Nerve Growth Factor Promotes the Activation of Phosphatidylinositol 3-Kinase and Its Association with the trk Tyrosine Kinase*

(Received for publication, January 16, 1992)

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We investigated the involvement of phosphatidylinositol 3-kinase (PtdIns 3-kinase) in the initiation of signal transduction by nerve growth factor (NGF) in the rat pheochromocytoma PC12 cell line. PtdIns 3-kinase catalyzes the formation of phosphoinositides with phosphate in the D-3 position of the inositol ring and previously has been found to associate with other activated protein tyrosine kinases, including growth factor receptor tyrosine kinases. Anti-phosphotyrosine immunoprecipitates had PtdIns 3-kinase activity that reached a maximum (9 times the basal activity) after a 5-min exposure of PC12 cells to NGF (100 ng/ml). Since NGF activates the tyrosine kinase activity of gp140trk, the product of the trk proto-oncogene, we also examined the association of PtdIns 3-kinase with gp140trk. Anti-gp140trk immunoprecipitates from NGF-stimulated PC12 cells had increased PtdIns 3-kinase activity compared to that of unstimulated cells, and larger increases were detected in cells overexpressing gp140trk, indicating that PtdIns 3-kinase associates with gp140trk. NGF produced large increases in [32P]phosphatidylinositol 3,4-bisphosphate and [32P]phosphatidylinositol 3,4,5-trisphosphate in PC12 cells labeled with [32P]orthophosphate, indicating an increase in PtdIns 3-kinase activity in intact cells. Using an anti-85-kDa PtdIns 3-kinase subunit antibody, we found that NGF promoted the tyrosine phosphorylation of an 85-kDa protein and two proteins close to 110 kDa. These studies demonstrate that NGF activates PtdIns 3-kinase and promotes its association with gp140trk and also show that NGF promotes the tyrosine phosphorylation of the 85-kDa subunit of PtdIns 3-kinase. Thus, PtdIns 3-kinase activation appears to be involved in differentiation as well as mitogenic responses.

Nerve growth factor (NGF)† was the first peptide growth

* This work was supported in part by grants from the Whitaker Foundation (to S. P. S.); National Institutes of Health Grant GM-41890 (to L. C. C.); and the National Cancer Institute, Department of Health and Human Services, under Contract N01-CO-74101 with Whitaker Foundation (to S. P. S.; National Institutes of Health Grant GM-41890 (to L. C. C.), and the National Cancer Institute, Department of Health and Human Services) to L. C. C. and the National Cancer Institute, Department of Health and Human Services, under Contract N01-CO-74101 with Whitaker Foundation to S. P. S. This work was supported in part by grants from the Whitaker Foundation (to S. P. S.; National Institutes of Health Grant GM-41890 (to L. C. C.), and the National Cancer Institute, Department of Health and Human Services, under Contract N01-CO-74101 with Whitaker Foundation to S. P. S. The abbreviations used are: NGF, nerve growth factor; PtdIns, phosphatidylinositol; PtdIns 3,4-P2, phosphatidylinositol-3,4-bisphosphate; PtdIns 3,4,5-P3, phosphatidylinositol-3,4,5-trisphosphate; DMEM, Dulbecco’s modified Eagle’s medium; PDGF, platelet-derived growth factor; BSA, bovine serum albumin; TBS, Tris-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography.

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‡ The abbreviations used are: NGF, nerve growth factor; PtdIns, phosphatidylinositol; PtdIns 3,4-P2, phosphatidylinositol-3,4-bisphosphate; PtdIns 3,4,5-P3, phosphatidylinositol-3,4,5-trisphosphate; DMEM, Dulbecco’s modified Eagle’s medium; PDGF, platelet-derived growth factor; BSA, bovine serum albumin; TBS, Tris-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography.
NGF Stimulates PI 3-Kinase and Its Association with gp140\(^{\text{trk}}\)

