Phospholipase C-γ1 is a guanine nucleotide exchange factor for dynamin-1 and enhances dynamin-1-dependent epidermal growth factor receptor endocytosis

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Summary

Phospholipase C-γ1 (PLC-γ1), which interacts with a variety of signaling molecules through its two Src homology (SH) 2 domains and a single SH3 domain has been implicated in the regulation of many cellular functions. We demonstrate that PLC-γ1 acts as a guanine nucleotide exchange factor (GEF) of dynamin-1, a 100 kDa GTPase protein, which is involved in clathrin-mediated endocytosis of epidermal growth factor (EGF) receptor. Overexpression of PLC-γ1 increases endocytosis of the EGF receptor by increasing guanine nucleotide exchange activity of dynamin-1. The GEF activity of PLC-γ1 is mediated by the direct interaction of its SH3 domain with dynamin-1. EGF-dependent activation of ERK and serum response element (SRE) are both up-regulated in PC12 cells stably overexpressing PLC-γ1, but knockdown of PLC-γ1 by siRNA significantly reduces ERK activation. These results establish a new role for PLC-γ1 in the regulation of endocytosis and suggest that endocytosis of activated EGF receptors may mediate PLC-γ1-dependent proliferation.

Supplemental data available online

Key words: Phospholipase C-γ1, Dynamin-1, Guanine nucleotide exchange factor (GEF), Endocytosis, Proliferation

Introduction

Phospholipase C (PLC) is involved in cellular proliferation and differentiation, and its enzymatic activity is upregulated by a variety of growth factors and hormones (Rhee et al., 1989). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, which are implicated in the mobilization of intracellular Ca^{2+} and protein kinase C activation, respectively (Berridge and Irvin, 1989). So far, eleven mammalian PLC-isozymes have been characterized; they can be divided into four isotypes, namely, β, γ, δ and ε (Fain, 1990; Rhee and Bae, 1997; Song et al., 2001). Many growth factors such as platelet-derived-growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) and nerve growth factor (NGF) elicit tyrosine phosphorylation of PLC-γ1 with stimulation of PtdIns(4,5)P_2 turnover in a wide variety of cells (Larose et al., 1993; Rotin et al., 1992; Peters et al., 1992; Obermeier et al., 1993).

Unlike other PLC isotypes, PLC-γ1 contains two Src homology (SH) 2 domains and one SH3 domain between the X and Y catalytic domains (Williams and Katan, 1996), which have been implicated in the regulation of cellular proliferation and growth. Overexpression of the SH2-SH2-SH3 domain of PLC-γ1 induced proliferation and transformation in 3Y1 rat fibroblasts (Chang et al., 1997). The SH2 domains of PLC-γ1 have been implicated in the association between PLC-γ1 and activated receptor tyrosine kinases, and the SH3 domain of PLC-γ1 has been reported to be responsible for the mitogenic effect of PLC-γ1. Overexpression of the SH2-SH2-SH3 domain of PLC-γ1 induces proliferation and transformation in 3Y1 rat fibroblasts, an effect mediated selectively by the SH3 domain (Chang et al., 1997; Smith et al., 1996). In addition, a PLC-γ1 mutant lacking the lipase activity still induced DNA synthesis, suggesting that PLC-γ1 may regulate the proliferation and mitogenic signaling regardless of its lipase activity (Huang et al., 1995). Thus, PLC-γ1 exerts other actions that are independent of its lipase activity and appears to be involved in the SH3 domain. Furthermore, PLC-γ1 augments agonist-induced calcium entry into cells and serves as a guanine nucleotide exchange factor (GEF) for PIKE, a nuclear protein that stimulates phosphatidylinositol 3-kinase (PI 3-kinase) activity (Patterson et al., 2002; Ye et al., 2002). Both

of these actions are lipase-independent and involve the SH3 domain of PLC-γ1.

Dynamin is a member of the GTPase superfamily that essentially participates in clathrin-mediated endocytosis in cells. Three mammalian isoforms of dynamin have been detected (Liu and Robinson, 1995; Urrutia et al., 1997). Dynamin-1 is exclusively expressed in neuronal cells (Nakata et al., 1991). But dynamin-2 is ubiquitously expressed (Cook et al., 1994) and dynamin-3 is primary expressed in Sertoli cells of the testis (Nakata et al., 1993). Dynamin's role in endocytosis was first revealed by phenotype analysis of a *Drosophila* homologue mutant, *shibire* (Urrutia et al., 1997; Warnock and Schmid, 1996). The role of dynamin in receptor-mediated endocytosis in mammalian cells has been confirmed by overexpression of dominant-negative mutants of dynamin (Herskovits et al., 1993; Damke et al., 1994). Furthermore, overexpression of mutant dynamin (K44A) inhibits not only uptake from clathrin-coated pits but also other types of endocytosis (Lamaze et al., 2001). However, its exact function remains controversial (Sever et al., 2000). There are some reports suggesting that dynamin participates in membrane fission mechanism as a mechanochemical enzyme (Hinshaw and Schmid, 1995; Smirnova et al., 1999; Stowell et al., 1999). However, recent studies indicate that dynamin functions as a regulatory molecule to activate effector molecules required for coated vesicle formation (Sever et al., 1999).

Dynamin has many functional domains (Liu and Robinson, 1995). In addition to the GTPase domain, dynamin also contains a pleckstrin homology domain (PH) implicated in membrane binding, a GTPase effector domain (GED) shown to be essential for self-assembly and stimulated GTPase activity. It has been reported that purified dynamin-1 self-assembles and forms rings and helical arrays (Hinshaw and Schmid, 1995). Self-assembly does not require guanine nucleotides, but does require the C-terminal proline-rich domain (PRD) of dynamin-1. Dynamin's PRD participates in the interaction with a large number of SH3 domain-containing proteins such as amphiphysin, intersectin, endophillin and Grb2 (David et al., 1996; Ringstad et al., 1997; Yamabhai et al., 1998; Gout et al., 1993). Dynamin's partners may either stimulate dynamin's GTPase activity or target dynamin to the plasma membrane. However, the function of these proteins in membrane trafficking and endocytosis remains to be determined.

Interestingly, some results indicate that GTP-bound dynamin controls a rate-limiting step in endocytosis by recruiting downstream effector molecules (Sever et al., 1999). GTP-bound dynamin controls the formation of constricted coated pits, and the stimulated rate of GTP-hydrolysis may switch dynamin-1 to off and release it from the membrane so that it does not impede membrane fission (Sever et al., 2000). We demonstrate that PLC-γ1 serves as a GEF for dynamin-1 through the direct interaction of its SH3 domain. Furthermore, the GEF activity of PLC-γ1 can regulate EGF-induced ERK activation and up-regulation of SRE-dependent transcription.

Materials and Methods

Plasmids

Src homology (SH) domains of PLC-γ1 were generated by PCR amplification using rat PLC-γ1 cDNA as a template (Suh et al., 1988), and subcloned into pGEX-4T2 plasmids (Pharmacia Biotechnology) for expression as glutathione S-transferase (GST)-fusion proteins. The mammalian expression vector for FLAG-epitope tagged wild-type, lipase-inactive mutant (LIM), deleting mutant (SH2-SH2 and SH3 domains) of PLC-γ1 was made by PCR. The amplified products were inserted in-frame with the FLAG-epitope tag of pFLAG-CMV-2 (Sigma). The mutation of Pro-842 to Leu, designated P842L was introduced into the FLAG-epitope tagged rat PLC-γ1 cDNA by PCR-directed mutagenesis. The construction of mutants of dynamin-1 was also performed by PCR-directed mutagenesis as previously described (Warnock et al., 1997).

Cell culture

Rat pheochromocytoma PC12 cells (Clontech, CA) were cultured in medium A (DMEM medium supplemented with 10% heat-inactivated horse serum and 5% bovine calf serum) at 37°C in a humidified incubator. The FLAG-tagged PLC-γ1 stably transfected PC12 cells (tet-off cell line) were cultured in medium B (DMEM supplemented with 10% heat-inactivated horse serum and 5% fetal calf serum, 100 μg/ml G418, 100 μg/ml hygromycin B, 2 μg/ml tetracyclin).

In vitro binding assay and co-immunoprecipitation

EGF- and NGF-stimulated PC12 cells were solubilized with lysis buffer A (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 20 mM NaF, 200 μM sodium orthovanadate, 1 mM PMSF, 1 μg/ml leupeptin, 5 μg/ml aprotinin and 2 μM pepstatin A), and cell lysates incubated with 5 μg of GST-fusion proteins were immobilized on glutathione-agarose beads for 1.5 hours at 4°C. PC12 cells were washed with PBS and lysed with lysis buffer A. The cell lysates were mixed with 3 μg of anti-dynamin-1 antibody pre-coupled to protein A-Sepharose for 30 minutes at 4°C. The immunocomplexes were collected by centrifugation and washed four times with cold lysis buffer A.

Purification of dynamin-1 from rat brain

Purification of dynamin-1 from rat brain was as previously described (Gout et al., 1993). After purification, we confirmed the purity of dynamin-1 by staining with Coomassie Brilliant Blue (CBB).

