Activation of phosphatidylinositol 3-kinase by epidermal growth factor, basic fibroblast growth factor, and nerve growth factor in PC12 pheochromocytoma cells

(transmembrane signaling/mitogenesis/phospholipids)

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ABSTRACT Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and nerve growth factor (NGF), which stimulate the phosphorylation of proteins on tyrosine in PC12 cells, initiate these modifications through ligand-specific cell surface receptors that contain the causative tyrosine kinases. One apparent substrate for these enzymes is phosphatidylinositol 3-kinase (PI 3-kinase), an enzyme that phosphorylates the D-3 position of the inositol ring and associates with several protein tyrosine kinases, as indicated by the fact that it is immunoprecipitated from EGF-, bFGF-, and NGF-stimulated PC12 cells by an anti-phosphotyrosine antibody. All three growth factors increase immunoprecipitable PI 3-kinase activity after 2 min of addition at concentrations able to stimulate either mitogenic or neurotrophic responses in PC12 cells. The level of stimulation of PI 3-kinase activity by EGF, bFGF, and NGF is 15- to 20-fold, 2- to 3-fold, and 8- to 10-fold, respectively. Moreover, tyrosine phosphorylation of PI 3-kinase was detected in EGF-, bFGF-, and NGF-stimulated PC12 cells, and the amount of the phosphorylation correlated with the level of stimulation of enzyme activity. In contrast, phosphatidylinositol 4-kinase, which produces the inositol phospholipids cleaved by phospholipase C- γ to yield diacylglycerol and inositol-1,4,5-trisphosphate, is not affected by these growth factors. The pattern of stimulation of PI 3-kinase does not correlate with the induction of neurite outgrowth but rather with the mitotic responses, suggesting that PI 3-kinase and its products may be more important for signaling in cell division than in trophic processes. However, the levels of phosphatidylinositol 3-phosphate do not coincide with the stimulation of [3 H]thymidine incorporation by these growth factors, rendering its role in mitotic functions, at least in PC12 cells, also uncertain.

Nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) induce reversible differentiation accompanied by neurite proliferation in the rat pheochromocytoma PC12 cell line, whereas epidermal growth factor (EGF) induces only mitogenic responses. As the receptors for all three growth factors contain tyrosine kinases, differences in protein tyrosine phosphorylation may be responsible for differences in intracellular signaling and phenotypic response generated by these receptors. Only few substrates for these induced tyrosine phosphorylations that are likely to be involved mechanistically have been identified in PC12 cells. Those include phospholipase C- γ and several other kinases (1–6).

Recently, several kinase-containing growth factor receptors have been demonstrated to physically associate with and activate an inositol phospholipid kinase, termed type I or phosphatidylinositol 3-kinase (PI 3-kinase). This enzyme

phosphorylates phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI-4-P), and phosphatidylinositol 4,5-bisphosphate (PI-4,5-P) at the D-3 position of the *myo*-inositol ring. PI 3-kinase is present in anti-phosphotyrosine immunoprecipitates from intact cells stimulated with various growth factors, and it specifically forms a complex with the receptors for platelet-derived growth factor, EGF, colony-stimulating factor 1, insulin, and interleukin 2 (7–11) and with the oncogene products of polyoma virus middle-sized tumor antigen (MTAg)/pp60^{c-src}, *v-src*, *v-fms*, *v-abl*, *v-yes*, and *v-ras* (12–17).

PI 3-kinase is a heterodimer comprised of 85- and 110-kDa protein subunits (18). cDNA clones encoding p85 have been isolated (19–21) and, on the basis of the deduced amino acid sequence, it appears that this protein represents a noncatalytic subunit of a PI 3-kinase enzyme, suggesting that the catalytic activity is associated with the 110-kDa protein. p85 contains two src-homology 2 domains (SH2 domains), which can act as linkers between tyrosine kinases and their substrates. Although tyrosine phosphorylation of p85 correlates with an increase in PI 3-kinase activity in intact cells, the role of D-3-phosphorylated inositol phospholipids in receptor-mediated signal transduction remains uncertain.

