Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 5650–5654, July 1991
Biochemistry

Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-γ1 by a kinase activity associated with the product of the *trk* protooncogene

(tyrosine kinase/growth factor/signal transduction/PC12 cells)

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Contributed by J. Michael Bishop, March 18, 1991

ABSTRACT **Nerve growth factor (NGF) promotes the survival and differentiation of specific populations of neurons. The molecular mechanisms by which cells respond to NGF are poorly understood, but two clues have emerged recently. First, NGF rapidly stimulates tyrosine phosphorylation of several unidentified proteins in the NGF-responsive pheochromocytoma cell line PC12 [Maher, P. (1988) *Proc. Natl. Acad. Sci. USA* 85, 6788–6791]. Second, the protein-tyrosine kinase encoded by the protooncogene *trk* ($p140^{trk}$), a member of the receptor class of tyrosine kinases, becomes activated and phosphorylated on tyrosine after NGF treatment of PC12 cells [Kaplan, D. R., Martin-Zanca, D. & Parada, L. F. (1991) *Nature (London)* 350, 158–160]. We now report that NGF rapidly induces tyrosine phosphorylation of phospholipase C-γ1 (PLC-γ1), and we present evidence that the responsible tyrosine kinase is either $p140^{trk}$ or a closely associated protein. Treatment of responsive cells with NGF elicited phosphorylation of PLC-γ1 on tyrosine and serine. PLC-γ1 immunoprecipitated from NGF-stimulated cells was phosphorylated *in vitro* by coprecipitating protein kinase activity, and the phosphorylations occurred principally on tyrosine. The responsible kinase could be depleted from cellular lysates by antibodies specific for $p140^{trk}$. This procedure also depleted a 140-kDa protein that normally coprecipitated with PLC-γ1 and became phosphorylated on tyrosine *in vivo* in response to NGF. Analysis of tryptic peptides from PLC-γ1 indicated that the residues phosphorylated *in vitro* by $p140^{trk}$-associated kinase activity were largely congruent with those phosphorylated *in vivo* after NGF treatment. Our findings identify PLC-γ1 as a likely substrate for the *trk*-encoded tyrosine kinase, and they provide a link between NGF-dependent activation of $p140^{trk}$ and the stimulation of intracellular second messenger pathways.**

Nerve growth factor (NGF) is a neurotrophic polypeptide required for the survival and differentiation of specific populations of vertebrate neurons (1–3). PC12 cells, cloned from a rat pheochromocytoma, have served as an important cell culture system for studying NGF action. When treated with NGF, PC12 cells differentiate, acquiring phenotypic properties characteristic of sympathetic neurons (4).

The molecular mechanisms that subserve NGF action on its target cells have not been well characterized, but evidence suggests that activation of a tyrosine kinase may be an important early component of the cellular response to NGF. Maher (5) demonstrated that NGF treatment of PC12 cells rapidly stimulates tyrosine phosphorylation of several unidentified proteins. To assess the role of protein-tyrosine kinase activation in mediating the cellular response to NGF we began identification of substrate proteins for the NGF-activated protein-tyrosine kinase. One clue to potential substrates was provided by the report that NGF stimulates inositolphospholipid hydrolysis in PC12 cells, suggesting activation of phospholipase C (6, 7). It has been shown that the phospholipase C-γ1 (PLC-γ1) isozyme becomes enzymatically activated when phosphorylated on tyrosine residues by the receptor for epidermal growth factor (EGF) (8). In this report we present evidence that NGF induces rapid phosphorylation of PLC-γ1 on tyrosine, in accord with a recent report from Kim *et al.* (9). In addition, after NGF stimulation PLC-γ1 coprecipitates with protein kinase activity and is phosphorylated *in vitro* predominantly on tyrosine residues.

