Phospholipase C-γ1 (PLC-γ1) hydrolyzes phosphatidylinositol 4,5-bisphosphate to the second messengers inositol 1,4,5-trisphosphate and diacylglycerol (DAG). PLC-γ1 is implicated in a variety of cellular signalings and processes including mitogenesis and calcium entry. However, numerous studies demonstrate that the lipase activity is not required for PLC-γ1 to mediate these events. Here, we report that the phospholipase activity of PLC-γ1 plays an essential role in nerve growth factor (NGF)-triggered Raf/MEK/MAPK pathway activation in PC12 cells. Employing PC12 cells stably transfected with an inducible form of wild-type PLC-γ1 or lipase inactive PLC-γ1 with histidine 335 mutated into glutamine in the catalytic domain, we show that NGF provokes robust activation of MAP kinase in wild-type but not in lipase inactive cells. Both Ras/C-Raf/MEK1 and Rap1/B-Raf/MEK1 pathways are intact in the wild-type cells. By contrast, these signaling cascades are diminished in the mutant cells. Pretreatment with cell permeable DAG analog 1-oleyl-2-acetylglycerol rescues the MAP kinase pathway activation in the mutant cells. These observations indicate that the lipase activity of PLC-γ1 mediates NGF-regulated MAPK signaling upstream of Ras/Rap1 activation probably through second messenger DAG-activated Ras and Rap-GEFs.

Growth factor stimulation triggers phospholipase C-γ1 (PLC-γ1) membrane translocation and association with receptor-tyrosine kinase through coupling between the phosphotyrosine docking site on receptor-tyrosine kinase and SH2 domain of PLC-γ1, resulting in tyrosine phosphorylation and activation of its enzymatic activity. When activated, PLC-γ1 provokes the hydrolysis of phosphatidylinositol 4,5-P2 to produce two second messenger molecules: inositol 1,4,5-trisphosphate and DAG, which regulate the intracellular Ca2+ and activation of protein kinase C, respectively. PLC-γ1 plays a critical role in cell proliferation and differentiation. In mouse, PLC-γ1 is essential for embryonic development, as disrupted pleg1 alleles produce embryonic lethality at approximately day 9.0 (1). Furthermore, disruption of the pleg1 gene in Drosophila is not lethal but leads to abnormal eye development (2). Microinjection of PLC-γ1 induces DNA synthesis in quiescent NIH 3T3 cells, and antibodies to PLC-γ1 can inhibit serum- and Ras-stimulated DNA synthesis in NIH 3T3 cells (3, 4). However, multiple studies demonstrate that the mitogenic activity of PLC-γ1 is not dependent on its phospholipase activity, but requires its SH3 domain (5–7). Recently, we have shown that PLC-γ1, through its SH3 domain, is a physiologic guanine nucleotide exchange factor (GEF) for PIKE (PI 3-kinase enhancer), a nuclear GTPase that activates nuclear phosphoinositide 3-kinase activity and mediates the physiologic activation by NGF for nuclear phosphoinositide 3-kinase activity (8, 9). Presumably, such mitogenic activity is associated with the activation by PIKE of nuclear phosphoinositide 3-kinase. In addition, the lipase activity is not needed for other major effects either, for example, the SH3 domain but not the lipase activity of PLC-γ1 has been shown to mediate agonist-induced Ca2+ entry in PC12 cells (10).