The association of PI 3-kinase with growth factor receptors and oncoproteins also implicates it as a mediator of cell growth and transformation. In this report, we show that anti-phosphotyrosine antibody immunoprecipitates from EGF-, bFGF-, and NGF-stimulated PC12 cells contain elevated levels of PI 3-kinase activity. We also show that under the same conditions tyrosine phosphorylation of PI 3-kinase occurs, suggesting that this enzyme is a direct substrate of EGF, bFGF, and NGF receptor kinases or of unidentified tyrosine kinases that are ligand activated.

MATERIALS AND METHODS

Materials. Mouse EGF and β -NGF were prepared by the methods of Savage and Cohen (22) and Mobley *et al.* (23), respectively. A bFGF analog in which all half-cystine residues were replaced by serines (24) was a gift from G. Michael Fox (Amgen). This analog has been shown to display activities indistinguishable from preparations of the natural sequence in PC12 cells (25). The anti-phosphotyrosine monoclonal antibody 1G2 coupled to agarose was purchased from Oncogene Science (Manhasset, NY); the anti-phosphotyrosine monoclonal antibody PY20 was from ICN; the anti-*v-src* monoclonal antibody (2–17) was from Microbiological

Abbreviations: MTAg, middle-sized tumor antigen; PI 3-kinase, phosphatidylinositol 3-kinase; PI, phosphatidylinositol; PI-3-P, PI-4-P, and PI-4,5-P, phosphorylated isomers of PI; SH2 domain, src-homology 2 domain; gPI, glycerophosphoinositol; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; NGF, nerve growth factor.

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Associates and the anti-rat PI 3-kinase polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY). PI was obtained from Avanti Polar Lipids; PI-4-*P* and phosphatidylserine were from Fluka and Sigma. High molecular weight protein electrophoresis standards were from Bio-Rad. Thin-layer silica gel plates were obtained from Analtech and Merck. [γ - 32 P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) was from DuPont/NEN, and [3 H]thymidine (41 Ci/mmol) and 125 I-labeled protein A (>30 μ Ci/ μ g) were from ICN. All other reagents were of analytical grade.

Cell Culture. PC12 cells were maintained in culture as described (26). For experiments, cells were plated in 150-mm Falcon culture dishes (Beckton Dickinson), precoated with poly(L-lysine) (Sigma), and grown to 60–70% confluency. The medium was removed and replaced with Dulbecco's modified Eagle's medium (DMEM) alone for 40–48 hr. Wild-type polyoma MTag-infected NIH 3T3 fibroblasts, τ -2 (27), obtained from B. Schaffhausen (Tufts University, Boston), were cultured in DMEM supplemented with 10% fetal bovine serum.

Mitogenesis Assay. PC12 cells were plated in 24-well Limbro plates (Flow Laboratories) precoated with poly(L-lysine). After 2 days they were washed three times with DMEM and kept in serum-free medium for at least 48 hr. At the end of this period, cells were treated with increasing concentrations of bFGF, EGF, and NGF in DMEM plus transferrin (30 μ g/ml), or complete medium, for 40 hr and labeled with 1 μ Ci of [3 H]thymidine per ml for the last 16 hr.

Immunoprecipitation and Immunoblotting. PC12 cells, grown as described above, were exposed to bFGF, EGF, and NGF in DMEM containing Hepes at the concentrations and the times indicated at 37°C. The cells were rinsed in cold phosphate-buffered saline containing 1 mM sodium orthovanadate and lysates (2 mg), prepared according to the protocol provided by Upstate Biotechnology, were incubated with 5 μ l of anti-PI 3-kinase antiserum for 2 hr at 4°C; the immunocomplexes were collected on protein A-Sepharose (Pharmacia LKB). After being washed, the complexes were boiled for 5 min in SDS sample buffer. All the samples were resolved by 7.5% SDS/PAGE and transferred to Immobilon P membranes (Millipore) by electroblotting at 60 V overnight. The membranes were probed with either anti-phosphotyrosine antibody or anti-PI 3-kinase polyclonal antiserum and then incubated with 125 I-labeled protein A. Autoradiography was performed with Kodak X-Omat films for 1–6 hr at –70°C.