Christopher Carpenter (Tufts University and Massachusetts General Hospital) against recombinant p85 that was cloned from rat liver by Dr. Mannheim or Genentech, Inc. (University of California, San Francisco) were grown in 100- or 150-mm dishes in DMEM plus 5% horse serum and 5% calf serum at 37°C in a 95/5% air/CO\(_2\) mixture. Where noted, in some experiments cells were switched to serum-free medium (DMEM plus 0.1% BSA) overnight prior to stimulation with NGF. PC12 cells overexpressing gp140\(^{\text{trk}}\) (line 6–15) were obtained by electroporation of cells with a retrovirus containing the human trk and neomycin resistance genes under control of the CMV promoter. The generation of this cell line was as described.\(^2\)

**Assay of PtdIns 3-Kinase Activity**

NGF was added to intact cells at 37°C. To assess the association of PtdIns 3-kinase with anti-P-Tyr, cells were treated with NGF (100 ng/ml) for the indicated time, washed twice with ice-cold buffer A (137 mM NaCl, 30 mM Tris, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 0.1 mM vanadate, pH 7.5), and lysed in lysis buffer (buffer A plus 10% glycerol (v/v), 1% Nonidet P-40 (v/v), and 1 mM phenylmethylsulfonyl fluoride). The lysates were vortexed and centrifuged at 14,000 RPM (Eppendorf 5414 centrifuge). The cleared supernatant was transferred to a fresh microcentrifuge tube, and incubated with anti-P-Tyr antibody (35 μg/ml lystate) for 2 h at 4°C. To assess the association of PtdIns 3-kinase with gp140\(^{\text{trk}}\), PC12 cells (2 × 10\(^5\)) were treated for 5 min with 100 ng/ml NGF at 37°C. Cells were washed twice in ice-cold Tris-buffer saline (TBS) (20 mM Tris, pH 8.0, 0.15 M NaCl) and lysed in buffer A without MgCl\(_2\) and CaCl\(_2\) as described (Kaplan et al., 1989). Lysates were clarified by centrifugation as described above and incubated with 4 μl of anti-trk antibody (203) for 3 h at 4°C. Protein-A-Sepharose (4 mg/ml lystate) was then added for 2 h at 4°C to the lysates containing the anti-trk antibody or anti-P-Tyr antibody.

The immunoprecipitates were washed three times in PBS/1% Nonidet P-40, two times in 0.1 M Tris/0.5 M LiCl, and two times in TNE (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5). All wash solutions contained 100 μM vanadate. The PtdIns kinase assay of the immunoprecipitate was performed by adding sonicated PtdIns (in 10 mM HEPES, 1 mM EDTA, pH 7.5, 0.5 μg/ml final concentration) and [γ\(^{32}\)P]ATP (30–40 μCi/sample) to the immunoprecipitates for 10 min at room temperature. The reaction was stopped by the addition of 80 μl of HCl (1 M) and 160 μl of methanol:chloroform (1:1 mixture). The lipid-containing organic phase was resolved on oxalate-coated thin-layer chromatography plates (Silica Gel 60, MCB reagents, Merck, Rahway, NJ) developed in chlorormomethanol: water:ammonium hydroxide (60:40:11:3:2). Radiolabeled spots corresponding to authentic PtdIns-4-P standards were excised and quantified by scintillation counting or Cerenkov radiation. In some experiments the PtdInsnspot was decayed and subjected to HPLC analysis to determine lipid identity and quantification, as previously described (Auger et al., 1989).

**Measurement of Polyphosphoinositides in Intact Cells**

Cells that were nearly confluent were cultured overnight in serum-free DMEM (0.1% BSA). The cells were washed once and incubated with phosphate-free DMEM/0.1% BSA for 15 min then exposed to 5 μl of the same medium plus carrier-free [32P]orthophosphate (100 μCi/ml) at 37°C for 4 h. NGF (100 ng/ml) was added to the cells for the designated time. The cells were washed twice with ice-cold buffer A and lysed in 1 M HCl: methanol (1:1, v/v), and the lipids were extracted with chloroform. The lipids were deacylated and analyzed by HPLC as described previously (Auger et al., 1989).