We were interested in identifying the tyrosine kinase that phosphorylates PLC-γ1 *in vivo* and *in vitro* in response to NGF stimulation. Recently, the product of the protooncogene *trk* ($p140^{trk}$) has been shown to become activated and phosphorylated on tyrosine after NGF treatment of PC12 cells (10). $p140^{trk}$ belongs to the receptor class of tyrosine kinases exemplified by the receptors for platelet-derived growth factor (PDGF) and EGF (11). In response to ligand binding, these receptors associate with and phosphorylate a number of cellular proteins, presumably regulating their activity (12, 13). We have found that PLC-γ1 coprecipitates with $p140^{trk}$ after NGF stimulation and is phosphorylated *in vitro* by tyrosine kinase activity associated with $p140^{trk}$. The sites of PLC-γ1 that become phosphorylated after NGF stimulation *in vivo* are largely consistent with those phosphorylated by $p140^{trk}$-associated activity *in vitro*, confirming that PLC-γ1 is likely a substrate for $p140^{trk}$ both *in vivo* and *in vitro*. These results elucidate some of the biochemical consequences of tyrosine kinase activation in NGF-responsive cells, and they support a critical role for the *trk* tyrosine kinase in mediating the cellular response to NGF.

MATERIALS AND METHODS

Materials. Murine monoclonal antibodies to PLC-γ1 were obtained from S. G. Rhee (14). The phosphotyrosine monoclonal antibody 4G10 was provided by D. Morrison, B. Drucker, and T. Roberts (15). $p140^{trk}$ antiserum was directed against a synthetic peptide corresponding to the 14 carboxyl-terminal amino acid residues of the *trk*-encoded protein (11).

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Abbreviations: NGF, nerve growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; PLC-γ1, phospholipase C-γ1; NP-40, Nonidet P-40.

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Nerve growth factor was obtained from Collaborative Research or from L. Reichardt (16) and was used at a concentration of 100 ng/ml.

Cell Culture and Extracts. PC12 cells and LA-N-5 cells were grown as previously described (17, 18). Cells were stimulated for the indicated times at 37°C by adding NGF directly to the growth medium, then lysed in 1% Nonidet P-40 (NP-40) lysis buffer as previously described (15). One hundred dorsal root ganglia were prepared by dissection from 14.5-day mouse embryos. Dorsal root ganglia were treated for 5 min with NGF in Hanks' salt solution, washed, and disrupted by treatment with a Dounce homogenizer in 1% NP-40 lysis buffer.

Immunoprecipitation, Western Blotting, and *in Vitro* Kinase Assay. PLC-γ1 was immunoprecipitated by using monoclonal antibodies specific for PLC-γ1 as previously described (15). Proteins were separated through an SDS/8% polyacrylamide gel, transferred to nitrocellulose, and probed for phosphotyrosine as previously described (15). $p140^{trk}$ was immunodepleted from PC12 cell lysates by two sequential immunoprecipitations with $p140^{trk}$ antiserum prior to immunoprecipitation of PLC-γ1. $p140^{trk}$ immunoprecipitations were carried out in the presence or absence of a competing peptide representing the epitope for the antiserum. For *in vitro* kinase assays, PLC-γ1 immunoprecipitates were washed, then incubated for 5 min at room temperature with 40 μl of kinase buffer (20 mM Tris·HCl, pH 7.6/5 mM $MnCl_2$/10 μM ATP) and 20 μCi of [γ-^{32}P]ATP (1 Ci = 37 GBq). The phosphorylated proteins were separated through an SDS/8% polyacrylamide gel, then transferred to Immobilon-P (Millipore).

Cell Labeling, Two-Dimensional Tryptic Peptide Mapping, and Phosphoamino Acid Analysis. PC12 cells were labeled with [^{32}P]orthophosphate (Amersham) at 2 mCi/ml for 5 hr in phosphate-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5% dialyzed fetal calf serum. Where indicated, cells were stimulated for 5 min with NGF prior to lysis in 1% NP-40 lysis buffer and immunoprecipitation of PLC-γ1. ^{32}P-labeled proteins were separated through an SDS/8% polyacrylamide gel, then transferred to Immobilon-P. For two-dimensional tryptic peptide mapping, ^{32}P-labeled PLC-γ1 bands were excised and digested with trypsin, and the phosphopeptides were separated on thin-layer cellulose plates by electrophoresis and chromatography as previously described (15). For phosphoamino acid analysis, the ^{32}P-labeled PLC-γ1 bands were excised and hydrolyzed in 6 M HCl at 110°C for 90 min, and the phosphoamino acids were separated by electrophoresis on thin-layer cellulose plates as previously described (19). ^{32}P-labeled phosphoamino acids were quantitated by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Unlabeled phosphoamino acid standards were visualized with ninhydrin.