Nucleotides exchange assay

The nucleotide exchange assay for binding of [^{35}S]GTP-γS to purified dynamin-1 and the dissociation of [^{3}H]GDP from dynamin-1 were as previously described (Zheng et al., 1995). Briefly, dynamin-1 (50 nM) and 50 nM GST-fused proteins were pre-incubated together in assay buffer at 37°C for 30 minutes, and the binding initiated by addition of 3 mM [^{35}S]GTP-γS (2 mCi/ml). The binding of [^{35}S]GTP-γS to dynamin-1 was examined in assay buffer (0.1 μg/μl BSA, 50 nM dynamin, 20 mM Tris, pH 7.6, 1 mM DTT, 5 mM $MgCl_2$, and 1 mM EDTA) and all reactions were carried out at 22°C and the aliquots were removed at the indicated times. Radioactive dynamin-1 was filtered through nitrocellulose filters. The GDP displacement assay was initiated by preloading dynamin-1 with 1 μM [^{3}H]GDP and the dissociation was triggered by adding 0.5 mM unlabelled GTP with 50 nM GST-fused proteins. All reactions were carried out at 25°C in assay buffer. Dissociation of [^{3}H]GDP was assayed by measuring the decrease in [^{3}H]GDP-dynamin-1 trapped on nitrocellulose filters.

GTP loading assay

PC12 cells were metabolically labeled in phosphate-free DMEM with 0.5 mCi/ml [^{32}P]H_3PO_4 for 4 hours at 37°C and treated with EGF for the indicated times. The cell lysates were incubated with 3 μg anti-dynamin antibody (BD Biosciences, CA, USA) for 30 minutes, and supernatant was added to 0.05 ml protein A Sepharose (50% slurry)

and rocked for 30 minutes at 4°C then washed twice with lysis buffer A and once with PBS. Subsequent steps were performed as described previously (Jeong et al., 2001; Rosen et al., 1994).

Receptor internalization assay

Receptor internalization assay was performed as previously described with minor modification (Lu et al., 2002). PC12 cells were cultured to 60-70% confluency prior to labeling with *N*-hydroxy-succinimide-biotin (Pierce, IL, USA) (1.5 mg/ml). After labeling with NHS-SS-biotin, the cells were incubated at 37°C for the indicated times in the presence or absence of EGF. Endocytosis was then stopped by transferring cells back to 4°C. After treatment with reducing solution (15.5 mg/ml glutathione, 75 mM NaCl, 75 mM NaOH, and 10% fetal bovine serum) and 5 mg/ml iodoacetamide in phosphate-buffered saline containing 0.8 mM $MgCl_2$, 1 mM $CaCl_2$ plus 1% bovine serum albumin, the cells were lysed in TNE (10 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40 and 1 mM EDTA). Equal amounts of cell lysates were used for precipitation of biotinylated proteins with streptavidin beads.

siRNA for PLC-γ1

Small interfering RNA (siRNA) of PLC-γ1 (PLC-γ1 siRNA #2) was purchased by Dharmacon (Lafayette, CO, USA). Other siRNA were synthesized. The sequences of siRNA for luciferase (control siRNA) were: sense, 5′-CUUACGCUGAGUACUUCGAdTdT-3′; antisense, 5′-UCGAAGUACUCAGCGUAAGdTdT-3′.

The sequences of siRNA for PLC-γ1 (PLC-γ1 siRNA #1), which did not affect the expression of PLC-γ1 were: sense, 5′-GAGCG-CCAUCAUCCAGAAUdTdT-3′; antisense, 5′-AUUCUGGAUGAU-GGCGCUCdTdt-3′. Double-stranded RNA was produced using conditions as described previously (Patterson et al., 2002). siRNA were transfected into PC12 cells using LipofecAMINE (Invitrogen, CA, USA).

Immunofluorescence analysis

PC12 cells on coverslips were treated with fluorescently labeled (rhodamine-conjugated)-EGF (Molecular Probe, OR, USA) for the indicated times. The cells were then washed with ice-cold Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline and fixed with freshly prepared 4% paraformaldehyde (Sigma) for 30 minutes at 4°C. The coverslips were mounted into chambers and the cells examined by confocal microscopy.

Reporter gene assay

PC12 cells were grown in poly-L-lysine-coated 24-well plates and transfected with serum responsive element (SRE)-luciferase plasmid using LipofectAMINE reagent (Invitrogen, CA, USA). After 36 hours, the cells were stimulated with EGF (10 ng/ml) for the indicated times. After washing with PBS and lysis, the luciferase activity in 1 μg of lysate was assayed using a luciferase assay kit (Promega, WI, USA) with a luminometer (Labsystems, UK).

Results

The SH3 domain of PLC-γ1 functions as guanine nucleotide exchange factor (GEF) for dynamin-1 in vitro

To understand how the SH2 or SH3 domains mediate the diverse actions of PLC-γ1, we sought putative binding partners using GST-fused proteins bearing the SH2-SH2 domain (GST-SH2-SH2) or SH3 domain (GST-SH3) of PLC-γ1. From MALDI-TOF mass spectrometry analysis, we identified that the proline-rich domain (PRD) of dynamin-1 directly interacts with the SH3 domain of PLC-γ1 (data not shown) (Gout et al., 1993; Scaife et al., 1994). Dynamin-1 is a GTPase whose GTP-bound form recruits downstream effectors (Sever et al., 1999). To test whether PLC-γ1 has guanine nucleotide exchange activity for dynamin-1, we examined the effect of the SH3 domain on dynamin-1's [^{35}S]GTP-γS binding and [^{3}H]GDP dissociation (Fig. 1A,B). The SH3 domain of PLC-γ1 (PLC-γ1-SH3) markedly increased the [^{35}S]GTP-γS-bound form of dynamin-1 and the dissociation of [^{3}H]GDP from dynamin-1, while the SH3 domains of Grb2 or amphiphysin had negligible effects (Fig. 1A,B). Also, purified full-length PLC-γ1 increased the GTP-binding activity of dynamin-1 in vitro (data not shown). However, a mutation of proline to leucine at position 842 in the SH3 of PLC-γ1 (P842L), which abolishes association with its effector proteins, resulted in no interaction with dynamin-1 and failed to stimulate binding by dynamin-1 of [^{35}S]GTP-γS or the dissociation of [^{3}H]GDP (Fig. 1A,B,D). When the amount of PLC-γ1-SH3 was increased, [^{35}S]GTP-γS binding to dynamin-1 was significantly enhanced, but P842L did not increase the binding of [^{35}S]GTP-γS to dynamin-1 (Fig. 1C). These results strongly indicate that PLC-γ1-SH3 specifically acts as a guanine nucleotide exchange factor (GEF) for dynamin-1 in vitro.

The SH3 domain of PLC-γ1 functions as GEF for dynamin-1 in vivo

We next explored GEF action of PLC-γ1 on dynamin-1 in vivo by overexpressing wild-type PLC-γ1 (PLC-γ1^{WT}) in PC12 cells using the tetracycline-off system after EGF treatment (Ye et al., 2002). EGF-activated GTP binding of dynamin-1 in PC12 cells peaking at 5 minutes (Fig. 2B). In addition, we tried to examine the influence of EGF by investigating the interaction between endogenous PLC-γ1 and dynamin-1 from PC12 cells. Dynamin-1 maximally co-precipitated with PLC-γ1 after EGF treatment for 5 minutes (Fig. 2A). Consistent with our results, it has been reported that dynamin-1 associated with PLC-γ1 after platelet growth factor (PDGF) stimulation (Scaife et al., 1994). These results indicate that the interaction between PLC-γ1 and dynamin-1 is regulated by EGF as well as PDGF and GEF activity of PLC-γ1 is induced by the association with dynamin-1. To investigate GEF action of endogenous PLC-γ1 on dynamin-1, we employed small interfering RNA (siRNA). Transfection of PLC-γ1-directed siRNA constructs completely diminished expression of PLC-γ1, but the expression of PLC-β1, -β3 and -γ2 did not change (Fig. 2C). Interestingly, after EGF treatment, the augmentation of GTP-bound dynamin-1 was diminished by PLC-γ1-directed siRNA constructs in PC12 cells at 5 minutes (Fig. 2D). From these results, we conclude that PLC-γ1 physiologically acts as a GEF on dynamin-1.

The interaction of PLC-γ1 with dynamin-1 is essential for its GEF activity

To check the effect of PLC-γ1 lipase activity on the GEF activity for dynamin-1, we established PC12 cell lines stably expressing PLC-γ1^{WT}, lipase-inactive mutants (PLC-γ1^{LIM}), PLC-γ1 lacking the SH3 domain (PLC-γ1$^{\Delta SH3}$), a point mutant of PLC-γ1 in the SH3 domain replacing proline-842 with leucine (PLC-γ1^{P842L}) (DeBell et al., 1999), and the SH3 domain of PLC-γ1 only (PLC-γ1^{SH3}). Overexpression of PLC-