**Immunoprecipitation and Western Blot Assays**

Anti-p65 Immunoprecipitations—A total of 2 × 10\(^5\) PC12, trk-PC12, or NIH-3T3 cells transfected with a rat trk gene (Kaplan et al., 1991b) were lysed in Lysis buffer, and cleared lysates were immunoprecipitated with 2 μl of anti-p65 for 3 h at 4°C. In some experiments, cells were lysed in RIPA buffer (NaCl, 150 mM; Nonidet P-40, 1%; deoxycholate, 0.5%; Trit, 50 mM; pH 8.0) containing 0.2% SDS. The immunoprecipitates were collected with 20 μl of protein A-Sepharose, washed three times with buffer A and once with distilled water, and electrophoresed on 8.5% SDS-polyacrylamide gels. Proteins were transferred to 0.2-μm nitrocellulose filters, the filters were blocked using TBS/2% BSA for 1 h at room temperature, and the filters were incubated with anti-P-Tyr for 16 h at 4°C. The filters were washed three times in TBS/0.2% Tween-20 (TTBS). Proteins were visualized using a chemiluminescence detection system (ECL, Amersham). Previous studies have focused upon the activation of PtdIns 3-kinase during cell proliferation or the activation of neutrophils and platelets (reviewed in Auger and Cantley, 1991). In the present study, we investigated the activation of PtdIns 3-kinase by NGF and the association of PtdIns 3-kinase with gp140\(^{\text{trk}}\), a tyrosine kinase that is associated with the initiation of cellular differentation. NGF produced an increase in PtdIns-3,4-P\(_2\) and PtdIns-3,4,5-P\(_3\), the products of PtdIns 3-kinase, and promoted the physical association of this kinase with gp140\(^{\text{trk}}\). NGF also stimulates the tyrosine phosphorylation of multiple cellular proteins, including the 85-kDa protein subunit of PtdIns 3-kinase. Thus, PtdIns 3-kinase appears to be directly activated by this NGF receptor.

**MATERIALS AND METHODS**

**Chemicals**

All chemicals were reagent grade or better. [\(^{32}\)P]ATP (specific activity, 3000 Ci/mmol) and [\(^{3}H\)orthophosphate (8500 Ci/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco Laboratories. NGF (2.58) was obtained from Boehringer-Mannheim or Genentech, Inc. Insulin was purchased from Sigma, and PDGF (BB) was obtained from Upstate Biochemicals Inc. Trk anti sera (203) was generated in rabbits to the 14 carboxyl-terminal amino acids of human gp140\(^{\text{ek}}\) as previously described (Martin-Zanca et al., 1988; Kaplan et al., 1991a). This trk antibody recognizes trk molecules from mouse, rat, and human (Kaplan et al., 1991a, 1991b). The anti-phosphotyrosine antibody, or anti-P-Tyr, was a murine monoclonal antibody generated using phosphotyramine as an immunogen and was kindly supplied by Dr. Brian Drucker (Dana Farber Cancer Institute, Boston, MA) and Dr. Deborah Morrison (National Cancer Institute-Frederick Cancer Research and Development Center). Antibody (anti-p85) to the 85-kDa subunit of PtdIns 3-kinase was raised in rabbits by Dr. Brian Schaffhausen (Tufts University) against recombinant p85 that was cloned from rat liver by Dr. Christopher Carpenter (Tufts University and Massachusetts General Hospital), and is commercially available from Upstate Biochemicals Inc.

