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Activation of Phosphatidylinositol-3 Kinase by Nerve Growth Factor Involves Indirect Coupling of the *trk* Proto-Oncogene with src Homology 2 Domains

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Summary

Growth factor receptor tyrosine kinases can form stable associations with intracellular proteins that contain src homology (SH) 2 domains, including the p85 regulatory subunit of phosphatidylinositol (PI)-3 kinase. The activation of this enzyme by growth factors is evaluated in PC12 pheochromocytoma cells and NIH 3T3 fibroblasts expressing the pp140$^{c\text{-}trk}$ nerve growth factor (NGF) receptor (3T3-c-*trk*). NGF causes the rapid stimulation of PI-3 kinase activity detected in anti-phosphotyrosine, but not in anti-trk, immunoprecipitates. This effect coincides with the tyrosine phosphorylation of two proteins, with molecular masses of of 100 kd and 110 kd, that coimmunoprecipitate with p85. Similar phosphorylation patterns are induced when an immobilized fusion protein containing the amino-terminal SH2 domain of p85 is used to precipitate tyrosine-phosphorylated proteins. Thus, although NGF produces the rapid activation of PI-3 kinase through a mechanism that involves tyrosine phosphorylation, there is no evidence for tyrosine phosphorylation of p85, or for its ligand-dependent association with the NGF receptor. Perhaps another phosphoprotein may link the NGF receptor to this enzyme.

Introduction

Although the mechanisms by which neurotrophins such as nerve growth factor (NGF) support the survival and differentiation of sympathetic and sensory neurons remain elusive, it is recognized that early changes in protein phosphorylation ultimately play an important role in the cellular actions of the growth factor. NGF increases the phosphorylation of several cellular proteins (Halegoua and Patrick, 1980; Nakanishi and Guroff, 1985; Landreth and Rieser, 1985) and is known to stimulate the activities of several serine/threonine kinases (Lee et al., 1985; Hama et al., 1986; Matsuda and Guroff, 1987; Rawland et al., 1987; Aletta et al., 1988; Heasley and Johnson, 1989a, 1989b; Vulliet et al., 1989; Miyasaka et al., 1990; Ohmichi et al., 1992b). Recent evidence (Kaplan et al., 1991a; Klein et al., 1991; Weskamp and Reichardt, 1991; Ohmichi et al., 1992b) indicates that these serine/threonine phosphorylations result from the direct binding of NGF to its high affinity receptor, pp140^{trk}. This receptor is a protein tyrosine kinase, and both its activity and tyrosine autophosphorylation are increased in response to NGF (Kaplan et al., 1991b; Ohmichi et al., 1991b).

The precise tyrosine phosphorylation substrates that initiate the differentiative responses of neurons to neurotrophins have yet to be identified. However, the many similarities in eary phosphorylation events produced by differentiative agents like NGF and mitogenic agents like epidermal growth factor (EGF) suggest that subtle differences in signaling events are required to distinguish different cellular fates. One mechanism for the divergence in signals initiated by tyrosine kinase receptors may be their differential interactions with effector proteins containing src homology 2 (SH2) domains. Three such signaling proteins, phospholipase C (PLC)-γ1 (Wahl et al., 1988; Margolis et al., 1989; Meisenhelder et al., 1989; Wahl et al., 1989a, 1989b), GTPase activating protein of *ras* (Molly et al., 1988; Trahey et al., 1988; Ellis et al., 1990), and phosphatidylinositol (PI)-3 kinase (Bjorge et al., 1990), are phosphorylated on tyrosine residues in response to numerous growth factors and can form stable associations with receptors in a growth factor-dependent manner (Kumjian et al., 1989; Kaplan et al., 1990; Kazlauskas et al., 1990; Margolis et al., 1990a; Cantley et al., 1991; Hu et al., 1992) via an interaction with the SH2 domain (Coughlin et al., 1989; Anderson et al., 1990; Margolis et al., 1990b; Moran et al., 1990; Escobedo et al., 1991a, 1991b; Otsu et al., 1991; Skolnik et al., 1991). We recently reported that NGF stimulates the association of its high affinity pp140$^{c\text{-}trk}$ receptor with the SH2 domains of PLC-γ1 in PC12 pheochromocytoma cells (Ohmichi et al., 1991b) and further that the constitutively active p70$^{c\text{-}trk}$ oncogene product is also associated with PLC-γ1 through its SH2 domains (Ohmichi et al., 1991a). Moreover, the NGF-dependent association of pp140$^{c\text{-}trk}$ with PLC-γ1 correlates with the tyrosine phosphorylation of a 38 kd phosphoprotein, which itself coprecipitates with both pp140$^{c\text{-}trk}$ and the SH2 domains of PLC-γ1 in response to NGF (Ohmichi et al., 1992c).