PI Kinase Assay. Lysates (1 mg) of unstimulated and stimulated PC12 cells, prepared according to the protocol provided by Oncogene Science, were incubated with 20 μ l of a 50% suspension of immobilized anti-phosphotyrosine antibody for 3–4 hr at 4°C. The immunoprecipitates were washed twice with lysis buffer and three times with 100 mM NaCl/1 mM EDTA/20 mM Tris-HCl, pH 7.5. The lipid kinase assay and lipid extraction were performed directly on the beads as described (28).

Deacylation and HPLC Analysis. For definitive identification of phosphatidylinositol phosphates (PIPs) detected on the TLC plates, the radioactive spots were excised, deacylated, and subjected to HPLC analysis using a 10- μ m Partisphere SAX column (Whatman) as described (28). A mixture of AMP and ADP was applied with the deacylated samples, and their 260-nm absorbance served to standardize elution times that were similar from one run to the next. Radioactivity was detected by assaying 300 μ l of eluate fractions in 10 ml of Hydrofluor (National Diagnostics, Manville, NJ) using an LS6000 IC Beckman scintillation counter. 3 H-labeled glycerophosphatidyl-4-*P* (3 H]gPI-4-*P*), prepared by deacylation of [3 H]PI-4-*P*, was used as an internal standard. The 32 P-labeled glycerophosphatidylinositol 3-phosphate (32 P]gPI-3-*P*) standard was prepared from anti-src immunoprecipitates of τ -2 cells.

RESULTS

Activation of PI Kinase by EGF, bFGF, and NGF. To examine the effect of three growth factors known to stimulate PC12 cells (albeit in different ways) on PI 3-kinase, serum-starved cells were treated with EGF, bFGF, and NGF for 2 min and lysed in detergent-containing buffer. Detergent-soluble proteins were immunoprecipitated with anti-phosphotyrosine antibody and the immunoprecipitates were incubated with PI-containing vesicles in the presence of Mg^{2+} and [γ - 32 P]ATP. As shown in Fig. 1, immunoprecipitates from EGF-, bFGF-, and NGF-stimulated PC12 cells contained elevated levels of a phospholipid kinase activity that phosphorylated PI to a product with a slightly lower chromatographic mobility than the PI-4-*P* standard. Furthermore, formation of this radiolabeled PI monophosphate was obtained under *in vitro* conditions optimized for the detection of PI 3-kinase activity rather than that of the ubiquitous PI 4-kinase. The increases in PI kinase activity following stimulation of PC12 cells with EGF, bFGF, and NGF were 15- to 20-fold, 2- to 3-fold, and 8- to 10-fold, respectively. The stimulation of PI kinase activity was observed at concentrations of EGF (1–10 ng/ml), bFGF (0.1–10 ng/ml), and NGF (10–100 ng/ml) that are similar to those required to elicit submaximal to maximal proliferative and neurotrophic responses for these cells (data not shown; see also Fig. 5). Concentrations of bFGF and EGF at 5 ng/ml and NGF at 100 ng/ml were used in all subsequent experiments.

Identification of the Lipid Products of the Induced PI Kinase Activity. Since the lipid kinase activity detected in anti-phosphotyrosine immunoprecipitates of stimulated PC12 cells by bFGF, EGF, and NGF phosphorylates PI and generates a product with TLC migration properties similar, but not identical, to PI-4-*P*, it was necessary to identify this product. The germane lipids were extracted from the thin-layer plate, deacylated, and analyzed by anion-exchange HPLC (Fig. 2). As shown in Fig. 2A, deacylated PI-3-*P* and PI-4-*P* (gPIPs) are readily separated by this method (28). The gPIP from control cells (Fig. 2B) showed detectable levels of both isomers, with a preponderance of the PI-3-*P* derivative. The gPIP from the growth factor-induced samples showed variable amounts of only the 3 isomer (Fig. 2 C–E). The 4-isomer was essentially undetectable. Thus the product formed by growth factor-stimulated PI kinase in PC12 cells appears to be the same as that formed in pp60^{c-src} immunoprecipitates from middle T-transformed cells, and all of the PI