**Cell Culture**

PC12 cells (from Dr. Larry Feig (Tufts University) or Dr. R. Kelly (University of California, San Francisco)) were grown in 100- or 150-mm dishes in DMEM plus 5% horse serum and 5% calf serum at 37°C in a 95/5% air/CO\(_2\) mixture. Where noted, in some experiments cells were switched to serum-free medium (DMEM plus 0.1% BSA) overnight prior to stimulation with NGF. PC12 cells overexpressing gp140\(^{\text{trk}}\) (line 6–15) were obtained by electroporation of cells with a retrovirus containing the human trk and neomycin resistance genes under control of the CMV promoter. The generation of this cell line was as described.\(^2\)

\(^2\) B. Hampstead, S. L. Rahin, L. Kaplan, S. Reid, L. Parada, and D. R. Kaplan, manuscript submitted.
Amersham) with secondary antibodies from Boehringer-Mannheim. To reprobe the filters, they were stripped of primary and secondary antibodies in 100 mM 2- mercaptetoethanol, 2% SDS, 0.5 mM Tris-HCl, pH 6.7, for 1 h at 60°C, washed three times in TBS, pH 8.0, reblocked, and incubated overnight with anti-p85 (1:5000 dilution in TTBS). Proteins were visualized using ECL as above.

Anti-P-Tyr Immunoprecipitations of p85—A total of \(10^7\) PC12 cells were lysed in Lysis buffer, and cleared lysates were immunoprecipitated with anti-P-Tyr (35 ng/ml) for 2 h at 4°C. The immunoprecipitates were collected with protein A-Sepharose, washed three times in PBS/1% Nonidet P-40, two times in 0.1 M Tris/0.5 M LiCl, and two times in TNE. All wash solutions contained 100 mM vanadate. The samples were boiled for 5 min, and the supernatants were electrophoresed on 7% SDS-polyacrylamide gels. Proteins were transferred to 0.2-μm nitrocellulose filters. The filter was blocked for 1 h in TBS/5% nonfat dry milk, washed two times with TTBS, and exposed to anti-p85 antibody (1:3000 dilution in TTBS/5% nonfat dry milk) for 1 h at room temperature. Filters were washed three times with TTBS. Filters were exposed to secondary anti-rabbit-AP antibodies (3 μl per 20 ml of TTBS/5% nonfat dry milk) from Promega and washed three times using TTBS, and proteins were identified by the alkaline phosphatase technique.

RESULTS

Anti-phosphotyrosine Immunoprecipitates of NGF-stimulated Cells Have PtdIns 3-Kinase Activity—Preliminary experiments demonstrated that exposure of PC12 cells to NGF produced an increase in anti-P-Tyr-immunoprecipitable PtdIns 3-kinase activity. To examine this more closely, we studied the time course of the effects of NGF on PC12 cells. As shown in Fig. 1, NGF (100 ng/ml) produced a time-dependent increase in PtdIns 3-kinase activity. The maximum stimulation occurred after 5 min of NGF exposure. At this time PtdIns 3-kinase activity was elevated to 9.2 ± 1.2 (mean ± S.E., n = 3) times the basal level of activity. After longer exposures of the cells to NGF, the activity declined to a lower level that remained stimulated above the basal level for at least 60 min of NGF exposure. At this time, PtdIns 3-kinase activity was 4.1 ± 0.9 (n = 3) times the basal activity. HPLC analysis of the deacylated product of the PtdInsP product confirmed that it is primarily (>90%) PtdIns-3-P (Fig. 1, inset). Thus, PtdIns 3-kinase was either phosphorylated on tyrosine or associated with a tyrosine phosphorylated protein in NGF-stimulated cells.