Although there was no detectable association of the NGF receptor with GTPase activating protein of *ras* (Ohmichi et al., 1991b), preliminary results indicated that NGF stimulated the phosphorylation of two proteins that specifically bound to the p85 subunit of PI-3 kinase (Ohmichi et al., 1992c). This enzyme phosphorylates PI on the D3 position of the inositol ring, leading to the formation of the novel phosphoinositides PI-3P, PI-3,4P_2, and PI-3,4,5P_3. Although the precise functions of these lipids remain unknown, the exposure of cells to growth factors (Kaplan et al., 1987; Whitman et al., 1987; Auger et al., 1989) or oncogenic transformation (Fukui et al., 1991) causes their accumulation. Moreover, mutational analyses of the platelet-derived growth factor (PDGF) (Coughlin et al.,

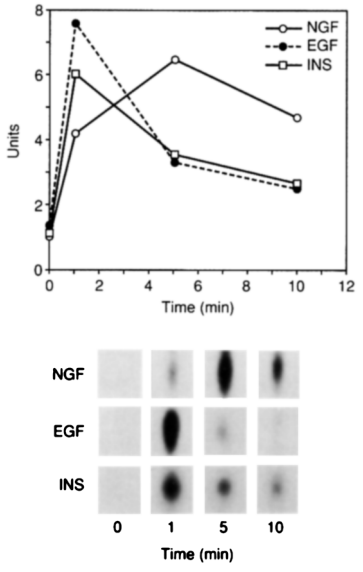

Figure 1. NGF, EGF, and Insulin Stimulate PI-3 Kinase Activity in Anti-Phosphotyrosine Immunoprecipitates of PC12 Cells

PC12 cells were grown in 150 mm dishes. NGF (100 nM), EGF (10 nM), or insulin (100 nM) was directly added to the medium and incubated for the indicated times at 37°C. Lysates were immunoprecipitated with anti-phosphotyrosine antiserum, and PI-3 kinase activity was measured by incubating with [γ-^{32}P]ATP and PI as described in Experimental Procedures. One unit of activity is defined as nmoles of PI-3P per min ($\times 10^{-2}$). In the lower panel are shown autoradiographs of the region of the thin-layer chromatograph containing PI-3P. Results are from a single experiment that was repeated three times.

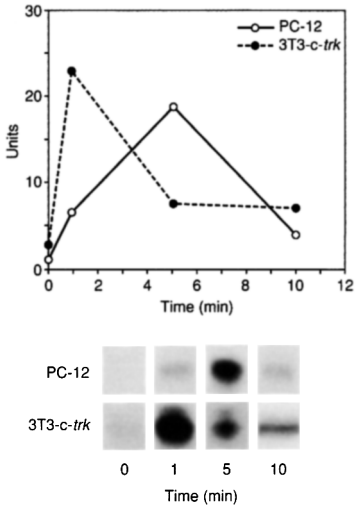

Figure 2. NGF Stimulates PI-3 Kinase Activity in Anti-phosphotyrosine Immunoprecipitates of PC12 and 3T3-c-*trk* Cells

PC12 and 3T3-c-*trk* cells were grown in 150 mm dishes. NGF (100 nM) was directly added to the medium and incubated for the indicated times at 37°C. Lysates were immunoprecipitated with anti-phosphotyrosine antiserum, and PI-3 kinase activity was measured as described in Figure 1. Autoradiographs are shown in a lower panel.