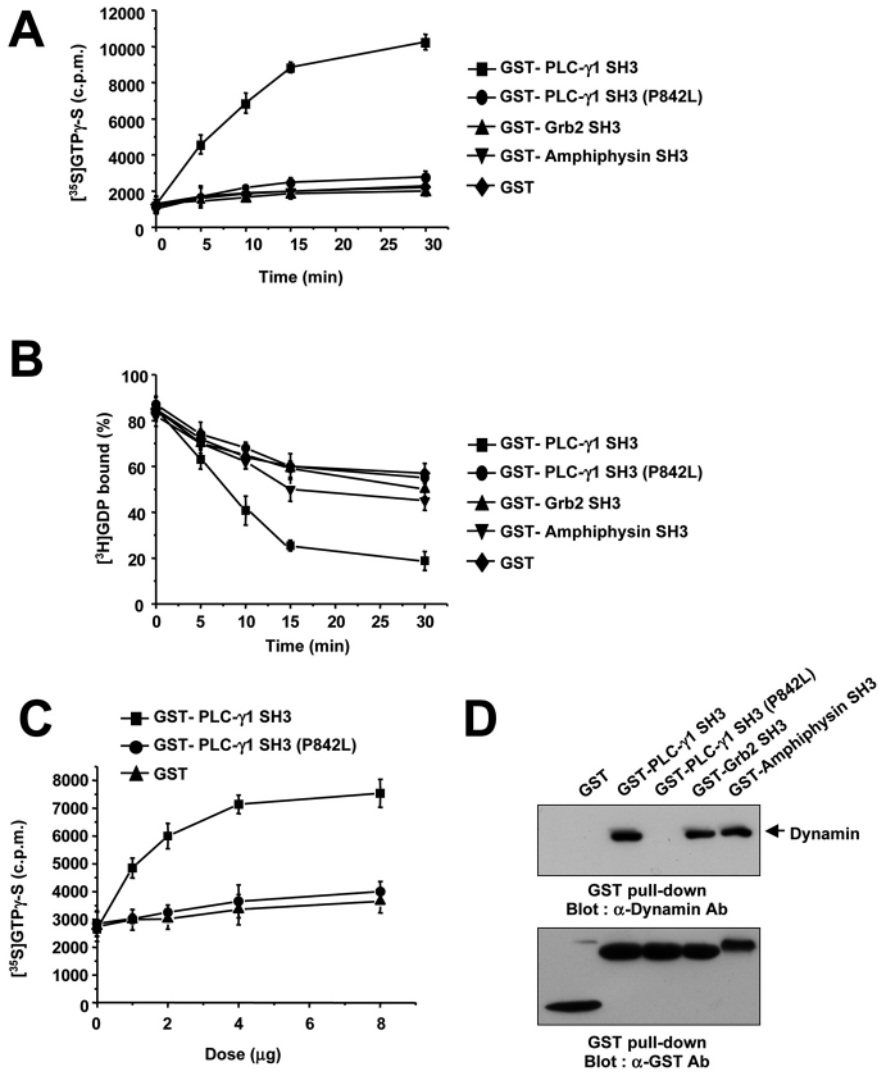

Fig. 1. The SH3 domain of PLC-γ1 functions as a guanine nucleotide exchange factor (GEF) for dynamin-1 in vitro. (A) Purified dynamin-1 (50 nM) together with purified GST (diamonds), GST-PLC-γ1 SH3 (squares), GST-PLC-γ1 SH3 (P842L) (circles), GST-Grb2 SH3 (triangles) or GST-amphiphysin SH3 fusion proteins (inverted triangles) (50 nM) were incubated with [^{35}S]GTP-γS. At the indicated times, radio-labeled dynamin-1 was measured by a nucleotide exchange assay as described in Materials and Methods. (B) Purified dynamin-1 (50 nM) was preloaded with 1 μM [^{3}H]GDP for 30 minutes at 22°C. Purified GST (diamonds), GST-PLC-γ1 SH3 (squares), GST-PLC-γ1 SH3 (P842L) (circles), GST-Grb2 SH3 (triangles) or GST-amphiphysin SH3 fusion proteins (inverted triangles) (50 nM) were added together with 0.5 mM unlabelled GTP at the start of the assay. At the time intervals indicated, the dynamin-1-bound radioactivity was measured by a filter-binding assay. The data is expressed as the percentage of [^{3}H]GDP bound to dynamin-1 before the addition of unlabelled GTP. (C) Purified dynamin-1 (1 μM) and purified GST (triangles), GST-PLC-γ1 SH3 (squares) or GST-PLC-γ1 SH3 (P842L) (squares) were incubated with radioactive labeled [^{35}S]GTP-γS. At the indicated dose of GST-fused proteins, radiolabeled dynamin-1 was measured by a nucleotide exchange assay. (D) Purified dynamin-1 was incubated with GST, GST-PLC-γ1 SH3, GST-PLC-γ1 SH3 (P842L), GST-Grb2 SH3 or GST-amphiphysin SH3 fusion proteins coupled to glutathione-Sepharose beads. Bound proteins were analyzed by immunoblotting with anti-dynamin-1 antibody.

γ1^{WT} or PLC-γ1^{LIM} augmented the GTP-binding activity of dynamin-1 indicating that lipase activity is not required for PLC-γ1 GEF activity. The importance of the SH3 domain is evident by the failure of PLC-γ1$^{\Delta SH3}$ or PLC-γ1^{P842L} expression to stimulate the GTP-binding activity of dynamin-1 (Fig. 3B). PLC-γ1^{LIM} as well as PLC-γ1^{WT} interacted with dynamin-1 in an EGF-dependent manner, whereas neither PLC-γ1$^{\Delta SH3}$ nor PLC-γ1^{P842L} interacted (Fig. 3A). Notably, overexpression of PLC-γ1^{SH3} also stimulated the GTP-binding activity of dynamin-1 (Fig. 3B). In addition, the PRD deleting mutant of dynamin-1 (dynamin-1$^{\Delta PRD}$) did not interact with PLC-γ1 after EGF treatment (Fig. 3C), and GTP-binding to dynamin-1$^{\Delta PRD}$ was inhibited in PC12 cells overexpressing PLC-γ1^{WT} (Fig. 3D). These results establish that the GEF activity of PLC-γ1 for dynamin-1 is independent of its lipase activity and specifically requires the SH3 domain of PLC-γ1.

GEF activity of PLC-γ1 for dynamin-1 enhances dynamin-1-dependent endocytosis

Dynamin-1 has been implicated in receptor-mediated endocytosis and in recycling of synaptic vesicles in neurons (McPherson et al., 2001; Hinshaw, 2000). Specifically, GTP-bound dynamin controls the formation of constricted coated pits and up-regulates receptor-mediated endocytosis (Sever et al., 2000). To assess the requirement of PLC-γ1 GEF activity for dynamin-1-dependent endocytosis, we quantified the amounts of internalized EGF receptor (EGFR) in PC12 cells after EGF stimulation. The increase of internalized EGFR was enhanced in PC12 cells transfected with either PLC-γ1^{WT} or PLC-γ1^{LIM}, but not in PC12 cells transfected with PLC-γ1$^{\Delta SH3}$ or PLC-γ1^{P842L} (Fig. 4A). These results suggest that PLC-γ1 accelerates dynamin-1-dependent endocytosis of EGFR by increasing the GTP-binding activity of dynamin-1.

Next, we examined the role of endogenous PLC-γ1 on receptor-mediated endocytosis. Transfection of PLC-γ1-directed siRNA constructs completely diminished expression of PLC-γ1 and significantly decreased EGF-induced EGFR internalization (Fig. 4B). Furthermore, overexpression of dynamin-1^{WT} enhanced EGF-induced EGFR endocytosis, but dynamin-1$^{\Delta PRD}$ diminished EGFR internalization (Fig. 4C). In contrast to EGFR, the internalization rate of transferrin receptors was not affected by overexpression of PLC-γ1^{WT} (data not shown), indicating the specific role of PLC-γ1 in EGFR endocytosis.