Association of PtdIns 3-Kinase with gp140trk—We next investigated whether gp140trk associated with PtdIns 3-kinase activity in wild-type PC12 cells and in PC12 cells that overexpressed gp140trk (trk-PC12 cells). Trk-PC12 cells express 20-fold more gp140trk than do wild-type cells, and show 10-fold higher levels of NGF-activated gp140trk tyrosine kinase activity. The association of PtdIns 3-kinase activity with gp140trk was measured in anti-trk immunoprecipitates of lysates prepared from untreated cells or cells treated for 5 min with NGF (Fig. 2A). In wild-type cells, NGF stimulated a slight increase (2-fold) in anti-trk-immunoprecipitable PtdIns 3-kinase activity. In contrast, NGF stimulated a much larger (10-fold) increase in anti-trk-immunoprecipitable PtdIns 3-kinase activity in trk PC12 cells. There was only a small amount of PtdIns 3-kinase activity present in precipitates prepared with the preimmune serum (Fig. 2B). Incuba-

![Fig. 1.](image1.png) **Fig. 1.** Exposure of PC12 cells to NGF increases anti-P-Tyr-immunoprecipitable PtdIns3-kinase activity. PC12 cells were serum-starved overnight and then were exposed to 100 ng/ml NGF for 0-60 min. Lysates were prepared in 1% Nonidet-P40 and immunoprecipitated for 2 h at 4°C with anti-P-Tyr. PtdIns 3-kinase activity was measured as the ratio of PtdIns 3-kinase activity to protein measured by BCA assay.

![Fig. 2.(A) PtdIns 3-kinase activity in wild-type PC12 cells and in PC12 cells overexpressing gp140trk.](image2.png) **Fig. 2.** A, PtdIns 3-kinase activity in wild-type PC12 cells and in PC12 cells overexpressing gp140trk. Wild-type PC12 cells (WT PC12) or PC12 cells overexpressing gp140trk (trk PC12) (2 x 10^7 cells) were treated for 5 min with NGF (100 ng/ml). Cell lysates were immunoprecipitated with 4 μl of anti-trk antibody 203 for 3 h at 4°C as described under "Materials and Methods." PtdIns 3-kinase activity of anti-trk immunoprecipitates was assayed using exogenous PtdIns as a substrate. B, the immunoprecipitation of PtdIns 3-kinase activity by anti-gp140trk (anti-trk) is specific. Trk PC12 cells were treated for 5 min with NGF (100 ng/ml), and immunoprecipitates were prepared with anti-trk serum (4 μl/ml), pre-immune serum (4 μl/ml), or anti-trk serum (4 μl/ml) plus the peptide (10 μg/ml) used to generate anti-trk.
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ion of anti-trk immunoprecipitates with a peptide (10 μg/ml) used to generate the trk antibody reduced significantly the association of PtdIns 3-kinase activity (Fig. 2B). In NGF-treated cells the amount of PtdIns 3-kinase activity observed in anti-trk immunoprecipitates represented less than 2% of the PtdIns 3-kinase activity in anti-P-Tyr immunoprecipitates when wild-type PC12 cells were assayed and less than 10% in assays of trk-PC12 cells (not shown). These results indicate that PtdIns 3-kinase associates with gp140°n. In conjunction with the observation that anti-P-Tyr antibody immunoprecipitates PtdIns 3-kinase activity (Fig. 1) and that NGF activates PtdIns 3-kinase activity in vivo (below), these results suggest that the activation of PtdIns 3-kinase by NGF occurs via stimulation of the gp140°n tyrosine kinase activity.

Analysis of Phosphoinositides in Intact PC12 Cells after NGF Stimulation—To investigate alterations in the levels of the various phosphoinositide products by NGF, we measured the in vivo levels of the D-3-phosphorylated polyphosphoinositides. Cells were prelabeled with [32P]orthophosphate and exposed to NGF for various times. The lipids were extracted and deacylated, and the glycerophosphoinositols polyphosphates were analyzed by HPLC (as in Fig. 1, inset). Unlike quiescent human vascular smooth muscle cells, which did not contain detectable amounts of PtdIns-3,4,5-P3, or PtdIns-3,4,5-P3 unless stimulated by growth factors (Auger et al., 1989), PC12 cells contained measurable radioactivity incorporated into PtdIns-3,4,5-P3 and PtdIns-3,4-P2 prior to stimulation by NGF. Small changes in these products were observed 1 min after NGF (100 ng/ml) exposure (Fig. 3A). Five min after NGF exposure, [32P] incorporation into PtdIns-3,4,5-P3 was increased to levels that were five times the basal level. After 15 min, the level of [32P]PtdIns-3,4,5-P3 had declined to near base levels and was maintained at this level after 30 min. The [32P]PtdIns-3,4-P2 level increased more than [32P] PtdIns-3,4,5-P3 and was maintained at a greater elevated level. After 5 min of NGF exposure, [32P] PtdIns-3,4-P2 increased to 13-fold the basal level, and further increased to a slightly greater level after 15 min. After 30 min, the level of [32P] PtdIns-3,4-P2 declined to a value that remained 8-fold that of the basal amount.