1989), EGF (Bjorge et al., 1990), and colony stimulating factor (Varticovski et al., 1989; Reedijk et al., 1990; Shurtleff et al., 1990) receptors indicated a strong correlation between stimulation of cell growth and PI-3 kinase activation. Since NGF, EGF, and insulin paradoxically share a number of common early signaling pathways (Saltiel and Decker, 1991), we have compared the actions of these three growth factors on stimulation of PI-3 kinase activity and association of tyrosine-phosphorylated proteins with its 85 kd regulatory protein. These results indicate that the high affinity NGF receptor differentially associates with SH2-containing proteins, via direct or indirect mechanisms.

Results

NGF Stimulates PI-3 Kinase Activity Via Its pp140$^{c\text{-}trk}$ Receptor

NGF binding to the pp140$^{c\text{-}trk}$ receptor stimulates its association with the SH2 domains of PLC-γ1 in both PC12 (Ohmichi et al., 1991b) and 3T3 fibroblasts expressing the human pp140$^{c\text{-}trk}$ (3T3-c-*trk*) (Ohmichi et al., 1992c) as well as its phosphorylation on tyrosine (Kim et al., 1991; Ohmichi et al., 1991b). To explore the involvement of other SH2 proteins in NGF action, we assayed the activity of PI-3 kinase in these cells after exposure to NGF, EGF, and insulin. Following hormonal treatment, lysates were precipitated with anti-phosphotyrosine antiserum, and the resulting immunoprecipitates were assayed for phosphorylation of PI (Figure 1). In PC12 cells, NGF rapidly activated PI-3 kinase activity that was detected in anti-phosphotyrosine immunoprecipitates. This effect was observed after 1 min of exposure and was maximal at 5 min, but declined thereafter. Similarly, both EGF and insulin also stimulated the activity of this enzyme in anti-phosphotyrosine immunoprecipitates. Effects of these hormones were maximal after only 1 min of exposure and declined thereafter.

The NGF-dependent activation of PI-3 kinase was also explored in 3T3 cells that were transfected with the pp140$^{c\text{-}trk}$ cDNA. These cells express the 140 kd high affinity receptor, but contain no low affinity p75 receptor (Ohmichi et al., 1992b). Exposure of 3T3-c-*trk* cells to NGF also caused an increase in PI-3 kinase activity, although the time course was slightly faster than that observed in PC12 cells, occurring maximally at 1 min (Figure 2). These data provide further evidence that the pp140$^{c\text{-}trk}$ receptor is both necessary and sufficient for the early signaling events in NGF action.

To explore further the mechanism of activation of PI-3 kinase in response to NGF, PC12 or 3T3-c-*trk* cells were treated with NGF and subsequently immunoprecipitated with anti-phosphotyrosine, anti-trk, or antisera raised against the 85 kd regulatory subunit of PI-3 kinase prior to assay of PI-3 kinase activity (Figure 3). Although NGF produced a marked increase in PI-3 kinase activity detected in anti-phosphotyrosine im-