RESULTS

NGF Induces Phosphorylation of PLC-γ1 on Tyrosine. Treatment of PC12 cells with NGF induced tyrosine phosphorylation of proteins with molecular masses of approximately 42, 45, 50, 60, 140, and 150 kDa (10). The 150-kDa tyrosine-phosphorylated band was of particular interest because PLC-γ1, a well-characterized substrate for the PDGF, EGF, and fibroblast growth factor (FGF) tyrosine kinase receptors, has a molecular mass of 148 kDa (20, 21). In addition, NGF is known to rapidly stimulate hydrolysis of inositol phospholipids in PC12 cells, suggesting activation of phospholipase C (6, 7).

To determine whether NGF treatment of PC12 cells induced tyrosine phosphorylation of PLC-γ1, we used a collection of monoclonal antibodies to immunoprecipitate PLC-γ1 from lysates of untreated and NGF-treated PC12 cells, then probed the immunoprecipitates by Western blotting with antibodies directed against phosphotyrosine. Induction of tyrosine phosphorylation in PLC-γ1 was detectable within 1 min of NGF treatment, reached a maximum at 2–5 min, decreased only slightly after 1 hr, and persisted at a diminished level after 5 days of continuous NGF treatment (Fig. 1*A* and data not shown). Approximately equal amounts of PLC-γ1 were present in all lanes, as demonstrated by immunoblot analysis with monoclonal antibodies specific for PLC-γ1 (data not shown).

The phosphorylation of PLC-γ1 on tyrosine in response to NGF was not specific to PC12 cells but appeared to be a general consequence of NGF action on responsive cells. For example, NGF elicited tyrosine phosphorylation of PLC-γ1 in the NGF-responsive human neuroblastoma cell line LA-N-5 (Fig. 1*A*), and PLC-γ1 was phosphorylated on tyrosine in sensory neurons from the dorsal root ganglia of mouse embryos maintained in the presence of NGF (Fig. 1*A*).

Phosphorylation of tyrosine residues in PLC-γ1 was confirmed by phosphoamino acid analysis of protein that had been immunoprecipitated after NGF treatment of PC12 cells labeled with [^{32}P]orthophosphate (Fig. 1*B*). NGF increased the incorporation of [^{32}P]phosphate into PLC-γ1 by 50%, as determined by scintillation counting (data not shown). Prior to NGF treatment, PLC-γ1 was phosphorylated exclusively on serine residues, whereas after NGF addition, PLC-γ1 became phosphorylated on tyrosine in addition to more extensive phosphorylation on serine (Fig. 1*C*). Half of the increased incorporation of ^{32}P into PLC-γ1 after NGF treat-