In contrast to the marked changes produced in [32P] PtdIns-3,4-P2, much smaller relative changes were produced in [32P] PtdIns-4,5-P2 following the addition of NGF (Fig. 3A). The largest change was observed at 30 min after NGF exposure, when the [32P]PtdIns-4,5-P2 level declined to about 60% of the basal amount. Relatively small changes were observed in the in vivo levels of [32P]PtdIns-3-P and [32P]PtdIns-4-P over the course of the NGF stimulation (Fig. 3B).

Tyrosine Phosphorylation of 85-kDa Protein in NGF-stimulated Cells—We next examined whether a component of PtdIns 3-kinase was phosphorylated on tyrosine in response to NGF treatment of cells. PtdIns 3-kinase activity co-purifies with proteins of 85 and 110 kDa (Carpenter et al., 1990). PC12 cells, trk-PC12 cells, or NIH-3T3 cells transfected with a rat trk cDNA (trk-3T3) were treated for 5 min with NGF (100 ng/ml), and cell lysates were immunoprecipitated with anti-p85 antibody. Protein blots of the immunoprecipitates were then probed with anti-P-Tyr antibody. Protein blots of these lysates were transferred to nitrocellulose and probed with anti-p85 antibody. Protein blots of the immunoprecipitates were also observed in NGF-treated PC12 cells or PDGF-treated NIH-3T3 cells that were lysed in a more stringent buffer (RIPA) that contained 0.2% SDS or in lysis buffer that contained 1% SDS. When anti-p85 immunoprecipitates of these lysates were transferred to nitrocellulose and probed with anti-P-Tyr, we detected a phosphorylated 85-kDa protein in growth factor-treated cells. An 85-kDa protein that co-localized with this protein was detected when the filter was stripped and reprobed with anti-p85 (not shown).

In trk-3T3 cells, NGF stimulated the tyrosine phosphorylation of an 85-kDa protein and of two proteins at approximately 110 kDa (Fig. 4A) that were immunoprecipitated using anti-p85. NGF also stimulated PtdIns 3-kinase activity by about 3-fold in anti-P-Tyr immunoprecipitates prepared from trk-3T3 cells (not shown). PDGF treatment of the trk-3T3 cells induced the tyrosine phosphorylation of 85- and 110-kDa proteins and a third protein of 185 kDa. The 185-kDa protein is most likely the PDGF receptor (Escobedo et al., 1991), and the 110-kDa protein may be a second component of PtdIns 3-kinase (Carpenter et al., 1990; Escobedo et al., 1991; Otsu et al., 1991). Immunoprecipitations and Western blot assays were also

![Figure 3. Effects of NGF on the levels of phosphoinositides of intact PC12 cells labeled in vivo using 32P-orthophosphate.](image-url)
performed using these antibodies in the reverse order from that described above. In these experiments, 85-kDa proteins were detected using anti-p85 antibody to blot the anti-P-Tyr immunoprecipitates from cell lysates of NGF-treated PC12 cells and PDGF-treated NIH-3T3 cells (Fig. 4C). p85 may appear as a doublet due to the lower percentage of acrylamide (7%) used in the resolving gel in this experiment. Taken together, these experiments indicate that the 85-kDa subunit of PtdIns 3-kinase is phosphorylated on tyrosine in growth factor-stimulated cells.