The mitogen-activated protein/extracellular signal-regulated kinases (MAP kinases or ERKs) regulate a diverse array of functions, such as cell growth and proliferation, differentiation, and apoptosis (11). Upon activation, the ERK/MAPKs translocate to the nucleus to phosphorylate several transcription factors including Elk-1, NF-IL6/C/EBP/NF-M, and Tal-1 (12–14). NGF treatment induces differentiation of PC12 cells into a sympathetic neuron-like phenotype, and this effect is regulated by the sustained activation of ERKs. In PC12 cells, NGF triggers both transient and sustained MAP kinase signalings, which are regulated through two distinct small GTPases Ras and Rap1. The activated Ras and Rap1 stimulate sequential activation of Raf serine/threonine kinases C-Raf and B-Raf, respectively, which in turn activate MEK1/ERKs. The transient activation of Ras is regulated through its association with an adaptor complex consisting of Shc/Grb2/Sos. While the prolonged activation of ERK signaling depends on a distinct pathway initiated through NGF receptor TrkA binding to fibroblast growth factor receptor substrate 2, whose tyrosine residues are phosphorylated and provide docking sites for adaptor protein Crk, which in turn binds and activates the Crk GEF. The activated Crk subsequently provokes Rap-1/B-Raf/MEK/ERK signaling (15).

Numerous studies indicate that there is cross-talk between the MAPK pathway and PLC-γ1 signaling cascade. For instance, elimination of the Shc binding site in NGF receptor TrkA reduces NGF-induced differentiation of PC12 cells. However, total abrogation of differentiation is observed when both
Shc and PLC-γ1 binding sites are eliminated (16, 17). Therefore, both Shc-mediated Ras-dependent and PLC-γ1-dependent pathways are essential for NGF-induced differentiation of PC12 cells. In addition, it has been shown before that PLC-γ1 mediates fibroblast growth factor-initiated C-Raf/MAPK activation through protein kinase C (18). Studies have also shown that it binds the Ras GEF SOS and enhances Ras activity (19). In addition, the SH2 domain of PLC-γ1 has been revealed to bind the proline-rich domain of SLP-76, and this interaction is required for T cell receptor-mediated activation of ERK, and nuclear factor of activated T cells (22). However, none of these studies explicitly defines the role of phospholipase activity of PLC-γ1 in MAPK signaling. Utilizing stable-inducible wild-type and lipase inactive PLC-γ1-(H335Q) PC12 cells, we demonstrate that the phospholipase activity of PLC-γ1 is required for NGF-triggered activation of the Raf/MEK/MAPK pathway. Our results indicate that the lipase activity of PLC-γ1 plays a critical role for some aspects of cellular signaling.

EXPERIMENTAL PROCEDURES

Cells and Reagents—PC12 cells were maintained in medium A (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 5% horse serum, and 100 units penicillin-streptomycin) at 37 °C with 5% CO2 atmosphere in a humidified incubator. The FLAG-tagged PLC-γ1 stably transfected PC12 cells (Tet-off cell line) were cultured in medium B (Dulbecco’s modified Eagle’s medium, 10% horse serum, 5% fetal bovine serum, 100 μg/ml G418, 100 μg/ml hygromycin B, 2 μg/ml tetracycline, and 100 units of penicillin-streptomycin). PLC-γ1 was induced in medium without tetracycline for 24 h. Mouse monoclonal anti-HA, anti-Myc, anti-Ras antibodies, Protein A/G-conjugated agarose beads, U73122, and U73343 were from Calbiochem. Anti-PLC-γ1 is required for NGF-triggered activation of the Ral/MEK/MAPK pathway. Our results indicate that the lipase activity of PLC-γ1 plays a critical role for some aspects of cellular signaling.