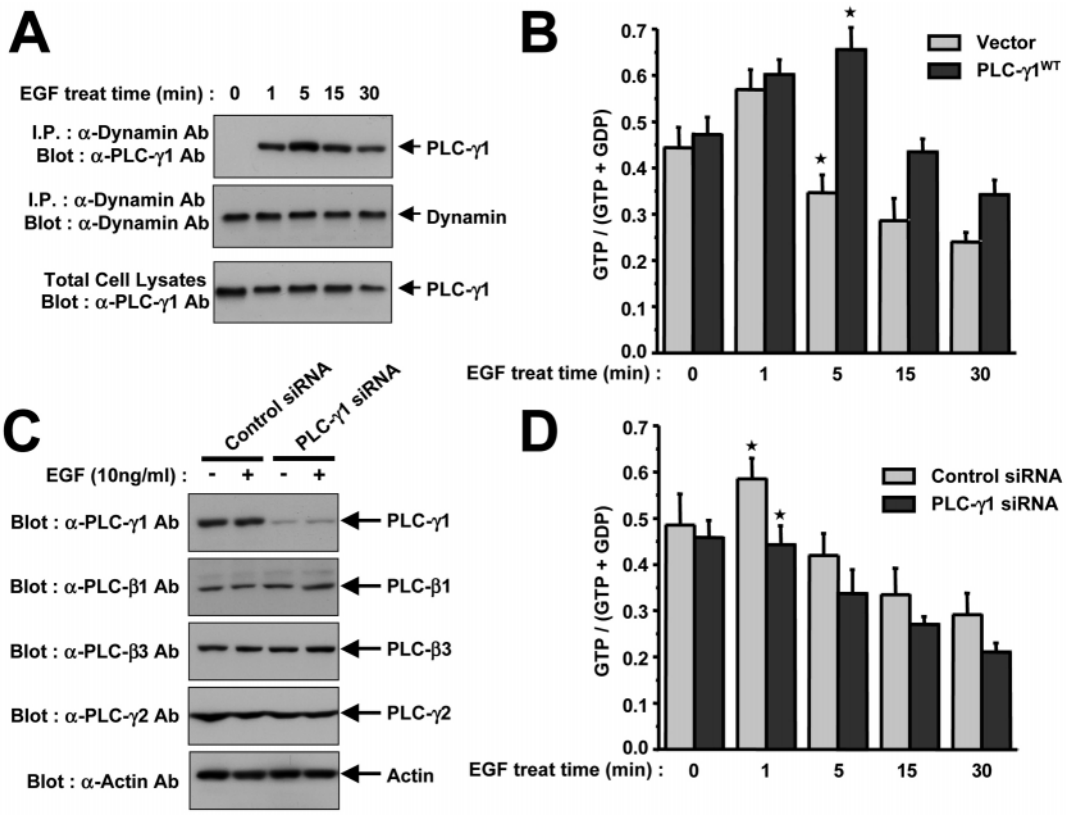

Fig. 2. The SH3 domain of PLC-γ1 functions as a guanine nucleotide exchange factor (GEF) for dynamin-1 in vivo. (A) PC12 cells stably transfected with PLC-γ1 constructs under a tet-off system were induced to express wild-type PLC-γ1. Cells were then treated with 10 ng/ml EGF for the indicated times. Co-immunoprecipitated PLC-γ1 was analyzed by immunoblotting with anti-PLC-γ1 antibody. (B) PC12 cells stably transfected with wild-type PLC-γ1 were metabolically labeled in phosphate-free DMEM with 0.5 mCi/ml $[^{32}P]H_3PO_4$ for 4 hours at 37°C and treated with EGF for 5 minutes. Dynamin-1 was immunoprecipitated with anti-dynamin-1 antibody, and bound guanine nucleotides were eluted with 1M KH_2PO_4 and separated on TLC plates. The ratio of GTP was calculated as GTP/(GTP+GDP) (Jeong et al., 2001; Rosen et al., 1994). (C) PC12 cells transfected with specific siRNA for PLCγ1 (described in Materials and Methods) were stimulated with EGF for the indicated times. The expression of PLC-β1, PLC-β3 and PLC-γ2 were analyzed by immunoblotting with anti-PLC-β1, PLC-β3 and PLC-γ2 antibodies. (D) PC12 cells were transfected with siRNA for PLC-γ1 metabolically labeled in phosphate-free DMEM with 0.5 mCi/ml $[^{32}P]H_3PO_4$ for 4 hour sat 37°C and treated with EGF for 5 minutes. The bound guanine nucleotides to dynamin-1 were analyzed by TLC. The ratio of GTP was calculated as GTP/(GTP+GDP). Stars indicate a significant difference compared with the control.

A previous report suggested that the SH3 domain of amphiphysin recruits dynamin to coated pits in vivo and inhibits EGF-induced EGFR endocytosis (Wigge et al., 1997). Therefore we investigated the effect of the SH3 domain of PLC-γ1 on dynamin-1-dependent endocytosis. As shown in Fig. 4D, overexpression of PLC-$\gamma1^{SH3}$ significantly increased EGFR endocytosis, but amphiphysin^{SH3} inhibited endocytosis. Consistent with this result, our nucleotide exchange data suggest that amphiphysin^{SH3} did not augment the binding of GTP by dynamin-1 or the dissociation of GDP (Fig. 1A,B). These results suggest that the GEF action of the SH3 domain of PLC-γ1 on dynamin-1 potentiates dynamin-1-dependent EGFR endocytosis.

We also examined the influence of PLC-γ1 on the EGF-induced internalization of EGF-EGFR complexes by using confocal microscopy. PC12 cells were stably transfected with empty vector, PLC-$\gamma1^{WT}$, or PLC-$\gamma1^{P842L}$, and the endocytic transport of fluorescently labeled (Rhodamine-conjugated) EGF was measured. Transfection of PLC-$\gamma1^{WT}$ augmented the amount of Rhodamine-labeled EGF in intracellular vesicular compartments after treatment with EGF, while PLC-$\gamma1^{P842L}$ had no effect (Fig. 5A). Furthermore, transfection of PLC-γ1-directed siRNA constructs attenuated the uptake of Rhodamine-EGF compared to the control siRNA constructs (Fig. 5B). Taken together, these results strongly suggest that PLC-γ1 acts as a physiological GEF for dynamin-1 through its SH3 domain and PLC-γ1 GEF activity potentiates dynamin-1-dependent endocytosis.

GEF activity of PLC-γ1 for dynamin upregulates dynamin-1-dependent ERK activation and SRE-dependent transcriptional activity

Previous reports showed that dynamin-1-dependent endocytosis is required for activation of the MAP kinase cascade (Kranenburg et al., 1999). We investigated whether PLC-γ1 regulates ERK activation by increasing dynamin-1-dependent EGFR endocytosis. Expression of both PLC-$\gamma1^{WT}$ and PLC-$\gamma1^{LIM}$ stimulated EGF-dependent ERK activation compared to empty vector-expression, while overexpression of PLC-$\gamma1^{\Delta SH3}$ or PLC-$\gamma1^{P842L}$ was ineffective (Fig. 6A). PLC-γ1 knock-down by siRNA substantially reduced ERK activation

(Fig. 6B). Furthermore, overexpression of PLC-γ1^{SH3} enhanced the activation of ERK, but not amphiphysin^{SH3} (Fig. 6C). As ERK activation stimulates serum response element (SRE)-dependent transcription (Whitmarsh et al., 1995), we examined the influence of PLC-γ1 on SRE-dependent transcription using a SRE-luciferase reporter gene assay. In parental PC12 cells, EGF-induced SRE activation elicited a 2-fold increase in luciferase activity, and overexpression of PLC-γ1^{WT} caused a 3-fold response (Fig. 7). However, overexpression of PLC-γ1^{P842L} failed to increase SRE-dependent transcriptional activity. Taken together, these results imply that potentiation of endocytic processes by PLC-γ1 GEF activity can mediate EGF-induced ERK activation and up-regulation of SRE-dependent transcription.

Discussion

The main finding of this study is that PLC-γ1 binds to dynamin-1 through its SH3 domain for which it serves as a GEF. This GEF activity regulates the influence of dynamin-1 upon EGFR endocytosis, ERK activation and SRE-dependent transcription. Interestingly, the SH3 domains of various proteins such as amphiphysin and endophilin interact with dynamin-1 and are involved in dynamin-1-dependent

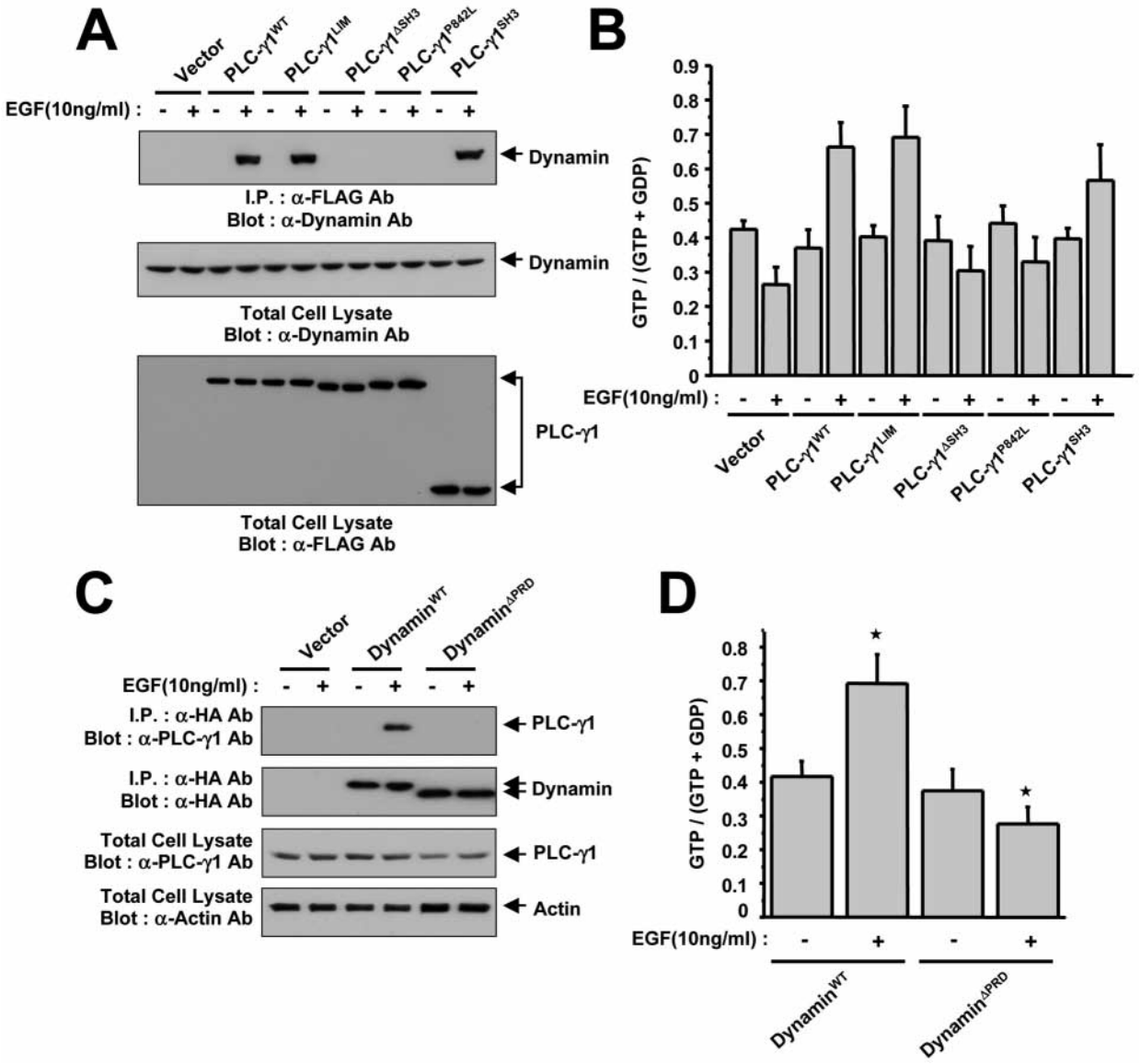

Fig. 3. The interaction of PLC-γ1 with dynamin-1 is essential for its GEF activity for dynamin-1. (A) PC12 cells stably transfected with various PLC-γ1 constructs were immunoprecipitated with anti-FLAG antibody. The cells were then immunoblotted with anti-dynamin antibody. (B) The stably transfected PC12 cells were metabolically labeled in phosphate-free DMEM with 0.5 mCi/ml [^{32}P]H_3PO_4 for 4 hours at 37°C and treated with EGF for the indicated times. The bound guanine nucleotides to dynamin-1 were analyzed by TLC. The ratio of GTP was calculated as GTP/(GTP+GDP). (C) PC12 cells overexpressing PLC-γ1^{WT} were transfected with HA-tagged dynamin-1^{WT} and dynamin-1^{ΔPRD} constructs. Immunoprecipitation with anti-HA antibody was followed by immunoblotting with anti-PLC-γ1 and anti-HA antibody. (D) PC12 cells overexpressing PLC-γ1^{WT} were transfected with HA-dynamin-1 and its mutant. The bound guanine nucleotides to dynamin-1 were analyzed by TLC. The ratio of GTP was calculated as GTP/(GTP+GDP). Stars indicate a significant difference compared with the control.