FIG. 1. PI kinase activity of anti-phosphotyrosine immunoprecipitates from bFGF- (F), EGF- (E), and NGF-stimulated (N) PC12 cells. Cells were exposed as indicated to growth factors for 2 min. Lipid kinase assays were performed using PI as substrate; [γ - 32 P]ATP was added to start the reaction. The products were extracted with chloroform/methanol, separated by TLC, and autoradiographed. The migration of the PI-4-*P* standard and the origin (ORI) are designated by arrows. The radioactivity at the origin is due to [γ - 32 P]ATP and other water-soluble 32 P-labeled materials that were not completely extracted from the chloroform layer. Results are representative of two or more independent experiments.

FIG. 2. HPLC analysis of deacylated PIP products extracted from anti-phosphotyrosine immunoprecipitates of bFGF-, EGF-, and NGF-treated PC12 cells. The $[^{32}\text{P}]\text{PIP}$ formed in immunoprecipitates of control and stimulated cells was separated by TLC, scraped, and deacylated. (A) Migration positions of a $[^{32}\text{P}]\text{gPi-3-P}$ standard (○), generated from polyoma virus MTag-transformed fibroblasts (τ -2), and a $[^3\text{H}]\text{gPi-4-P}$ standard (△). (B–E) Deacylated extracts of PC12 cells that were unstimulated (B) and stimulated with bFGF (C), EGF (D), and NGF (E). The positions of two internal standards (i.e., AMP and ADP) are indicated by solid vertical arrows. The position of $[^3\text{H}]\text{gPi-4-P}$ in B–E is indicated by an open arrow.

kinase activity induced in PC12 cells by EGF, bFGF, and NGF appears to be PI 3-kinase.

Onset and Duration of EGF, bFGF, and NGF Action. Following addition of bFGF, EGF, and NGF, increased immunoprecipitable PI 3-kinase activity in PC12 cells reached maximum levels after 2 min and decreased considerably after 15 min (Fig. 3); with bFGF this latter level reached control values of unstimulated cells. The PI 3-kinase

FIG. 3. Time-dependent stimulation of PI 3-kinase in PC12 cells by bFGF, EGF, and NGF. Anti-phosphotyrosine immunoprecipitates prepared from untreated (C), bFGF- (F), EGF- (E), and NGF- (N) treated cells, either for 2 min (open bars) or 15 min (solid bars), were incubated with PI and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The quantity of ^{32}P incorporated into PI-3-P is shown as cpm. The data points and error bars represent the mean thousands of cpm $\pm$ standard deviations from triplicate samples of a single representative experiment.

activity detected in anti-phosphotyrosine immunoprecipitates of EGF-stimulated cells was the greatest, followed by NGF and bFGF. Although some variability in the utilization of the PI substrate was observed, the general profile shown was found in four independent experiments.

Tyrosine Phosphorylation of PI 3-Kinase. Serum-starved PC12 cells were stimulated with bFGF, EGF, and NGF for 5 min and lysates were prepared and subjected to immunoprecipitation with anti-PI 3-kinase antibodies. Tyrosine phosphorylation of PI 3-kinase was assessed by probing the immunocomplexes with anti-phosphotyrosine antibody (Fig. 4A) and the level of enzyme present in these complexes with anti-PI 3-kinase antibodies (Fig. 4B). Clearly, the 85-kDa subunit of PI 3-kinase is phosphorylated on tyrosine in response to all three growth factors. The level of this modification appears to be markedly different, with EGF inducing the most and bFGF inducing the least, which parallels the level of stimulation of PI 3-kinase activity by the three growth factors. Furthermore, a 110-kDa protein is heavily phosphorylated under the same conditions and likely corresponds to the catalytic subunit of PI 3-kinase. In the case of the EGF stimulation, the 170-kDa phosphorylated protein probably corresponds to the EGF receptor. The amount of the 85-kDa protein present in PC12 cells stimulated by bFGF, NGF, and EGF was verified by probing the immunoprecipitates with anti-PI 3-kinase antibodies using a partial purified preparation of the rat liver enzyme as standard (18).