RESULTS

H335Q Mutation in the Catalytic Domain of PLC-γ1 Diminishes NGF-mediated Phospholipase Activity—To explore the effect of phospholipase activity of PLC-γ1 in NGF signaling, we employed tet-off PC12 cells stably transfected with the inducible form of FLAG-tagged PLC-γ1-WT and PLC-γ1-(H335Q), designated as PLC-γ1-LIM. A point mutation in the X-catalytic domain of PLC-γ1 (His335→Gln) is catalytically inactive when expressed in mammalian cells (7). To examine whether the inducible PLC-γ1-LIM could inhibit NGF-triggered phospholipase activity, we performed an in vitro lipase activity assay utilizing [3H]phosphatidylinositol 4,5-P2 as a substrate, and normalized the enzymatic activity against empty vector-uninduced cells. When induced in the absence of tetracycline, cells transfected with wild-type PLC-γ1 display almost 1-fold augmentation of lipase activity even in the absence of NGF treatment compared with empty vector-transfected control cells. NGF treatment for 5 min for these cells leads to a further enhancement of PLC-γ1 activity, and the activity decreases somewhat at 30 min. By contrast, the lipase inactive mutant displays only 50% activity compared with control cells even without NGF stimulation, consistent with earlier findings (26). Compared with the control and PLC-γ1-WT cells, the lipase activity in the PLC-γ1-LIM cells is markedly inhibited in response to NGF stimulation (Fig. 1A). However, we observed essentially similar lipase activity in the uninduced PC12 cells (Fig. 1B). Expression of induced FLAG-tagged PLC-γ1 was verified (Fig. 1C). Tyrosine phosphorylation plays a critical role for the activation of PLC-γ1 (27). To determine whether cells transfected with PLC-γ1-LIM possess distinct tyrosine phosphorylation, we monitored tyrosine phosphorylation of PLC-γ1 immunoprecipitated by anti-FLAG antibody (Fig. 1D). We observed a similar tyrosine phosphorylation effect upon NGF treatment in both PLC-γ1-WT and -LIM cells, indicating that the upstream signaling from NGF receptor TrkA to PLC-γ1 is intact in the lipase inactive mutant-transfected cells. To further evaluate the phospholipase activity in intact cells, we cultured both induced and uninduced cells in inositol-free medium supplemented with myo-[2-3H]inositol, and stimulated with NGF for various time points. PLC-γ1 activity analysis reveals similar results as in the in vitro assay (Fig. 1, E and F). The expression of PLC-γ1 in both induced and uninduced cells is shown (Fig. 1, G and H). We have observed similar effects in different clones of cells (data not shown). These data indicate that the exogenously expressed PLC-γ1 mutant acts in a dominant-negative manner despite expression of endogenous PLC-γ1.
MEK1/MAP Kinase Signaling Is Abrogated in PC12 Cells Transfected with PLC-γ1-LIM—To evaluate the effect of phospholipase activity of PLC-γ1 on the NGF-mediated MAP kinase cascade, we monitored the activation of MAP kinase using anti-phospho-ERK1/2 antibody. Cells transfected with PLC-γ1-WT display higher MAP kinase activity compared with control cells even in the absence of NGF, and NGF treatment elicits further stimulation. In contrast, MAP kinase activity is

![Image](https://via.placeholder.com/150)
diminished in cells transfected with PLC-γ1-LIM (Fig. 2A). Consistent with this observation, pretreatment with 10 μM PLC inhibitor U73122 but not its inactive analog U73433 also inhibits NGF-stimulated MAP kinase activation (data not shown). However, other MAP kinase family members including JNK1 and p38 are not activated in any of these cells. Moreover, phosphoinositide 3-kinase signaling is intact in these cells, as demonstrated by the phosphorylation of Akt (Fig. 2B). Equal amounts of FLAG-tagged PLC-γ1 constructs are expressed (Fig. 2C). Different stable clones display similar effect upon NGF treatment (Fig. 2D). This observation, combined with diminished phospholipase activity in PLC-γ1-LIM cells, suggests that the overexpressed PLC-γ1-LIM(H335Q) mutant is acting in a dominant-negative manner. Moreover, PLC-γ1 metabolic products diacylglycerol and inositol 1,4,5-trisphosphate might somehow contribute to NGF-elicited MAP kinase activation through mediating the upstream machinery. For example, NGF activates a DAG-regulated protein kinase, protein kinase C-δ, which appears to act between Raf and MAPK/ERK and is required for activation of MAPK cascade (28).