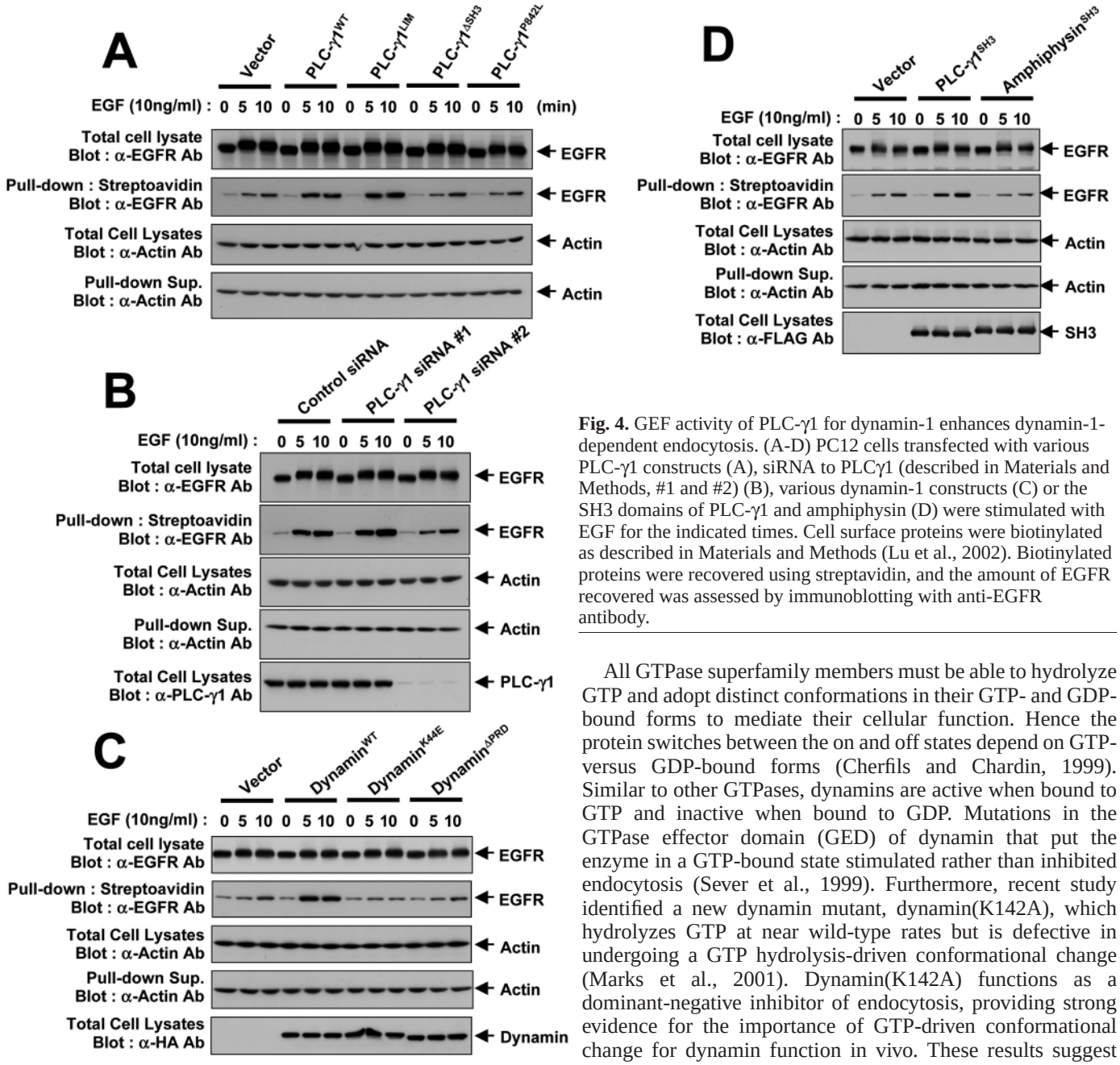

Fig. 4. GEF activity of PLC-γ1 for dynamin-1 enhances dynamin-1-dependent endocytosis. (A-D) PC12 cells transfected with various PLC-γ1 constructs (A), siRNA to PLCγ1 (described in Materials and Methods, #1 and #2) (B), various dynamin-1 constructs (C) or the SH3 domains of PLC-γ1 and amphiphysin (D) were stimulated with EGF for the indicated times. Cell surface proteins were biotinylated as described in Materials and Methods (Lu et al., 2002). Biotinylated proteins were recovered using streptavidin, and the amount of EGFR recovered was assessed by immunoblotting with anti-EGFR antibody.

endocytosis (Schmid et al., 1998). Amphiphysin has been implicated in the endocytic process by studies where an inhibition of endocytosis was observed when its SH3 domain was microinjected into the synapse of the giant lamprey or transfected into fibroblasts (Shupliakov et al., 1997; Wigge et al., 1997). Furthermore, the SH3 domain of endophilin and syndapin inhibit coated-vesicle formation in vitro (Simpson et al., 1999). However, there is no evidence that the proteins interacting with dynamin can switch the conformation of GTP- or GDP-bound forms. We have demonstrated that the PLC-γ1 SH3 domain significantly facilitates the nucleotide exchange reaction of dynamin-1 and EGF-induced EGFR endocytosis. These results suggest that PLC-γ1 appears to be a key component of the dynamin-1-dependent endocytic machinery.

All GTPase superfamily members must be able to hydrolyze GTP and adopt distinct conformations in their GTP- and GDP-bound forms to mediate their cellular function. Hence the protein switches between the on and off states depend on GTP- versus GDP-bound forms (Cherfils and Chardin, 1999). Similar to other GTPases, dynamins are active when bound to GTP and inactive when bound to GDP. Mutations in the GTPase effector domain (GED) of dynamin that put the enzyme in a GTP-bound state stimulated rather than inhibited endocytosis (Sever et al., 1999). Furthermore, recent study identified a new dynamin mutant, dynamin(K142A), which hydrolyzes GTP at near wild-type rates but is defective in undergoing a GTP hydrolysis-driven conformational change (Marks et al., 2001). Dynamin(K142A) functions as a dominant-negative inhibitor of endocytosis, providing strong evidence for the importance of GTP-driven conformational change for dynamin function in vivo. These results suggest that GTP-bound dynamin controls a rate-limiting step in its functions such as the formation of constricted clathrin-coated pits. In the present study, we demonstrate that the SH3 domain of PLC-γ1 is a robust GEF for dynamin-1 by assaying guanine nucleotide exchange activity in vitro (Fig. 1). Furthermore, specific reduction of endogenous PLC-γ1 by siRNA reduced the contents of GTP-bound dynamin-1 (Fig. 2D). From these results, we suggest that the SH3 domain of PLC-γ1 may physiologically regulate the function of dynamin-1 by stimulating the dissociation of bound GDP from dynamin-1 and the association of GTP with dynamin-1.

GEFs are critical regulators of the timing and localization of the activation of GTPases. GEFs stimulate the dissociation of tightly bound GDP nucleotide from GTP-binding proteins in response to upstream signals (Cherfils and Chardin, 1999). The catalytic domains of the different classes of GEFs share no

sequence homology and are structurally unrelated. However, they have very similar substrates and the functions. GEFs are critical regulators of timing and localization of the activation of GTPases. Their complex domain structure enables them to activate their repressed exchange activity (Quilliam et al., 2002; Hoffman and Cerione, 2002). More than 50 identified