EGF, bFGF, and NGF-Dependent Stimulation of $[^3\text{H}]\text{Thymidine Incorporation into PC12 Cells}$. To compare the activation of PI 3-kinase with the mitogenic responses of PC12 cells stimulated by these growth factors, the incorporation of $[^3\text{H}]\text{thymidine}$ was measured as a function of growth factor concentration. As shown in Fig. 5, not only EGF, which has been reported to be a weak mitogen for this cell line (29), but also bFGF and NGF induced thymidine incorporation as a measure of DNA synthesis. In fact, NGF and bFGF were

FIG. 4. Identification of tyrosine phosphorylation of PI 3-kinase in PC12 cells. Anti-PI 3-kinase immunoprecipitates were prepared from untreated (lanes 1), bFGF- (lanes 2), NGF- (lanes 3), and EGF- (lanes 4) treated PC12 cells for 5 min. As control a partially purified preparation of rat liver PI 3-kinase (lanes 5) was included using the same conditions as described in the text. The immunoprecipitates were separated by SDS/PAGE and probed with anti-phosphotyrosine antibody (A) or anti-PI 3-kinase antiserum (B). Arrows indicate the positions of proteins with apparent molecular masses of 170, 110, and 85 kDa. The 190-kDa protein present in the rat liver immunoprecipitate (B) may correspond to the complex of the 110- and 85-kDa subunits of PI 3-kinase. The 55-kDa band, present in all lanes, corresponds to the heavy chain of IgG. Molecular mass markers are indicated in kDa.

more potent than either EGF or the serum control at concentrations of 10–100 ng/ml. However, the stimulation of DNA synthesis by bFGF or NGF does not preclude the subsequent formation of neurites in these cells (data not shown).

DISCUSSION

As with many cells, FGF, NGF, and EGF induce various responses in PC12 cells that stem from the activation of receptor-bound tyrosine kinases. Some of the processes are direct—i.e., they result from immediate tyrosine phosphorylations induced by these kinases—and others are indirect, occurring from the action of other entities downstream from the initial phosphorylation events. The activation of protein kinase C by diacylglycerol, which arises from the hydrolysis of certain inositol phospholipids by phospholipase C- γ , is such an example. Although many of these various signals eventually reach the nucleus, where they are translated into effects on gene expression, a clear definition of the pathways involved is not yet available. At best, various components, acting as messengers (or message generators), have been identified but not their precise organization. This situation is further complicated by the fact that these stimulations can produce either mitogenic or trophic responses or both, making it difficult to determine which events (and messengers) are related to which activity. The PC12 cell paradigm provides an excellent means for evaluating the contributions of different signaling pathways to the mitotic and differentiative responses, because of the several growth factors that variably activate these cells.

In this study, we have identified another potential messenger for growth factor-induced responses in PC12 cells—i.e., PI-3-P. This molecule apparently arises from the activation, presumably through tyrosine phosphorylation, of a PI 3-kinase, which was identified in immunoprecipitates by phosphotyrosine-specific antibodies in growth factor-treated cells. The modest stimulations observed are in keeping with previous studies (30) that suggested that NGF induces small amounts of inositol phospholipid synthesis in these cells.

FIG. 5. Comparison of growth factor-induced mitogenic responses of PC12 cells. Cells were plated in complete medium into 24-well dishes at 2.5×10^5 cells per well. Two days after removal of serum, the cells were given the indicated concentrations of bFGF (Δ), EGF ($\bullet$), and NGF ($\blacksquare$) in DMEM or complete serum for 40 hr. Each point represents the average of quadruplicate samples, expressed as percentage of maximal stimulation of cells grown in complete serum. Similar results were obtained in at least three independent experiments.

