphates in COS-7 cells

Total inositol phosphates in COS-7 cells were quantitated essentially as described (Kelley *et al.*, 2001). Briefly, COS-7 cells cultured in 12-well culture plates were transfected with expression plasmids using LipofectAMINE 2000 (Gibco-BRL) after reaching 90–95% confluency. Twenty-four hours after transfection, medium was changed to inositol-free DMEM supplemented with 4 μ Ci per well of *myo*-[³H]inositol (84 Ci/mmol, NEN). After 12 h incubation, 10 mM LiCl was added to the culture medium, and cells were incubated for additional 1 h. The reaction was stopped by adding ice-cold perchloric acid to the final concentration of 4.5% (v/v),

and [3 H]inositol phosphates were separated by column chromatography using Dowex AG1-X8 (200–400 mesh, formate form). Radioactivities of total inositol phosphates were quantitated by liquid scintillation counting.

In vitro binding assay

Maltose-binding protein (MBP)-PLC ϵ RA(2094–2302) was previously described (Song *et al.*, 2001). Glutathione *S*-transferase (GST)-Rap1A, GST-Rap2A and GST-Rap2B were expressed in *Escherichia coli* by using the expression vector pGEX-5X (Amersham), and purified with glutathione-Sepharose (Amersham). *In vitro* binding of GST-tagged Rap GTPases (10 pmol) preloaded with GTP γ S or GDP to MBP-PLC ϵ RA(2094–2302) (50 pmol) was assayed in 100 μ l of the reaction mixture as described (Song *et al.*, 2001). GST-tagged Rap GTPases were detected by immunoblotting using an anti-GST antibody (Shima *et al.*, 1997).

In vitro GEF assay

FLAG-tagged PLC ϵ was expressed in *Spodoptera frugiperda* Sf9 cells, and affinity-purified by the use of anti-FLAG M2 resin (Sigma) as described previously (Song *et al.*, 2001). GST-tagged Rap GTPases (4 pmol) preloaded with [3 H]GDP (3000 c.p.m./pmol, NEN) were incubated with PLC ϵ (1 pmol) in 50 μ l of the reaction mixture, and *in vitro* GEF activities were assayed as described (Jin *et al.*, 2001).

In vivo GEF (pull-down) assay

BaF3-PDGFR(Y977F/Y989F)/PLC ϵ cells transfected with pEF-BOS-HA-Ha-Ras by electroporation (for Ha-Ras) or

BaF3-PDGFR(Y977F/Y989F)/PLC ϵ cells (for Rap1) were serum-starved in RPMI 1640 supplemented with bovine serum albumin (1 mg/ml) for 2 h. Thereafter, cells were stimulated with PDGF (50 ng/ml), and GTP-bound forms of Ha-Ras and Rap1 were detected by pull-down assays using GST-Raf-1-Ras-binding domain (RBD) (for Ha-Ras) or GST-Ral guanine nucleotide dissociation stimulator (RalGDS)-Rap1-interacting domain (RID) (for Rap1) as previously described (Gao *et al.*, 2001). Ha-Ras and Rap1 were visualized by immunoblotting using anti-HA (12CA5, Roche) and anti-Rap1 (sc-65, Santa Cruz) antibodies, respectively.

Measurement of IP $_3$ in BaF3-derived transfectants

BaF3-derived transfectants were serum-starved in RPMI 1640 supplemented with bovine serum albumin (1 mg/ml) for 2 h, and stimulated with PDGF (50 ng/ml). Intracellular IP $_3$ concentrations were determined by using the IP $_3$ [3 H] radioreceptor assay kit (NEN) according to the manufacturer's instructions.

Acknowledgements

We thank Dr Nagahiro Minato for the SPA-1 cDNA and Dr Garry Nolan for helpful advice. This investigation was supported by Grants-in-aid for Scientific Research in Priority Areas and for Scientific Research (B) and (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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