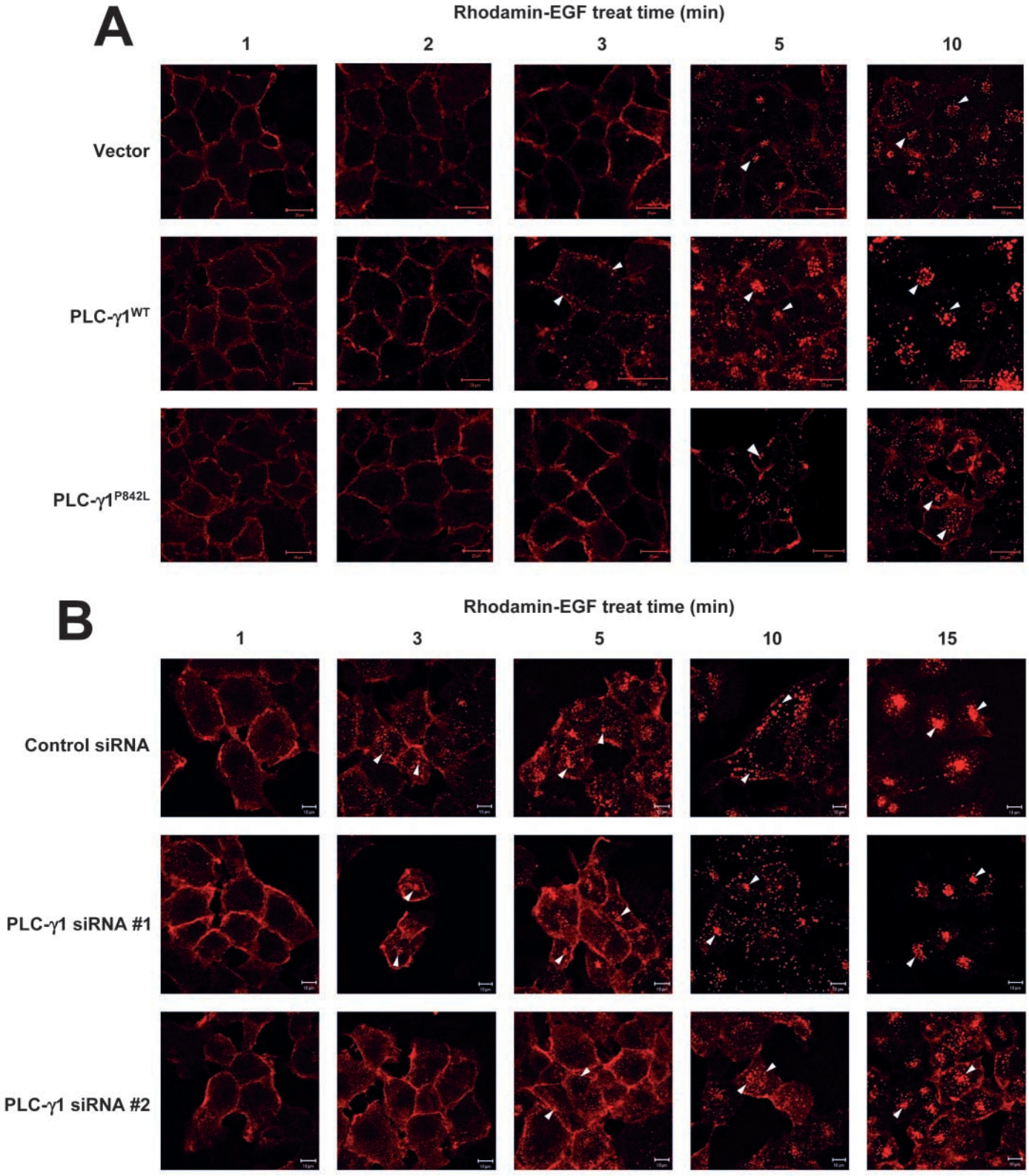

Fig. 5. PLC-γ1's GEF activity potentiates dynamin-1-dependent EGFR endocytosis. PC12 cells transfected with various PLC-γ1 constructs (A) or PLC-γ1 siRNA duplexes (B) were incubated with Rhodamine-conjugated EGF at the indicated times and fixed cells were examined by confocal microscopy. Arrowheads indicate the internalized EGF-EGFR vesicles.

GEFs contain various combinations of protein-protein interaction domains including Dbl homology (DH) and pleckstrin homology (PH) domains (Schmidt and Hall, 2002). DH domains are responsible for binding to Rho proteins and facilitating GDP-GTP exchange (Hart et al., 1991). PLC-γ1 lacks a DH domain, but has GEF activity for dynamin-1. Also, we previously reported that the SH3 domain of PLC-γ1 increases GTP loading activity of Ras and PIKE (Kim et al., 2000; Ye et al., 2002). Consistent with our findings, recent report suggests that a new domain in DOCK180 called Docker, which does not contain any homology to a DH domain, acts as a GEF for Rac and is both necessary and sufficient for Rac activation (Brugnera et al., 2002). Taken together, these results suggest that non-conventional GEFs participate in a different mechanism for interacting with and activating GTPases.

The mechanisms of dynamin's self-assembly are well understood. The PRD promotes dynamin self-assembly in vitro (Scaife et al., 1998). After deleting the PRD, dynamin no longer self-assembles under these conditions. Furthermore, it has been reported that dynamin is a tetramer and interdomain interactions are possible (Muhlberg et al., 1997). These interactions are proposed to occur between adjacent, antiparallel polypeptides. Through domain exchange between subunits, conformational change caused by GTP hydrolysis in one tetramer could be propagated throughout an assembled dynamin ring to affect concerted activity of the collar. Furthermore, Smirnova et al. suggested a series of binding interactions between three domains of dynamin: the GTPase domain, the middle domain and the assembly domain (Smirnova et al., 1999). Studies on the antiviral Mx proteins, which have low homology to dynamin, have suggested that the C-terminal domain of the protein folds back on to the GTPase domain and regulates its activity (Schwemmle et al., 1995). From these results, the regulation of the GTPase domain of one polypeptide by the GED and PRD of another polypeptide in the tetramer could allow all the molecules in a collar to 'talk' to each other, making such a concerted activity possible. In the present study, we observed that dynamin-1 no longer self-assembles by deletion of its PRD. Furthermore, the fragment flanking the GTPase domain associates with the C-terminal region of dynamin-1 and these two fragments still interact with the SH3 domain of PLC-γ1 (supplemental data Fig. S1, http://jcs.biologists.org/supplemental/). Taken together, these results provide evidence that the PLC-γ1 SH3 domain binds to the C-terminal region of dynamin-1, which associates with the GTPase domain. Thus, the SH3 domain of PLC-γ1 may directly stimulate the nucleotide exchange activity for dynamin-1.

Dynamin-1 mediates clathrin-dependent endocytosis of numerous proteins including transferrin receptors and various growth factor receptors such as EGFR, PDGFR and NGFR (Schmid et al., 1998). Cells expressing dynamin-1 mutants deficient in GTP binding (K44A, K44E or S45N) fail to internalize transferrin or EGF receptors (Damke et al., 1994). In our study, PLC-γ1 specifically regulated the EGF-induced endocytosis of EGFR by acting as a GEF for dynamin-1. Consistent with these results, a single tyrosine that serves as a docking site for PLC-γ1 on the FGF receptors mediates their endocytosis perhaps by the actions of PLC-γ1, such as reported here (Sorokin et al., 1994). The selective influence of PLC-γ1 on EGFR might reflect recruitment of PLC-γ1 to activated growth factor receptors through its SH2 domain, and interaction of its SH3 domain with dynamin-1. The direct interaction of PLC-γ1 with dynamin-1 increases the GEF activity of dynamin, and thus specifically accelerates the endocytosis of EGFR endocytosis.

Endocytosis has been proposed as an integral component of signaling cascades such as the MAP kinase pathway (Wiley and Burke, 2001), as expression of the GTP-binding and hydrolysis-defective K44A dynamin-1 mutant attenuates EGF-induced ERK1/2 activation (Vieira et al., 1996). Internalized EGFR is autophosphorylated and catalytically active (Lai et al., 1989), suggesting that the internalized EGF-EGFR complex maintains its ability to generate cell signaling from endosomes. This fits with our finding that the GEF

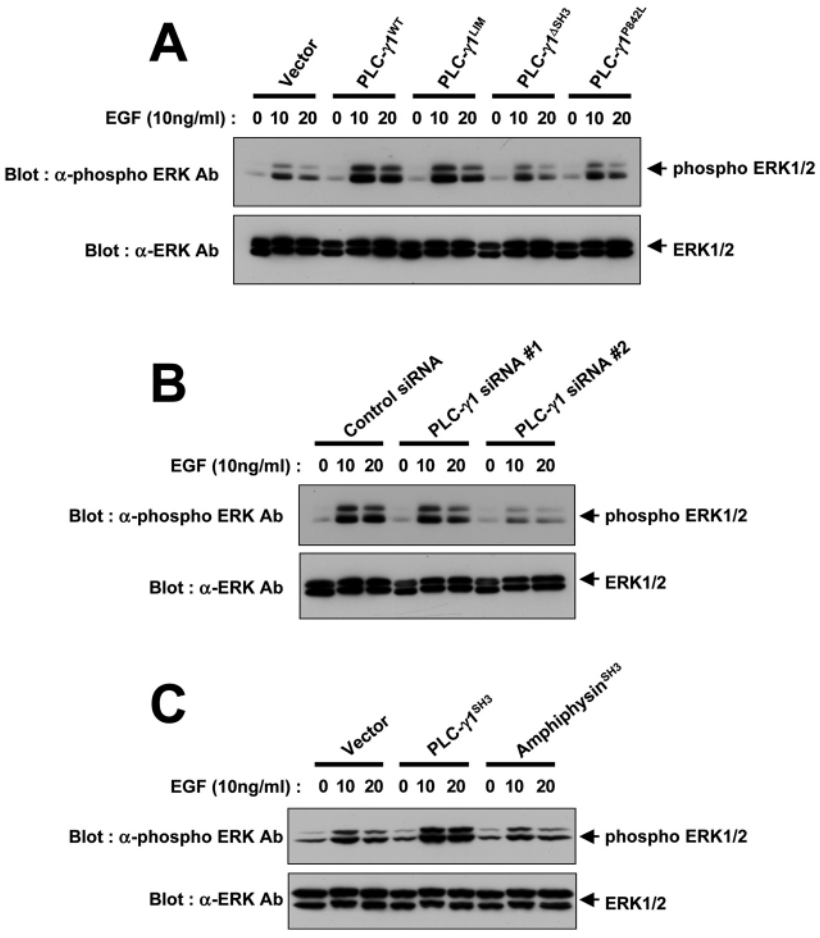

Fig. 6. GEF activity of PLC-γ1 for dynamin-1 upregulates dynamin-dependent ERK activation. PC12 cells transfected with various PLC-γ1 constructs (A), PLC-γ1 siRNA duplexes (#1 and #2) (B) or the SH3 domains of PLC-γ1 and amphiphysin (C) were treated with EGF for indicated times. Activation of ERK was measured by immunoblotting with phospho-ERK1/2 antibody. The total cell lysates were analyzed by immunoblotting with ERK1/2 antibody.