However, the synthesis is entirely of the nonhydrolyzed isomer, a result that was unexpected in view of previous findings that this same factor induces diacylglycerol formation, which, at least initially, arises from inositol phospholipid pools (31). Clearly, the production of this material depends on preexisting pools and not on material formed from growth factor stimulation.

The observed tyrosine phosphorylation of PI 3-kinase by bFGF, NGF, and EGF supports the view that this protein is a substrate for receptor tyrosine kinases in PC12 cells. Interestingly, the 85-kDa regulatory subunit and the 110-kDa putative catalytic subunit show similar patterns of tyrosine phosphorylation. Similar observations have been reported in an *in vitro* system using an immunopurified MTA γ /pp60^{c-src} complex and purified PI 3-kinase (32). The interaction of the 85-kDa subunit of PI 3-kinase has been reported for the receptors of platelet-derived growth factor (7) and EGF (8) but not for those of either NGF or FGF. The presence of a tyrosine-phosphorylated 170-kDa protein in the EGF-stimulated PC12 cells, which precipitates with anti-PI 3-kinase antibodies, supports the view that this receptor–enzyme association also occurs in PC12 cells. Using synthetic peptides, it has recently been suggested (33) that the C terminus of the FGF receptor can interact directly with PI 3-kinase. Moreover, the presence of a tyrosine kinase domain in the *trk* A protein (34), now recognized to be at least a part of the NGF receptor in these cells, is also consistent with such an activation, albeit that it does not contain good consensus sequences for direct PI 3-kinase binding. Thus, all of these receptor systems have the potential to activate PI 3-kinase. However, it cannot be ruled out that activation of PI 3-kinase in PC12 cells is regulated by stimulation of a nonreceptor protein-tyrosine kinase such as a member of the *src* family.

The role of PI-3-P as a signal transducer in any cell system has not yet been defined. Since the molecules presumably remain membrane bound, it is most likely that they function to activate membrane-bound entities analogous to diacylglyc-

erol and protein kinase C. Equally uncertain is the role of this activation in mitotic versus trophic responses. Since the stimulation of PI 3-kinase has already been observed in mitogenic responses (35), it would seem to be at least related in part to signals that dictate cell division. The stimulation of PI 3-kinase in PC12 cells by EGF, bFGF, and NGF is not inconsistent with this view. First, the EGF response is significantly greater than that seen with either NGF or bFGF. EGF only stimulates cell division in PC12 cells, albeit modestly (29). Second, NGF and bFGF do induce [³H]thymidine incorporation, in keeping with the observations that these factors can stimulate a round of cell division prior to the onset of the neurotrophic response (36), thus providing a rationale for the PI-3-P response observed for these growth factors as well. Finally, the activation of PI 3-kinase occurs in the same range (and at the same cell density) as the stimulation of [³H]thymidine incorporation. However, the levels of PI-3-P induced are not quantitatively correlative with the mitotic effect either, as judged by the levels of [³H]thymidine incorporation induced (Fig. 5). Rather, they are more closely parallel to the receptor concentration or perhaps the relative affinity of PI 3-kinase for the autophosphorylation of the receptor kinases.

The rather substantial stimulation of DNA synthesis by NGF is in contrast with previous results that reported no effect of NGF on thymidine incorporation or increase in cell number in serum-starved PC12 cells (37). It is possible that the results obtained in this study are related to a less complete synchronization of the cells. However, it should be stressed that the incorporation of [³H]thymidine may not accurately reflect cell division, and therefore mitotic activity, as it has been reported that PC12 cells, treated with NGF for up to 14 days, continue to synthesize DNA and either divide or become polyploid (38). Thus, the lack of correlation of PI-3-P levels with the amount of [³H]thymidine incorporation may be due to other pathways of DNA synthesis that can proceed even in differentiated cells. This could obscure the relationship between growth factor-induced production of the messenger and true mitotic events.

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