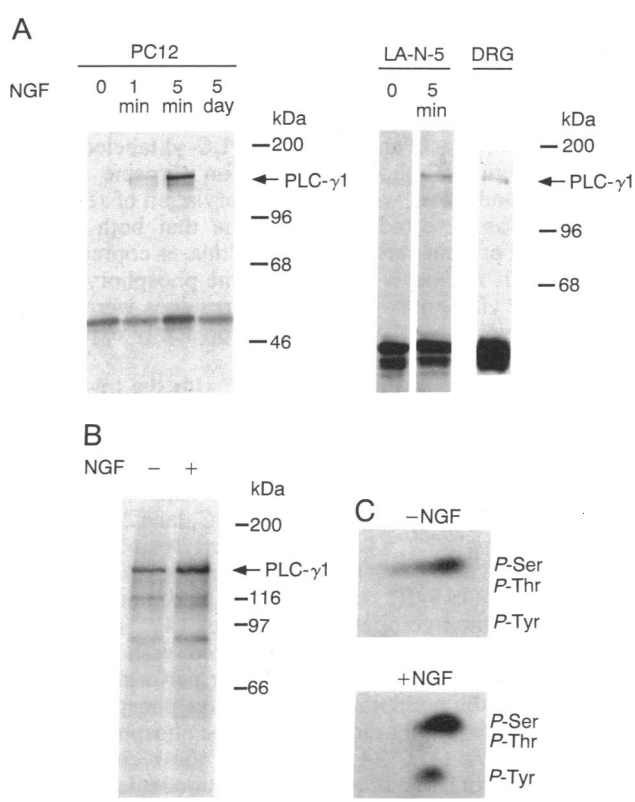

FIG. 1. NGF stimulates rapid tyrosine phosphorylation of PLC-γ1 in responsive cells. (*A*) Anti-phosphotyrosine immunoblot of PLC-γ1 immunoprecipitated from lysates of PC12 cells, LA-N-5 cells, and mouse dorsal root ganglia (DRG). Cells were stimulated with NGF for the indicated times. PLC-γ1 band is indicated. (*B*) ^{32}P-labeled PLC-γ1 immunoprecipitated from lysates of NGF-treated (+) or untreated (–) PC12 cells labeled *in vivo* for 5 hr with [^{32}P]orthophosphate. PLC-γ1 band is indicated. (*C*) Phosphoamino acid analysis of ^{32}P-labeled PLC-γ1 before (–) and after (+) NGF treatment. The positions of phosphoserine (*P*-Ser), phosphothreonine (*P*-Thr), and phosphotyrosine (*P*-Tyr) are indicated.

ment could be accounted for by the appearance of phosphotyrosine and half by the increase in phosphoserine.

To determine the proportion of PLC-γ1 phosphorylated on tyrosine after NGF treatment of PC12 cells, we compared the amount of PLC-γ1 recovered by clearing lysates with anti-phosphotyrosine antibodies to that recovered by using antibodies specific for PLC-γ1. This procedure reproducibly indicated that approximately 10–15% of PLC-γ1 in PC12 cells became phosphorylated on tyrosine in response to NGF (data not shown).

To ascertain whether NGF stimulates tyrosine phosphorylation of PLC-γ1 at physiologically relevant concentrations of NGF, a dose-response experiment was performed. Maximal phosphorylation of PLC-γ1 was seen at NGF concentrations of 50–100 pM (data not shown). Concentrations of NGF in this range are able to elicit complete differentiation of PC12 cells (4).

PLC-γ1 Is Phosphorylated *in Vitro* by Coprecipitating Kinase Activity. Recently it has been observed that some substrates coprecipitate with protein kinase activities after growth factor treatment of cells (13, 15, 22). Therefore, we immunoprecipitated PLC-γ1 from lysates of NGF-stimulated or unstimulated PC12 cells and incubated the immunoprecipitates with [γ-^{32}P]ATP in an attempt to detect protein kinase activity. Immunoprecipitates prepared from NGF-treated cells phosphorylated PLC-γ1 *in vitro*, whereas those from untreated cells did not (Fig. 2*A*, lanes 1 and 2). Similar results were obtained when PLC-γ1 was immunoprecipitated from lysates of NGF-stimulated LA-N-5 cells (data not shown). No other substrates for the coprecipitating protein kinase activity were detected. Although other phosphorylated proteins were often apparent (Fig. 2*A*), their phosphorylation was variable and did not depend upon NGF stimulation.

Phosphoamino acid analysis of the PLC-γ1 labeled *in vitro* showed prominent phosphorylation on tyrosine residues (Fig. 2*B*). In addition, weaker phosphorylation of serine and threonine was detected. We conclude that both protein-tyrosine and protein-serine/threonine kinases coprecipitated with PLC-γ1. Although PLC-γ1 became phosphorylated on threonine *in vitro*, phosphothreonine residues were not apparent *in vivo* (Fig. 1*C*). We cannot presently explain this discrepancy.

Tyrosine Kinase Activity Associated with the *trk*-Encoded Protein Phosphorylates PLC-γ1 *in Vitro*. We wished to determine the identity of the tyrosine kinase activity that coprecipitated with PLC-γ1. Since a 140-kDa protein coprecipitated with PLC-γ1 and was apparent in Western blots probed for phosphotyrosine (Fig. 1*A* and Fig. 2*C*, lane 2), a possible candidate was p140^{trk}, a transmembrane tyrosine kinase that becomes activated and phosphorylated on tyrosine after NGF treatment of PC12 cells (10). To explore this possibility, we used immunoprecipitation to deplete p140^{trk} from lysates of PC12 cells that had been stimulated with NGF, then immunoprecipitated PLC-γ1 and either performed a kinase reaction with the precipitate or probed for phosphotyrosine. This procedure greatly reduced both the phosphorylation of PLC-γ1 *in vitro* (Fig. 2*A*, lane 4) and the recovery of the 140-kDa protein (Fig. 2*C*, lane 4). Immunodepletion of both the 140-kDa protein and the kinase activity was prevented by a peptide representing the epitope for the p140^{trk} antiserum (Fig. 2*A*, lane 3, and Fig. 2*C*, lane 3).