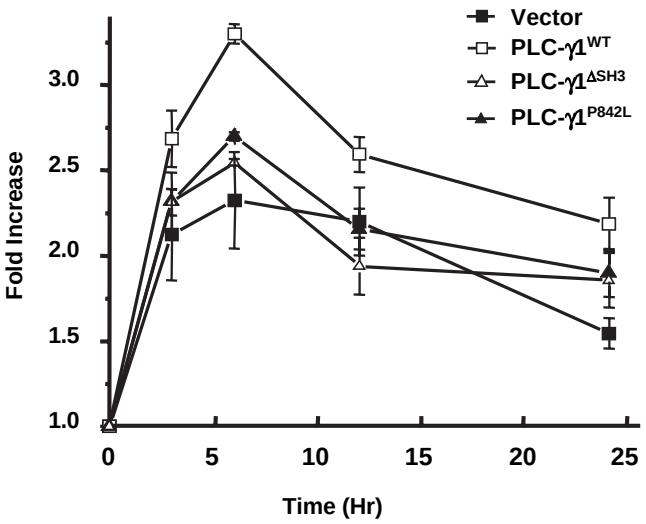

Fig. 7. GEF activity of PLC-γ1 for dynamin-1 upregulates dynamin-dependent SRE-dependent transcriptional activity. The SRE-luciferase reporter gene was transfected in PC12 cells which were stably transfected with indicated PLC-γ1 mutant genes (vector, closed squares; PLC-$\gamma1^{WT}$, open squares; PLC-$\gamma1^{\Delta SH3,}$ open triangles; PLC-$\gamma1^{P842L}$, closed triangles). EGF-induced increase of luciferase activity was quantified as mean ± s.d.

activity of PLC-γ1 for dynamin-1 leads to an enhanced rate of EGF-induced EGFR endocytic vesicular trafficking, and subsequently up-regulates ERK and SRE-dependent transcriptional activity. Furthermore, our experiments on PLC-γ1 depletion by siRNA of PLC-γ1 establish a role for PLC-γ1 in receptor-mediated endocytosis. Depletion of PLC-γ1 was significantly reduced in EGF-induced receptor internalization (Fig. 4B and Fig. 5B). Taken together, these results strongly suggest that PLC-γ1 GEF activity upregulates ERK and SRE-dependent signaling via EGFR endocytic vesicular trafficking. These results imply that endocytic processes can mediate activation of ERK.

It has been well known that PLC-γ1 plays a central role in growth factor-mediated signal transduction through SH domains (Nishibe et al., 1990). Microinjection of the SH3 domain of PLC-γ1 into NIH-3T3 cells induced mitogenesis, suggesting that the SH3 domain of PLC-γ1 is involved in proliferation (Smith et al., 1996). However, the means by which the growth factor-induced signaling is regulated by SH3 domain has not been elucidated. In the present study, we suggest a possible mechanism by which the SH3 domain of PLC-γ1 may be involved in the mitogenic actions. Cells expressing mutants in the SH3 domain of PLC-γ1 fail to up-regulate the activity of ERK or SRE-dependent transcription (Fig. 6A and Fig. 7). In addition, the PLC-γ1 SH3 domain alone potentiates GTP-binding to dynamin-1 and the activity of ERK (Fig. 3 and Fig. 6C). These results suggest that the GEF function of PLC-γ1 for dynamin-1 may link with PLC-γ1's mitogenic actions.

In conclusion, our results provide evidence that PLC-γ1 directly functions as a physiological GEF for dynamin-1 in vivo. Through acting as a GEF for dynamin-1, PLC-γ1 enhances EGFR-mediated endocytosis and therefore up-regulates activation of ERK and SRE-dependent transcriptional activity. These results provide the first evidence of physiological coupling between PLC-γ1-mediated signaling and dynamin-1-mediated endocytosis suggesting that PLC-γ1 may function as a key molecule in growth factor-induced proliferation through regulation of the endocytosis of growth factor receptors.

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References

Berridge, M. J. and Irvin, R. F. (1989). Inositol phosphates and cell signalling. *Nature* **341**, 197-205.

Brugnera, E., Haney, L., Grimsley, C., Lu, M., Walk, S. F., Tosello-Trampont, A. C., Macara, I. G., Madhani, H., Fink, G. R. and Ravichandran, K. S. (2002). Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex. *Nat. Cell. Biol.* **4**, 574-582.

Chang, J. S., Noh, D. Y., Park, I. A., Kim, M. J., Song, H., Ryu, S. H. and Suh, P. G. (1997). Overexpression of phospholipase C-gamma1 in rat 3Y1 fibroblast cells leads to malignant transformation. *Cancer Res.* **57**, 5465-5468.

Cherfils, J. and Chardin, P. (1999). GEFs: structural basis for their activation of small GTP-binding proteins. *Trends Biochem. Sci.* **24**, 306-311.

Cook, T. A., Urrutia, R. and McNiven, M. A. (1994). Identification of dynamin 2, an isoform ubiquitously expressed in rat tissues. *Proc. Natl. Acad. Sci. USA* **91**, 644-648.

Damke, H., Baba, T., Warnock, D. E. and Schmid, S. L. (1994). Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell Biol.* **127**, 915-934.

David, C., McPherson, P. S., Mundigl, O. and de Camilli, P. (1996). A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals. *Proc. Natl. Acad. Sci. USA* **93**, 331-335.

DeBell, K. E., Stoica, B. A., Veri, M. C., di Baldassarre, A., Miscia, S., Graham, L. J., Rellahan, B. L., Ishiai, M., Kurosaki, T. and Bonvini, E. (1999). Functional independence and interdependence of the Src homology domains of phospholipase C-gamma1 in B-cell receptor signal transduction. *Mol. Cell. Biol.* **19**, 7388-7398.

Fain, J. N. (1990). Regulation of phosphoinositide-specific phospholipase C. *Biochim. Biophys. Acta Mol. Cell Res.* **1053**, 81-88.

Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booker, G. W., Campbell, I. D. and Waterfield, M. D. (1993). The GTPase dynamin binds to and is activated by a subset of SH3 domains. *Cell* **75**, 25-36.

Hart, M. J., Eva, A., Evans, T., Aaronson, S. A. and Cerione, P. A. (1991). Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the dbl oncogene product. *Nature* **354**, 311-314.

Herskovits, J. S., Burgess, C. C., Obar, R. A. and Vallee, R. B. (1993). Effects of mutant rat dynamin on endocytosis. *J. Cell Biol.* **122**, 565-578.

Hinshaw, J. E. (2000). Dynamin and its role in membrane fission. *Annu. Rev. Cell. Dev. Biol.* **16**, 483-519.

Hinshaw, J. E. and Schmid, S. L. (1995). Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. *Nature* **374**, 190-192.

Hoffman, G. R. and Cerione, R. A. (2002). Signaling to the Rho GTPases: networking with the DH domain. *FEBS Lett.* **513**, 85-91.

Huang, P. S., Davis, L., Huber, H., Goodhart, P. J., Wegrzyn, R. E., Oliff, A. and Heimbrook, D. C. (1995). An SH3 domain is required for the mitogenic activity of microinjected phospholipase C-gamma 1. *FEBS Lett.* **358**, 287-292.

Jeong, M. J., Lee, S. S., Lee, K. I., Cho, A., Kwon, B. M., Moon, M. J., Park, Y. M. and Han, M. Y. (2001). Increased GTP-binding to dynamin II does not stimulate receptor-mediated endocytosis. *Biochem. Biophys. Res. Commun.* **283**, 136-142.

Kim, M. J., Chang, J. S., Park, S. K., Hwang, J. I., Ryu, S. H. and Suh, P. G. (2000). Direct interaction of SOS1 Ras exchange protein with the SH3 domain of phospholipase C-gamma1. *Biochemistry* **39**, 8674-8682.

Kranenburg, O., Verlaan, I. and Moolenaar, W. H. (1999). Dynamin is required for the activation of mitogen-activated protein (MAP) kinase by MAP kinase kinase. *J. Biol. Chem.* **274**, 35301-35304.

Lai, W. H., Cameron, P. H., Doherty, J. J., II, Posner, B. I. and Bergeron, J. J. (1989). Ligand-mediated autophosphorylation activity of the epidermal growth factor receptor during internalization. *J. Cell Biol.* **109**, 2751-2760.

Lamaze, C., Dujeancourt, A., Baba, T., Lo, C. G., Benmerah, A. and Dautry-Varsat, A. (2001). Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Mol. Cell* **7**, 661-671.

Larose, L., Gish, G., Shoelson, S. and Pawson, T. (1993). Identification of residues in the beta platelet-derived growth factor receptor that confer specificity for binding to phospholipase C-gamma 1. *Oncogene* **8**, 2493-2499.

Liu, J. P. and Robinson, P. J. (1995). Dynamin and endocytosis. *Endocr. Rev.* **16**, 590-607.

Lu, Z., Murray, J. T., Luo, W., Li, H., Wu, X, Lu, H., Backer, J. M. and Chen, Y. G. (2002). Transforming growth factor β activates Smad2 in the absence of receptor endocytosis. *J. Biol. Chem.* **277**, 29363-29368.