NGF induces rapid and sustained MAPK signaling, whereas EGF stimulates transient MAP kinase activation in PC12 cells. To determine whether phospholipase activity of PLC-γ1 plays any role in EGF-provoked MAPK pathway, we examined the activation of MAP kinase using anti-phospho-ERK antibody. Cells transfected with empty vector, PLC-γ1-WT, or PLC-γ1-LIM were cultured in the absence of tetracycline for 24 h, before 50 ng/ml EGF was added. MAP kinase activation was analyzed with phospho-ERK antibody (top panel). An equal amount of MEK1/2 was employed in this analysis (bottom panel).
MEK1/2 is completely inhibited in cells transfected with PLC-\(\gamma\)-LIM, although equal levels of MEK1/2 are expressed in these cells (Fig. 2F), suggesting that upstream Raf signaling of MEK1/2 is affected by the phospholipase activity of PLC-\(\gamma\)-1.

**NGF-stimulated Raf Kinase Activation Is Blocked in PLC-\(\gamma\)-LIM Cells**—PC12 cells express two members of the Raf family, B-Raf and C-Raf, both of which have been shown to be activated by NGF as a consequence of their membrane translocation (29, 30). To explore the effect of phospholipase activity of PLC-\(\gamma\) on Raf kinases, we examined the activity of both B-Raf and C-Raf kinases utilizing Syntide II peptide as a substrate. NGF-triggered Raf kinase activity is markedly blocked in PLC-\(\gamma\)-LIM cells compared with empty vector or PLC-\(\gamma\)-WT-expressing PC12 cells (Fig. 3A). Both B-Raf and C-Raf kinases are substantially inhibited in cells expressing PLC-\(\gamma\)-LIM even in the absence of NGF treatment, indicating that phospholipase activity of PLC-\(\gamma\) determines the basal, physiologic activity of Raf kinases as well as being responsible for its response to growth factor stimulation. B-Raf kinase displays higher activity than C-Raf in both cells, consistent with previous findings that NGF-stimulated MAP kinase activation is predominantly contributed by B-Raf (31). However, we observed similar Raf kinase activity in uninduced PC12 cells with higher B-Raf activity than C-Raf (data not shown). These observations suggest that signaling upstream of the Raf kinase is influenced by phospholipase activity of PLC-\(\gamma\).

**Phospholipase Activity of PLC-\(\gamma\) Mediates NGF-stimulated Ras and Rap1 Activation**—In NGF-stimulated PC12 cells, C-Raf activation is essentially mediated by Ras, whereas B-Raf is predominantly regulated by Rap1 (15, 32). To investigate whether small GTPases Ras and Rap1 are interfered by the phospholipase activity of PLC-\(\gamma\), we performed in vitro Ras and Rap1 activation assays employing RBDs of C-Raf and B-Raf to bind the activated, GTP-bound forms of Ras and Rap1. We observed robust association between Ras/Rap1 and GST-C-Raf-RBD/GST-RalGDS in cells expressing PLC-\(\gamma\)-WT even without NGF stimulation. NGF treatment further augments the interaction, and binding activity decreases after 30 min stimulation. However, we observed very faint Ras/Rap1 activity in cells expressing PLC-\(\gamma\)-LIM compared with cells transfected with control vector or PLC-\(\gamma\)-WT whether or not the cells were stimulated with NGF (Fig. 4). Metabolic labeling of PC12 cells with \(^{[32P]}\) orthophosphate reveals similar results, NGF stimulation incurs more GTP bound Ras/Rap1 in cells expressing PLC-\(\gamma\)-WT than PLC-\(\gamma\)-LIM (data not shown). These observations demonstrate that phospholipase activity of PLC-\(\gamma\) mediates NGF-stimulated GTPase Ras and Rap1 activation in PC12 cells.