The most direct interpretation of these results is that p140^{trk} itself is responsible for the phosphorylation of PLC-γ1 on tyrosine in response to NGF. Alternatively, the responsible kinase might be tightly associated with p140^{trk}, as reported previously for the PDGF receptor and several cytoplasmic protein-tyrosine kinases (23). This is likely to be the case for the serine/threonine kinase that we observe coprecipitating with PLC-γ1, since virtually all phosphorylation of PLC-γ1 *in vitro* was eliminated by immunodepletion with antibodies specific for p140^{trk}.

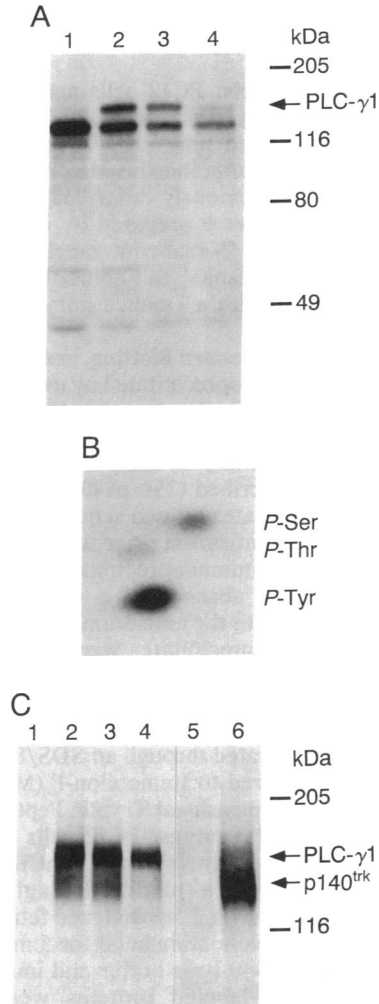

FIG. 2. NGF elicits coprecipitation of PLC-γ1 with protein kinase activity associated with p140^{trk}. (*A*) *In vitro* kinase assay of PLC-γ1 immunoprecipitates. Lanes 1 and 2, PLC-γ1 was immunoprecipitated directly from lysates of unstimulated (lane 1) or NGF-stimulated (lane 2) PC12 cells. Lanes 3 and 4, prior to the immunoprecipitation of PLC-γ1, NGF-stimulated PC12 cell lysates were incubated with antibodies specific for p140^{trk} either in the presence (lane 3) or in the absence (lane 4) of a competing peptide representing the epitope for the antiserum. The PLC-γ1 band is indicated. (*B*) Phosphoamino acid analysis of the ^{32}P-labeled PLC-γ1 band from lane 2 in *A*. The positions of phosphoserine (*P*-Ser), phosphothreonine (*P*-Thr), and phosphotyrosine (*P*-Tyr) are indicated. (*C*) Anti-phosphotyrosine Western blot of PLC-γ1 and p140^{trk} immunoprecipitates. Lanes 1 and 2, PLC-γ1 was immunoprecipitated directly from lysates of unstimulated (lane 1) or NGF-stimulated (lane 2) PC12 cells. Lanes 3 and 4, prior to the immunoprecipitation of PLC-γ1, NGF-stimulated PC12 cell lysates were incubated with antibodies specific for p140^{trk} (11) either in the presence (lane 3) or in the absence (lane 4) of a competing peptide representing the epitope for the antiserum. Lanes 5 and 6, tyrosine phosphorylated protein recovered by immunoprecipitation of p140^{trk} in the presence (lane 5) or absence (lane 6) of a competing peptide representing the epitope for the antiserum. The PLC-γ1 and p140^{trk} bands are indicated.

Comparison of the Sites of PLC-γ1 Phosphorylated *in Vivo* and *in Vitro*. Tryptic peptide mapping of PLC-γ1 was performed to compare the sites of PLC-γ1 phosphorylated *in vivo* and *in vitro*. PLC-γ1 isolated from unstimulated PC12 cells gave rise to seven phosphopeptides (Fig. 3*A*). The pattern of phosphopeptides resembled that found previously for PLC-γ1 isolated from mouse NIH-3T3 cells, so we have