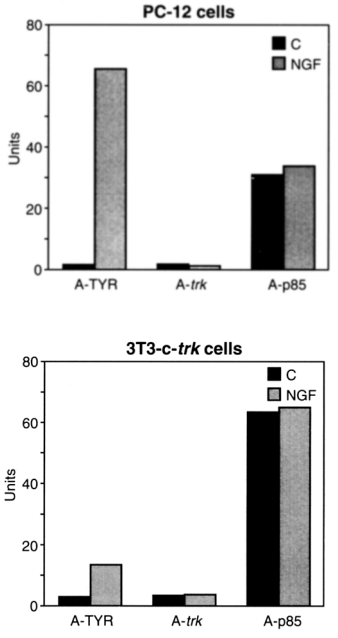

Figure 3. NGF-Stimulated PI-3 Kinase Activity Is Not Detected in Anti-trk or Anti-p85 Immunoprecipitates from PC12 or 3T3-c-*trk* Cells

PC12 and 3T3-c-*trk* cells were grown in 150 mm dishes. NGF (100 nM) was directly added to the medium and incubated for 5 min in PC12 cells or 1 min in 3T3-c-*trk* cells at 37°C. Lysates were immunoprecipitated with anti-phosphotyrosine, anti-trk, or anti-p85 antisera, and PI-3 kinase activity was measured as described in Figure 1.

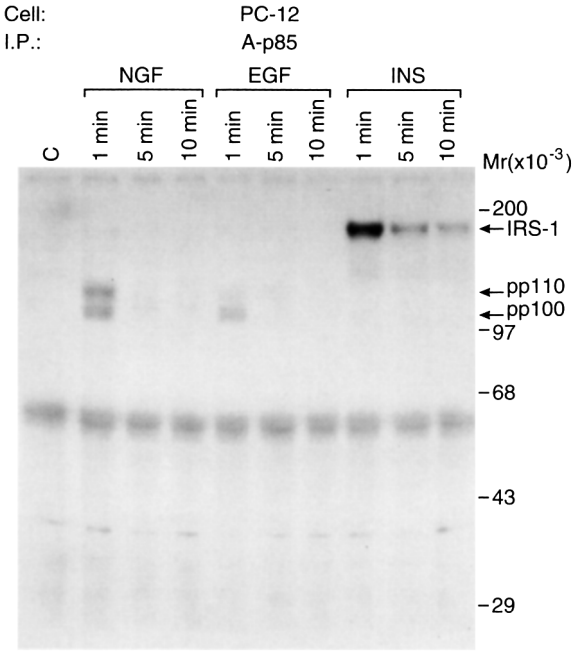

Figure 4. NGF, EGF, and Insulin Stimulate the Tyrosine Phosphorylation of Proteins in PC12 Cells That Coprecipitate with p85

PC12 cells were grown in 150 mm dishes. NGF (100 nM), EGF (10 nM), or insulin (100 nM) was directly added to the medium and incubated for the indicated times at 37°C. Lysates were immunoprecipitated with anti-p85 antiserum and subject to SDS–PAGE, followed by immunoblotting with anti-phosphotyrosine antiserum, as described in Experimental Procedures.

munoprecipitates from both cell types, no significant increase was observed in anti-trk immunoprecipitates. In addition, precipitation with a glutathione S-transferase (GST) fusion protein containing the SH2 domain of p85 also brought down NGF-stimulated PI-3 kinase activity (data not shown). Immunoprecipitation with anti-p85 antisera brought down significant enzyme activity in the absence of NGF; however, there was no significant increase upon NGF treatment. The elevated activity observed in these immunoprecipitates may reflect activation of the enzyme by antibody binding to the p85 subunit, mimicking the activation produced by binding of tyrosine-phosphorylated proteins to the p85 SH2 domain.