**Diacylglycerol Analog 1-Oleyl-2-acetylgllycerol (OAG) Rescues MAPK Signaling Cascade in PLC-\(\gamma\)-LIM Cells**—Ras/C-Raf/MEK1/2/MAPK and Rap1/B-Raf/MEK1/2/MAPK signaling cascades are impaired in cells transfected with PLC-\(\gamma\)-LIM but not PLC-\(\gamma\)-WT, indicating that enzymatic activity of PLC-\(\gamma\) is required for NGF to provoke the complete activation of the MAPK pathway. To explore whether addition of the PLC-\(\gamma\) hydrolysate product could restore MAPK signaling in PLC-\(\gamma\)-LIM-transfected PC12 cells, we utilized a cell permeable synthetic diacylglycerol analog, OAG. FLAG-PLC-\(\gamma\)-LIM was induced in the absence of tetracycline for 24 h, and pretreated with 5 \(\mu\)M OAG or vehicle solvent Me2SO as a control for 30 min before NGF was introduced. Rap1 and Ras activation assays reveal marked activation of Rap1 and Ras even in the absence of NGF. NGF treatment further enhances both Rap1 and Ras activity. By contrast, NGF triggers modest augmentation of Rap1 and Ras activity in Me2SO-treated control cells (Fig. 5A). Quantitative analysis from three independent experiments reveals that OAG triggers Ras/Rap1 activation is still NGF dependent (Fig. 5B). These data demonstrate that the metabolic product of PLC-\(\gamma\) is required to rescue NGF-mediated small GTPase Rap1 and Ras activation. To further test whether the downstream Raf kinases activation is also rescued by OAG, we monitored B-Raf and C-Raf kinase activity in the presence of OAG and Me2SO. Pretreatment with OAG but not Me2SO renders PLC-\(\gamma\)-LIM cells to activate both B-Raf and C-Raf in response to NGF treatment (Fig. 5C). Consequently,

**Fig. 4.** Phospholipase activity of PLC-\(\gamma\) mediates NGF-stimulated GTPase Ras and Rap1 activation. Cells transfected with PLC-\(\gamma\)-WT or PLC-\(\gamma\)-LIM were induced in the absence of tetracycline for 24 h, before 100 ng/ml NGF was added. At different time points, cells were lysed and analyzed with in vitro Ras and Rap1 activation assays employing GST-C-Raf-RBD and GST-RalGDS.

**Fig. 3.** NGF-stimulated Raf kinase activation is blocked in PLC-\(\gamma\)-LIM cells. Raf kinase activity is reduced in PLC-\(\gamma\)-LIM cells. Cells transfected with empty vector, PLC-\(\gamma\)-WT, or PLC-\(\gamma\)-LIM were induced for 24 h, before NGF was introduced. B-Raf and C-Raf were, respectively, immunoprecipitated and analyzed with in vitro kinase assay utilizing Syntide II peptide as a substrate in the presence of 0.1 \(\mu\)Ci of \(^{[32P]}\)ATP.

**Fig. 2.** NGF-stimulated MAPK Signaling Cascade in PLC-\(\gamma\)-LIM Cells—Cells transfected with PLC-\(\gamma\)-WT or PLC-\(\gamma\)-LIM were induced for 24 h, before 100 ng/ml NGF was added. At different time points, cells were lysed and analyzed with in vitro kinase assay utilizing Syntide II peptide as a substrate.
MAP kinase is also robustly activated by NGF in OAG but not Me2SO pretreated PLC-/H92531-LIM cells (Fig. 5D, top panel). As a control, we observed the same activity of MAP kinase in empty vector-transfected control cells pretreated with Me2SO or OAG (Fig. 5D, bottom panel). In the absence of NGF, pretreatment with OAG alone slightly stimulates the MAPK cascade presumably through activating DAG binding RasGRF and RapGRF. Quantitative analysis from three independent experiments reveals that OAG-stimulated ERK activation is still NGF dependent (Fig. 5E). These data strongly suggest that phospholipase activity of PLC-/1 mediates NGF-stimulated MAPK cascade in PC12 cells.