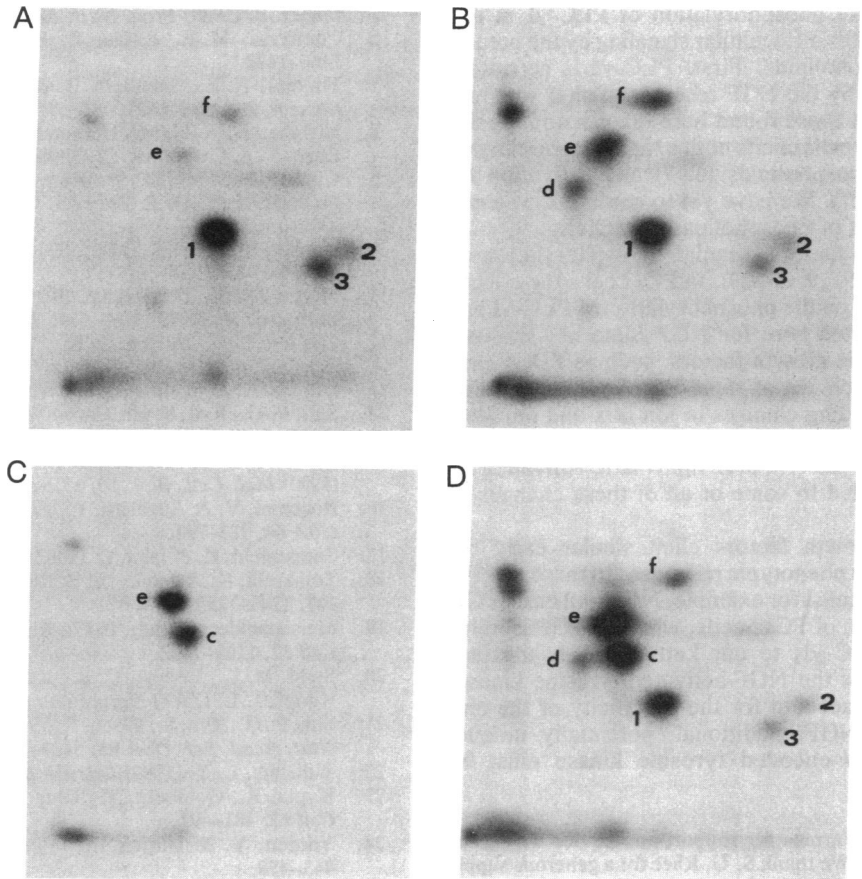

FIG. 3. Tryptic phosphopeptides of PLC-γ1 labeled *in vivo* and *in vitro*. Peptides were analyzed on thin-layer cellulose plates by electrophoresis (horizontal axis) and chromatography (vertical axis). (*A*) Tryptic phosphopeptides of PLC-γ1 immunoprecipitated from lysates of unstimulated PC12 cells labeled *in vivo* for 5 hr with [^{32}P]orthophosphate. (*B*) Tryptic phosphopeptides of PLC-γ1 immunoprecipitated from lysates of PC12 cells labeled *in vivo* for 5 hr with [^{32}P]orthophosphate then stimulated for 5 min with NGF. (*C*) Tryptic phosphopeptides of PLC-γ1 phosphorylated *in vitro*. PLC-γ1 immunoprecipitates from lysates of NGF-treated PC12 cells were used for an immune complex kinase assay in the presence of 20 μCi of [γ-^{32}P]ATP. (*D*) Mixture of samples from *B* and *C*.

designated four of the prominent peptides according to previous convention (19) (peptides e and 1–3; peptide f is unique to the present pattern). NGF treatment resulted in the enhanced phosphorylation of two of these peptides (e and f) and the appearance of a novel phosphopeptide (d) (Fig. 3*B*). The changes in peptides d and e appear to correspond to those reported previously after activation of the EGF and PDGF receptors (15, 19), whereas the increased phosphorylation of peptide f does not. PLC-γ1 phosphorylated *in vitro* contained two prominently labeled phosphopeptides (e and c) (Fig. 3*C*) and three additional phosphopeptides (2, 3, and d) observed only in longer exposures (data not shown). One of the prominently labeled peptides (e) and the three minor phosphopeptides (2, 3, and d) comigrated with peptides from PLC-γ1 phosphorylated *in vivo* (Fig. 3*D* and data not shown).