NGF Stimulates the Association of Tyrosine-Phosphorylated Proteins with the 85 kd Subunit of PI-3 Kinase

Recent studies indicate that binding of certain growth factors to their receptors can induce a stable association with p85 of PI-3 kinase (Kaplan et al., 1987; Escobedo et al., 1991a) as well as the phosphorylation of this protein (Kazlauskas and Cooper, 1989, 1990). To determine whether the activation of PI-3 kinase by NGF occurs through a similar mechanism, PC12 cells were treated with NGF, EGF, or insulin, and lysates were precipitated with anti-p85 antiserum (Figure 4). The resulting immunoprecipitates were electrophoresed on polyacrylamide gels, and tyrosine-phosphorylated proteins were detected by immunoblotting with anti-phosphotyrosine antiserum. In cells treated with NGF, two tyrosine-phosphorylated proteins were detected that migrated with apparent M_r values of 100 and 110. These phosphoproteins were observed within 1 min of exposure to NGF and rapidly declined thereafter. EGF also stimulated the tyrosine phosphorylation of pp100 and pp110, although not as effectively as NGF. In insulin-treated PC12 cells, pp100 and pp110 were not detected, although a 185 kd tyrosine-phosphorylated protein, tentatively identified as insulin receptor substrate 1 (Sun et al., 1991), was found in anti-p85 immunoprecipitates within 1 min of exposure. The phosphorylation of this protein also declined thereafter, although it appeared to be more stable than the NGF-dependent phosphorylation of the 100 and 110 kd proteins.

To evaluate the cell specificity of the NGF- and EGF-dependent tyrosine phosphorylations of pp100 and pp110, the effects of these growth factors were evaluated in other cell types expressing their respective receptors. 3T3-c-*trk* cells and NIH 3T3 cells transfected with the human EGF receptor (3T3/hEGFR) (Decker et al., 1990) were treated with NGF or EGF, and the lysates were precipitated with anti-p85 antiserum (Figure 5). The addition to 3T3-c-*trk* cells of 100 nM NGF for 1 min

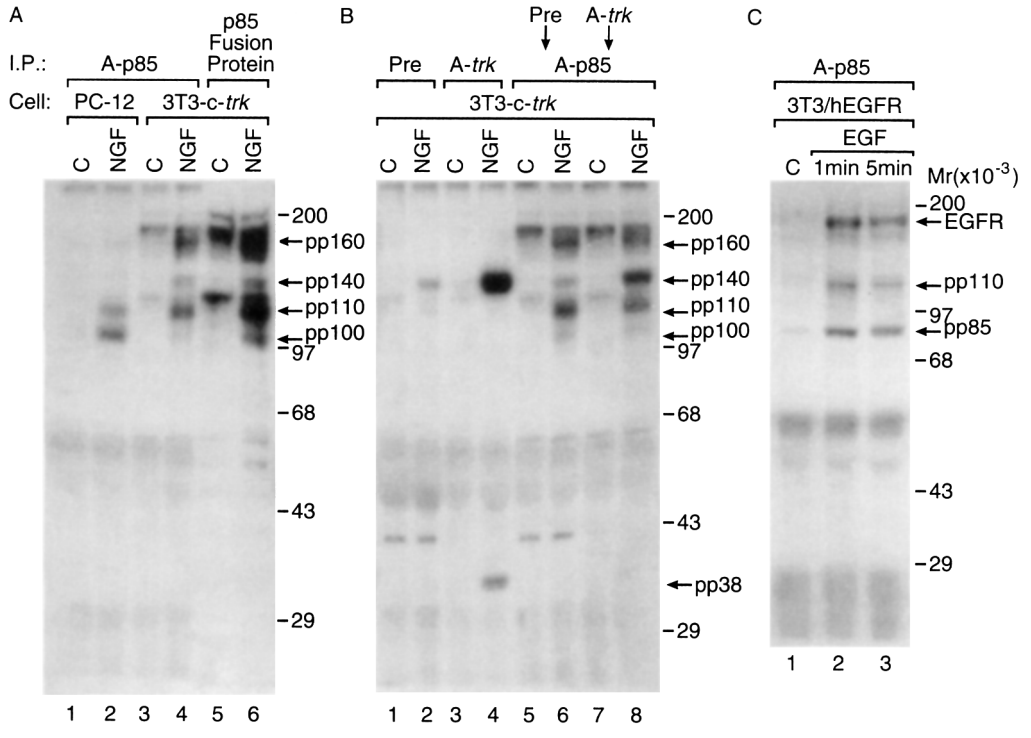

Figure 5. NGF Stimulates the Tyrosine Phosphorylation of Proteins in 3T3-c-*trk* Cells That Associate with the p85 SH2 Domain