**DISCUSSION**

The results presented in this paper demonstrate that 1) PtdIns 3-kinase is activated by NGF in PC12 cells, 2) PtdIns 3-kinase associates with the trk proto-oncogene product, gp140trak, and 3) an 85-kDa protein is phosphorylated on tyrosine in NGF- (and PDGF-) treated cells. Immunoprecipitation and Western blot assays using an antibody raised against the p85 subunit of PtdIns 3-kinase demonstrate that this protein is the 85-kDa PtdIns 3-kinase subunit.

While enhancements in inositol phosphate levels have been previously demonstrated in NGF-treated PC12 cells, the types of phosphoinositides generated were not characterized. We show that NGF treatment of cells induces an increase in the levels of PtdIns-3,4-P2 and PtdIns-3,4,5-P3, the expected products of PtdIns 3-kinase. Increases in the levels of PtdIns-4,5-P2, a substrate for PLC-γ1 activities, were not observed in these studies. The association of PtdIns 3-kinase with gp140trak and the increased cellular levels of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 occurred within 5 min of NGF addition to cells, suggesting that PtdIns 3-kinase activation is a primary response to gp140trak activation.

Our results demonstrate an activation of PtdIns 3-kinase activity in cells responding to NGF, an anti-mitogenic and differentiative factor. Increases in PtdIns 3-kinase activity previously have been observed only in growth factor-treated and -transformed cells and in neutrophils and platelets exposed to activating agents (reviewed in Auger and Cantley, 1991; and Cantley et al., 1991). It is unlikely that activation of PtdIns 3-kinase by NGF alone is sufficient to induce differentiation of PC12 cells. Both EGF and insulin, which are mitogenic agents for PC12 cells, stimulate the appearance of PtdIns 3-kinase activity in anti-P-Tyr immunoprecipitates from these cells. EGF also stimulates the production of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 in PC12 cells labeled with [32P]Pi. Neither EGF nor insulin promotes the morphological differentiation of PC12 cells. Therefore, although the activation of PtdIns 3-kinase is one of the first events involved in NGF-mediated signal transduction in PC12 cells, its role in differentiation remains unclear. The distinction between the involvement of PtdIns 3-kinase in differentiation from that of growth processes could conceivably involve the relative amounts of or temporal response of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 to the activating agent. In fact, the kinetics and relative levels of the in vivo production of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 in NGF-treated cells are different from those measured in EGF-treated cells. These differences may denote a distinction between a growth response and a differentiative response and are the focus of current studies. Alternatively, the promotion of cell growth and transformation (and differentiation) may require multiple cellular responses of which the activation of PtdIns 3-kinase may be but one. This may involve the recruitment of multiple cytosolic enzymes to the plasma membrane and the generation of multiple signals in the cell (reviewed in Cantley et al., 1991).

The association of PtdIns 3-kinase with receptor protein-tyrosine kinases has been a hallmark observation of this family of tyrosine kinases (Cantley et al., 1991). PtdIns 3-kinase is a heterodimer consisting of 85- and 110-kDa proteins (Carpenter et al., 1990). The 85-kDa subunit has two src homology-2 (SH-2) domains, a src homology-3 (SH-3) domain, and a bcr homology domain (Otsu et al., 1991). SH-2 domains have recently been shown to confer special association with tyrosine-phosphorylated proteins (Cantley et al., 1991). The tyrosine phosphorylation domains of some receptor and nonreceptor proteins that bind PtdIns 3-kinase are highly conserved and have a phospho-Tyr-Met/Val-Asp/Pro-Met consensus sequence (Cantley et al., 1991). Phosphopeptides based on this sequence can block the in vitro association between PtdIns 3-kinase and the PDGF receptor (Escobedo et al., 1991) or polyoma middle T/p60src (Auger et al., 1992). Certain ligand-activated tyrosine kinases such as the insulin receptor do not contain a good consensus PtdIns 3-kinase association sequence, yet bind PtdIns 3-kinase (Ruderman et al., 1990). The amount of PtdIns 3-kinase binding to insulin receptors represents only a small proportion of anti-P-Tyr immunoprecipitable PtdIns 3-kinase activity (Ruderman et al., 1990), suggesting that the association between the receptor and PtdIns 3-kinase is of a low affinity.