**DISCUSSION**

In this report, we show that the phospholipase activity of PLC-/ is critical for the NGF-mediated MAPK signaling pathway in PC12 cells. Induction of stably transfected lipase inactive PLC-/1-(H335Q) markedly decreases the activation of Ras/C-Raf/MEK/MAPK and Rap1/B-Raf/MEK/MAPK cascades compared with the wild-type PLC-/1-transfected cells (Figs. 2–4). Treatment of PC12 cells with fibroblast growth factor or NGF leads to outgrowth of neurites and cessation of cell division (33), whereas treatment with EGF incurs cell proliferation. NGF stimulation results in a transient and sustained MAPK activation. By contrast, EGF provokes the evanescent activation of MAPK. Consistent with this idea, we failed to observe any defects in the activation of MAPK signaling in lipase inactive mutant cells upon EGF treatment (Fig. 2E), indicating that the phospholipase activity of PLC-/ is not implicated in EGF-initiated MAPK signaling.

It has been shown before that a fibroblast growth factor receptor mutant (Tyr-766) unable to activate PLC-/1 elicits reduced activation of C-Raf/MAPK signaling probably through
inhibiting Raf phosphorylation, which is mediated by PLC-γ1-dependent protein kinase C (18). Moreover, pretreatment with U73122 results in 87% inhibition of NGF-mediated MAPK phosphorylation in TrkA-transfected Chinese hamster ovary cells, whereas it has no effect on NGF-stimulated Akt activation (34). NGF activates a DAG-regulated protein kinase, protein kinase C-δ, which is required for activation of the MAPK cascade and for neurite outgrowth. Protein kinase C-δ appears to act between Raf and MAPK/ERK in the pathway (28). These data strongly support our observations that the phospholipase activity of PLC-γ1 is implicated in the NGF-mediated MAPK pathway.

Addition of OAG, a cell-permeable synthetic DAG analog, rescues Ras, Rap1, and the MAP kinase cascade in PLC-γ1-LIM cells (Fig. 5), suggesting that PLC-γ1 regulates MAPK signaling upstream Ras/Rap1 activation probably through second messenger-mediated GEF. Several mammalian GEFs capable of activating Ras or Rap1 have been identified so far. For example, Sos, RasGRF (35, 36), and CalDAG-GEFII (also called RasGRF) are GEFs for Ras (37). The RasGRF family appear to be specialized for activating Ras in response to calcium signaling, via their calmodulin-binding IQ motifs (36, 38). C3G, cAMP-GEF (also called Epac), and CalDAG-GEF I are GEFs for Rap (39). CalDAG-GEFII can bind directly to and be activated by second messengers such as calcium and DAG. The second messenger-mediated GEF may represent a new class of GEFs, distinct from receptor-tyrosine kinase/adaptor molecule-associated GEFs. In addition, these GEFs display very different and restricted central nervous system expression patterns, suggesting that they may act as distinct regulators of neuronal signaling (11). NGF receptor TrkA may induce multiple signals to activate Ras and Rap1, one of which might involve PLC-γ1 activation. Complete activation of Ras and Rap1 pathways needs both Shc/fibroblast growth factor receptor substrate 2 and PLC-γ1-dependent stimulation of upstream GEFs.

At glutamatergic synapses, Ras has been implicated as a major target for calcium signaling to MAP kinases, through stimulation of RasGRF (38) or via tyrosine kinases such Src or Pyk2 (40). Stimulation of both metabotropic and N-methyl-D-aspartate-a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid postsynaptic receptors leads to calcium influx and elevation of cAMP and DAG, which can regulate both Ras and Rap-dependent action through RasGRF and CalDAG-GEF (11). In parallel to the receptor-tyrosine kinase phosphotyrosine docking site for the SH2 domain containing adaptor molecules (e.g. Grb2, Shc, and Crk), it has been shown that PLC-γ1 can associate directly with phosphotyrosine sites on N-methyl-D-aspartate channels via its SH2 domain (41). Existence of multiple MAPK signaling in postsynaptic density might allow neurons to regulate the kinetics of ERK activation. The ability to temporally regulate Ras- and Rap-dependent signaling has been demonstrated to regulate NGF-mediated gene expression and cell physiology of PC12 cells (32).

Acknowledgments—We are indebted to Dr. J. L. Bos at Utrecht University, The Netherlands, and Dr. Mike Gold at the University of British Columbia, Canada, for GST-RalGDS construct.