PC12, 3T3-c-*trk*, and 3T3/hEGFR cells were grown in 150 mm dishes. NGF (100 nM) or EGF (10 nM) was directly added to the medium and incubated for the indicated times at 37°C. (A) In lanes 1–4, lysates were immunoprecipitated with anti-p85 antiserum and subject to SDS–PAGE, followed by immunoblotting with anti-phosphotyrosine antiserum. In lanes 5 and 6, a GST–p85 SH2 fusion protein immobilized with glutathione–Sepharose beads was mixed with lysates from cells. Bound proteins were subject to SDS–PAGE followed by antiphosphotyrosine immunoblotting. (B) Lysates were immunoprecipitated with preimmune rabbit serum (lanes 1 and 2) or anti-trk antiserum (lanes 3 and 4). The resulting supernatants were subsequently precipitated with anti-p85 antiserum (lanes 5–8) and subject to SDS–PAGE, followed by immunoblotting with anti-phosphotyrosine antiserum. (C) Lysates from 3T3/hEGFR cells were immunoprecipitated with anti-p85 antiserum and subject to SDS–PAGE, followed by immunoblotting with anti-phosphotyrosine antiserum.

induced the tyrosine phosphorylation of the two proteins (pp100 and pp110) as well as additional proteins with molecular masses of 160 kd and 140 kd (Figure 5A). To explore whether the 140 kd phosphoprotein identified in anti-p85 immunoprecipitates of 3T3-c-*trk* cells was the *trk* proto-oncogene, lysates from control and NGF-treated cells were preimmunoprecipitated with preimmune or anti-trk antisera, and the resulting supernatants were subsequently precipitated with anti-p85 antiserum (Figure 5B). Preimmunoprecipitation with anti-trk antiserum brought down the NGF-dependent tyrosine phosphorylated pp140$^{c\text{-}trk}$ and its associated 38 kd protein (Ohmichi et al., 1992c) (Figure 5B, lane 4), but did not clear the 140 kd protein from the anti-p85 immunoprecipitates (Figure 5B, lane 8), suggesting that this 140 kd band is not the *trk* proto-oncogene.

In contrast to PC12 cells, treatment of 3T3/hEGFR cells with EGF caused the coprecipitation of its tyrosine-phosphorylated receptor in the anti-p85 immunoprecipitate (Figure 5C). These results suggested that the association of the EGFR with p85 occurs only in cells overexpressing this receptor, similar to what was observed for the association of this receptor with PLC-γ1 (Ohmichi et al., 1991b). Furthermore, the phosphorylation of p85 in response to NGF was not observed in either PC12 or 3T3-c-*trk* cells, despite the marked increase in PI kinase activity detected in the anti-phosphotyrosine immunoprecipitates. The presence of the p85 protein in anti-p85 immunoprecipitates from both cell types was confirmed by Western blotting with the same antiserum (Figure 6). Moreover, this antiserum can recognize phosphorylated p85, since the EGF-stimulated tyrosine phosphorylation of p85 was detected in immunoprecipitates from 3T3/hEGFR cells (Figure 5C). Interestingly, Western blotting of anti-trk immunoprecipitates from NGF-treated PC12 or 3T3-c-*trk* cells with anti-p85 revealed no evidence for p85. Additionally, blotting of anti-p85 immunoprecipitates from NGF-treated cells with anti-trk antiserum revealed no evidence for pp140$^{c\text{-}trk}$ (data not shown). However, the results in 3T3 cells overexpressing EGFR suggest that overexpression of the NGF receptor in cells may lead to NGF-dependent phosphorylation of p85 and direct association with its receptor.