Marks, B., Stowell, M. H., Vallis, Y., Mills, I. G., Gibson, A., Hopkins, C. R. and McMahon, H. T. (2001). GTPase activity of dynamin and resulting conformation change are essential for endocytosis. *Nature* **410**, 231-235.

McPherson, P. S., Kay, B. K. and Hussain, N. K. (2001). Signaling on the endocytic pathway. *Traffic* **2**, 375-384.

Muhlberg, A. B., Warnock, D. E. and Schmid, S. L. (1997). Domain structure and intramolecular regulation of dynamin GTPase. *EMBO J.* **16**, 6676-6683.

Nakata, T., Iwamoto, A., Noda, Y., Takemura, R., Yoshikura, H. and Hirokawa, N. (1991). Predominant and developmentally regulated expression of dynamin in neurons. *Neuron* **7**, 461-469.

Nakata, T., Takemura, R. and Hirokawa, N. (1993). A novel member of the dynamin family of GTP-binding proteins is expressed specifically in the testis. *J. Cell Sci.* **105**, 1-5.

Nishibe, S., Wahl, M. I., Hernandez-Sotomayor, S. M., Tonks, N. K., Rhee, S. G. and Carpenter, G. (1990). Increase of the catalytic activity of phospholipase C-gamma 1 by tyrosine phosphorylation. *Science* **250**, 1253-1256.

Obermeier, A., Halfter, H., Wiesmuller, K. H., Jung, G., Shclessinger, J. and Ullrich, A. (1993). Tyrosine 785 is a major determinant of Trk-substrate interaction. *EMBO J.* **12**, 933-941.

Patterson, R. L., Rossum, D. B., Ford, D. L., Hurt, K. J., Bae, S. S., Suh, P. G., Kurosaki, T., Snyder, S. H. and Gill, D. L. (2002). Phospholipase C-gamma is required for agonist-induced Ca2+ entry. *Cell* **111**, 529-541.

Peters, K. G., Marie, J., Wilson, E., Ives, H. E., Escobedo, J., del Rosario, M., Mirda, D. and Williams, L. T. (1992). Point mutation of an FGF receptor abolishes phosphatidylinositol turnover and Ca2+ flux but not mitogenesis. *Nature* **358**, 678-681.

Quilliam, L. A., Rebhun, J. F. and Castro, A. F. (2002). A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases. *Prog. Nucleic Acid Res. Mol. Biol.* **71**, 391-444.

Rhee, S. G. and Bae, Y. S. (1997). Regulation of phosphoinositide-specific phospholipase C isozymes. *J. Biol. Chem.* **272**, 15045-15048.

Rhee, S. G., Suh, P. G., Ryu, S. H. and Lee, S. Y. (1989). Studies of inositol phospholipid-specific phospholipase C. *Science* **244**, 546-550.

Ringstad, N., Nemoto, Y. and de Camilli, P. (1997). The SH3p4/Sh3p8/SH3p13 protein family: binding partners for synaptojanin and dynamin via a Grb2-like Src homology 3 domain. *Proc. Natl. Acad. Sci. USA* **94**, 8569-8574.

Rosen, L. B., Ginty, D. D., Weber, M. J. and Greenberg, M. E. (1994). Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. *Neuron* **12**, 1207-1221.

Rotin, D., Margolis, B., Mohammadi, M., Daly, R. J., Daum, G. and Li, N. (1992). SH2 domains prevent tyrosine dephosphorylation of the EGF receptor: identification of Tyr992 as the high-affinity binding site for SH2 domains of phospholipase C gamma. *EMBO J.* **11**, 559-567.

Scaife, R., Gout, I., Waterfield, M. D. and Margolis, R. L. (1994). Growth factor-induced binding of dynamin to signal transduction proteins involves sorting to distinct and separate proline-rich dynamin sequences. *EMBO J.* **13**, 2574-2582.

Scaife, R., Venien-Bryan, C. and Margolis, R. L. (1998). Dual function C-terminal domain of dynamin-1: modulation of self-assembly by interaction of the assembly site with SH3 domains. *Biochemistry* **37**, 17673-17679.

Schmid, S. L., McNiven, M. A. and de Camilli, P. (1998). Dynamin and its partners: a progress report. *Curr. Opin. Cell Biol.* **10**, 504-512.

Schmidt, A. and Hall, A. (2002). Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev.* **16**, 1587-1609.

Schwemmle, M., Richter, M. F., Herrmann, C., Nassar, N. and Staeheli, P. (1995). Unexpected structural requirements for GTPase activity of the interferon-induced MxA protein. *J. Biol. Chem.* **270**, 13518-13523.

Sever, S., Muhlberg, A. B. and Schmid, S. L. (1999). Impairment of dynamin's GAP domain stimulates receptor-mediated endocytosis. *Nature* **398**, 481-486.

Sever, S., Damke, H. and Schmid, S. L. (2000). Garrotes, springs, ratchets, and whips: putting dynamin models to the test. *Traffic* **1**, 385-392.

Shupliakov, O., Low, P., Grabs, D., Gad, H., Chen, H., David, C., Takei, K., de Camilli, P. and Brodin, L. (1997). Synaptic vesicle endocytosis impaired by disruption of dynamin-SH3 domain interactions. *Science* **276**, 259-263.

Simpson, F., Hussain, N. K., Qualmann, B., Kelly, R. B., Kay, B. K., McPherson, P. S. and Schmid, S. L. (1999). SH3-domain-containing proteins function at distinct steps in clathrin-coated vesicle formation. *Nat. Cell Biol.* **1**, 119-124.

Smirnova, E., Shurland, D. L., Newman-Smith, E. D., Pishvaee, B. and van der Bliek, A. M. (1999). A model for dynamin self-assembly based on binding between three different protein domains. *J. Biol. Chem.* **274**, 14942-14947.

Smith, M. R., Liu, Y. L., Kim, S. R., Bae, Y. S., Kim, C. G., Kwon, K. S., Rhee, S. G. and Kung, H. F. (1996). PLC gamma 1 Src homology domain induces mitogenesis in quiescent NIH 3T3 fibroblasts. *Biochem. Biophys. Res. Commun.* **222**, 186-193.

Song, C., Hu, C. D., Masago, M., Kariyai, K., Yamawaki-Kataoka, Y., Shibatohge, M., Wu, D., Satoh, T. and Kataoka, T. (2001). Regulation of a novel human phospholipase C, PLCepsilon, through membrane targeting by Ras. *J. Biol. Chem.* **276**, 2752-2757.

Sorokin, A., Mohammadi, M., Huang, J. and Schlessinger, J. (1994). Internalization of fibroblast growth factor receptor is inhibited by a point mutation at tyrosine 766. *J. Biol. Chem.* **269**, 17056-17061.

Stowell, M. H., Marks, B., Wigge, P. and McMahon, H. T. (1999). Nucleotide-dependent conformational changes in dynamin: evidence for a mechanochemical molecular spring. *Nat. Cell. Biol.* **1**, 27-32.

Suh, P. G., Ryu, S. H., Moon, K. H., Suh, H. W. and Rhee, S. G. (1988). Inositol phospholipid-specific phospholipase C: complete cDNA and protein sequences and sequence homology to tyrosine kinase-related oncogene products. *Proc. Natl. Acad. Sci. USA* **85**, 5419-5423.

Urrutia, R., Henley, J. R., Cook, T. and McNiven, M. A. (1997). The dynamins: redundant or distinct functions for an expanding family of related GTPases? *Proc. Natl. Acad. Sci. USA* **94**, 377-384.

Vieira, A. V., Lamaze, C. and Schmid, S. L. (1996). Control of EGF receptor signaling by clathrin-mediated endocytosis. *Science* **274**, 2086-2089.

Warnock, D. E. and Schmid, S. L. (1996). Dynamin GTPase, a force-generating molecular switch. *BioEssays* **18**, 885-893.

Warnock, D. E., Baba, T. and Schmid, S. L. (1997). Ubiquitously expressed dynamin-II has a higher intrinsic GTPase activity and a greater propensity for self-assembly than neuronal dynamin-I. *Mol. Biol. Cell* **8**, 2553-2562.

Whitmarsh, A. J., Shore, P., Sharrocks, A. D. and Davis, R. J. (1995). Integration of MAP kinase signal transduction pathways at the serum response element. *Science* **269**, 403-407.

Wigge, P., Vallis, Y. and McMahon, H. T. (1997). Inhibition of receptor-mediated endocytosis by the amphiphysin SH3 domain. *Curr. Biol.* **7**, 554-560.

Wiley, H. S. and Burke, P. M. (2001). Regulation of receptor tyrosine kinase signaling by endocytic trafficking. *Traffic* **2**, 12-18.

Williams, R. L. and Katan, M. (1996). Structural views of phosphoinositide-specific phospholipase C: signalling the way ahead. *Structure* **4**, 1387-1394.

Yamabhai, M., Hoffman, N. G., Hardison, N. L., McPherson, P. S., Castagnoli, L., Cesareni, G. and Kay, B. K. (1998). Intersectin, a novel adaptor protein with two Eps15 homology and five Src homology 3 domains. *J. Biol. Chem.* **273**, 31401-31407.

Ye, K., Aghdasi, B., Luo, H. R., Moriarity, J. L., Wu, F. Y., Hong, J. J., Hurt, K. J., Bae, S. S., Suh, P. G. and Snyder, S. H. (2002). Phospholipase C gamma 1 is a physiological guanine nucleotide exchange factor for the nuclear GTPase PIKE. *Nature* **415**, 541-544.

Zheng, H., Hart, M. J. and Cerione, P. A. (1995). Guanine nucleotide exchange catalyzed by dbl oncogene product. *Methods Enzymol.* **256**, 77-84.