Phosphoamino acid analysis of peptide e phosphorylated *in vitro* detected only phosphotyrosine (data not shown). Peptide c labeled *in vitro* was also phosphorylated on tyrosine, but it did not correspond to any of the phosphopeptides labeled *in vivo*. Phosphopeptide f, which was prominent after NGF treatment *in vivo*, was not apparent *in vitro*; none of the phosphopeptides from *in vivo* were sufficiently labeled to permit analysis of phosphoamino acids. These discrepancies are not surprising, since additional protein kinases that do not coprecipitate with PLC-γ1 may phosphorylate PLC-γ1 *in vivo*. It is also possible that the behavior of the tyrosine kinase *in vitro* differs from its behavior *in vivo* due to the effects of solubilization or loss of accessory cellular components. Nevertheless, the sites of PLC-γ1 phosphorylated in response to NGF *in vivo* were largely congruent with those phosphorylated by p140^{trk}-associated kinase activity *in vitro*.

DISCUSSION

The data presented here indicate that binding of NGF to cell surface receptors elicits phosphorylation of PLC-γ1 on tyrosine and serine, a finding in accord with the recent work of Kim *et al.* (9). Immunodepletion experiments with antibodies specific for p140^{trk} indicate that the enzyme responsible for tyrosine phosphorylation is likely to be p140^{trk} or a closely associated kinase. This is in accord with the ability of NGF to activate the kinase of p140^{trk} (10). Further experiments with purified components will be necessary to confirm that the *trk*-encoded tyrosine kinase phosphorylates PLC-γ1 directly. NGF also induces serine phosphorylation of PLC-γ1, although the kinase responsible has not yet been identified.

NGF induces tyrosine phosphorylation of p140^{trk} (10), an event that from precedent seems likely to be autocatalytic (24). Yet no NGF-dependent *in vitro* phosphorylation of a 140-kDa protein was observed in the PLC-γ1 immunoprecipitates from PC12 cells (see Fig. 2*A*, lane 2). Two possible explanations come to mind. First, autophosphorylation of p140^{trk} may be relatively feeble in the presence of substrate and, thus, may be below the level of detection in our assay. Second, most of the susceptible tyrosine residues in p140^{trk} may have been phosphorylated *in vivo*, prior to preparation of the immunoprecipitates.

We presume that the phosphorylation of PLC-γ1 in response to NGF facilitates intracellular signaling by the breakdown of inositolphospholipids. First, PLC-γ1 is activated when phosphorylated by the EGF receptor at sites that are largely congruent with those found here (8). Second, application of NGF to PC12 cells elicits both a rapid increase in the turnover of inositolphospholipids (6, 7) and activation of protein kinase C (25–27). We have yet to confirm the suspicion by direct analysis of phospholipase C activity.

A variety of growth factors stimulate phosphorylation of PLC-γ1 on tyrosine (15, 19, 28, 29). In PC12 cells the effects of EGF and basic FGF on the phosphorylation of PLC-γ1 are similar to those reported here for NGF (data not shown). NGF and the mitogenic growth factors, such as PDGF and EGF, induce a common set of rapid metabolic changes in responsive cells, including changes in ion flux and intracellular pH, cytoskeletal rearrangements, and induction of cellular genes, such as c-*fos* (13, 30). Enzymatic activation of PLC-γ1 may be coupled to some or all of these changes in cellular metabolism.

Although many growth factors elicit similar early responses, the long-term phenotypic responses to these diverse ligands vary. In PC12 cells, for example, NGF and basic FGF provoke differentiation of PC12 cells, whereas EGF is mitogenic (4, 31, 32). PLC-γ1, to our knowledge, is the first identified substrate for the NGF-activated tyrosine kinase, $p140^{trk}$. However, to account for the specificity of the biological response to NGF, additional, potentially unique, substrates for the *trk*-encoded tyrosine kinase must be sought.

We acknowledge H. Varmus for support and advice during the inception of this project. We thank S. G. Rhee for a generous supply of anti-PLC-γ1 antibodies; D. Morrison, B. Drucker, and T. Roberts for 4G10 antibody; L. Reichardt for PC12 cells and NGF; W. Mobley for NGF; D. Morrison, M. McMahon, and R. Finney for critical reading of the manuscript; and S. Rabin for technical assistance. M.L.V. is a Howard Hughes Medical Institute Predoctoral Fellow. This work was supported by National Institutes of Health Grant CA 44338, by funds from the G.W. Hooper Foundation, and by the National Cancer Institute under contract N01-C0-74101 with Advanced BioScience Laboratories.

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