The concentration dependence of the effect of NGF on the appearance of pp100/110 in the anti-p85 immu-

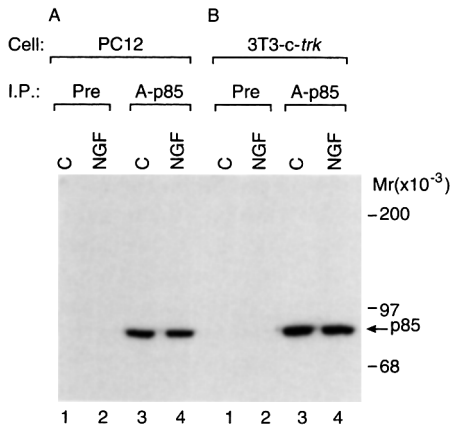

Figure 6. Detection of p85 in Immunoprecipitates by Western Blotting

PC12 (A) and 3T3-c-*trk* (B) cells were grown in 150 mm dishes. NGF (100 nM) was directly added to the medium and incubated for 1 min at 37°C, as described in Figure 4. Lysates were immunoprecipitated with preimmune rabbit serum (lanes 1 and 2) or anti-p85 antiserum (lanes 3 and 4) and subject to SDS-PAGE, followed by immunoblotting with anti-p85 antiserum, as described in Experimental Procedures.

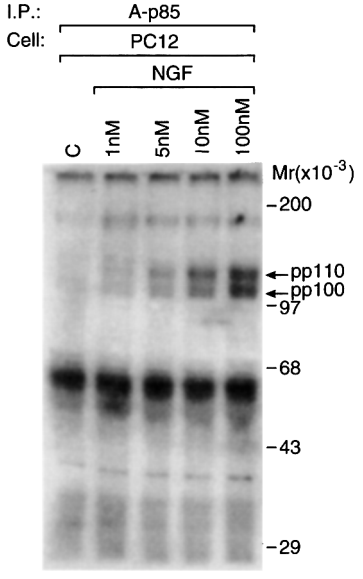

Figure 7. The Dose Dependence of NGF-Induced Tyrosine Phosphorylation of pp100 and pp110 in PC12 Cells

PC12 cells were treated with the indicated concentrations of NGF for 1 min at 37°C. Lysates were immunoprecipitated with anti-p85 antiserum and subject to SDS-PAGE, followed by immunoblotting with anti-phosphotyrosine antiserum.

noprecipitates was evaluated in PC12 cells (Figure 7). NGF caused a dose-dependent increase in the phosphorylation of these proteins, which was observed with as little as 1 nM NGF and increased up to concentrations of 100 nM.

To explore further the nature of the association of these NGF- and EGF-dependent phosphoproteins with the p85 subunit of PI-3 kinase, we examined the effect of NGF on the binding of tyrosine-phosphorylated proteins to an immobilized fusion protein containing the amino-terminal SH2 domain of p85 (Figure 8). Cells were treated with the growth factors, and lysates were incubated with the immobilized fusion protein prior to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting with anti-phosphotyrosine antiserum. The addition to PC12 cells of NGF or EGF induced the association of both pp110 and pp100 with the p85 fusion protein, although neither receptor was detected in these precipitates. Moreover, the addition to 3T3-c-*trk* cells of NGF induced the association of pp110 and pp100 as well as pp140 and pp160 (Figure 5A, lanes 5 and 6). In contrast, treatment of 3T3/hEGFR cells with EGF caused the association of its tyrosine-phosphorylated receptor with the fusion protein, as well as additional proteins, including pp110. Additionally, treatment of Swiss 3T3 cells with PDGF also stimulated the association of its tyrosine-phosphorylated receptor (molecular mass, 190 kd) with the SH2 domain fusion protein. In insulin-treated PC12 and HIRc cells (overexpressing the human insulin receptor), both the β subunit of the insulin receptor (molecular mass, 95 kd) and a protein tentatively identified as insulin receptor substrate 1 (molecular mass, 185 kd) were detected, as well as the 110 kd protein, although the 100 kd NGF- and EGF-sensitive protein was not found.