The characteristics of the association of PtdIns 3-kinase
with gp140<sup>trk</sup> are similar to that of the insulin receptor. The amount of PtdIns 3-kinase activity observed in anti-trk immunoprecipitates represents only a small fraction (<2%) of the PtdIns 3-kinase activity in anti-P-Tyr immunoprecipitates, indicating that in the case of the insulin receptor, the association of PtdIns 3-kinase activity with gp140<sup>trk</sup> in detergent-containing buffer is unstable. Consistent with this result, we did not observe the presence of significant quantities of gp140<sup>trk</sup> in anti-p85 immunoprecipitates prepared from NGF-treated cells.<sup>4</sup> The association of these proteins may be below the level of sensitivity of the anti-gp140<sup>trk</sup> and anti-p85 antibodies. Another possibility is that gp140<sup>trk</sup> may interact with other subunits of PtdIns 3-kinase. However, we do observe the association of baculovirus-expressed trk with SH2 domains of p85/PtdIns 3-kinase in <i>vitro</i>,<sup>4</sup> indicating that trk is capable of forming complexes with p85. Alternatively, PtdIns 3-kinase may become activated in NGF-treated PC12 cells by interacting with other receptor binding sites or other proteins, such as the recently identified insulin receptor substrate IRS-1 (Sun et al., 1991). In insulin-treated PC12 cells, IRS-1 directly binds PtdIns 3-kinase.<sup>5</sup> However, IRS-1 does not co-immunoprecipitate with PtdIns 3-kinase from NGF-treated PC12 cells,<sup>6</sup> and IRS-1 is not a substrate of gp140<sup>trk</sup>,<sup>7</sup> indicating that the activation of PtdIns 3-kinase by ligand-activated gp140<sup>trk</sup> receptors involves mechanisms distinct to that utilized by insulin receptors.

REFERENCES

Auger, K. R., Serunian, L. A., Soltsoff, S. P., Libby, P., and Cantley, L. C. (1989) Cell 57, 167-175
Auger, K. R., and Cantley, L. C. (1991) Cancer Cells 3, 263-270
Auger, K. R., Carpenter, C. L., Shoelson, S. E., Pwnica-Worms, H., and Cantley, L. C. (1992) J. Biol. Chem. 267, 5408-5415
Barde, Y. A. (1989) Neuron 2, 1525-1534
Bienle, J., and Erikson, R. L. (1986) EMBO J. 5, 3441-3447
Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., Depinho, R. A., Panayootatos, N., Cobb, M. H., and Vancopouges, G. D. (1991) Cell 65, 665-675
Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltsoff, S. (1991) Cell 64, 293-302
Carpenter, C. L., and Cantley, L. C. (1990a) Biochemistry 29, 11147-11156.
Carpenter, C. L., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B., and Cantley, L. C. (1990b) J. Biol. Chem. 265, 19704-19711
Consters, M. L., and Guroff, G. (1987) J. Neurochem. 48, 1466-1472
Escobedo, J. A., Navancasatutas, S., Kavanagh, W. M., Milly, D., Fried, V. A., and Williams, L. T. (1991) Cell 65, 75-82
Gotoh, Y., Nishida, E., Yamashita, T., Hoshi, M., Nakawami, M., and Sakai, H. (1990) Eur. J. Biochem. 193, 661-669

4 D. R. Kaplan, unpublished results.
5 V. Cleghow, T. Pawson, B. Schaffhausen, and D. R. Kaplan, unpublished results.
6 D. R. Kaplan and M. White, unpublished results.