Discussion

Like many other growth factors with tyrosine kinase receptors, NGF rapidly stimulates the activity of PI-3

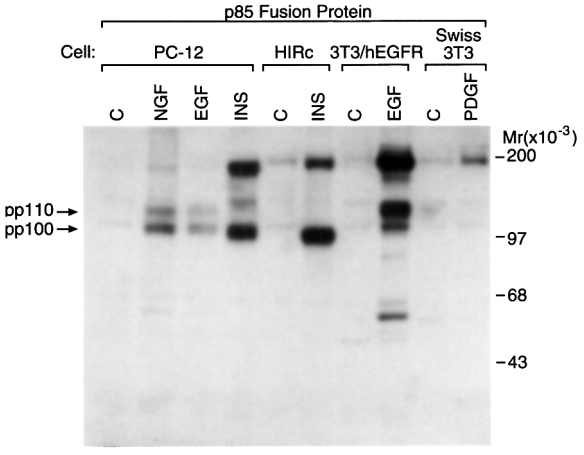

Figure 8. NGF, EGF, and Insulin Induce the Association of Tyrosine-Phosphorylated Proteins with the p85 SH2 Domain

PC12, HIRc, 3T3/hEGFR, and Swiss 3T3 cells were grown in 150 mm dishes. NGF (100 nM), EGF (10 nM), insulin (100 nM), or PDGF (10 nM) was directly added to the medium and incubated for 1 min at 37°C. Lysates were incubated with a GST-p85 fusion protein immobilized with glutathione-Sepharose beads. The association of tyrosine-phosphorylated proteins with the fusion protein was evaluated as described in Figure 5.

kinase. Although the role of the novel lipid products of this enzyme, as well as the mechanism of activation, remains poorly understood, there is little doubt that cellular tyrosine phosphorylation plays an important role (Kaplan et al., 1987; Auger et al., 1989; Coughlin et al., 1989; Bjorge et al., 1990; Endermann et al., 1990; Kazlauskas and Cooper, 1990; Hu et al., 1992). NGF-, EGF-, and insulin-stimulated PI-3 kinase activities were detected in anti-phosphotyrosine immunoprecipitates, suggesting that the enzyme is itself phosphorylated or can associate with one or more growth factor–dependent tyrosine-phosphorylated proteins. However, in the case of NGF, there is no evidence that the PI-3 kinase itself can directly associate with the tyrosine-phosphorylated NGF receptor. Although it is possible that the anti-trk antibody employed in these studies blocks PI-3 kinase activity or association with the receptor, this antiserum did not precipitate PI-3 kinase activity. Moreover, immunoblotting of anti-trk immunoprecipitates from NGF-treated cells with anti-p85 antiserum, or blotting of anti-p85 immunoprecipitates with anti-trk antiserum, revealed no evidence for the association of p85. In addition, the pp140$^{c\text{-}trk}$ protein was not detected in anti-p85 or p85 SH2 fusion protein precipitates from NGF-treated cells, in contrast to the identification of the tyrosine-phosphorylated receptor in anti-PLC-γ1 or PLC-γ1 SH2 fusion protein precipitates (Ohmichi et al., 1991b). The lack of NGF receptor association with p85 is not surprising, since the tyrosine kinase domain of trk lacks the nucleotide consensus sequence (EYMXM) thought to be required for p85 binding, although it does have a region of some similarity (FEYMRHGD) around Tyr-595. Additionally, there is also no evidence that p85 itself undergoes phosphorylation on tyrosine in response to NGF, since an 85 kd tyrosine-phosphorylated band was not detected in anti-p85 immunoprecipitates in response to the growth factors, despite the presence of this protein, as verified by immunoblotting.

Interestingly, NGF did cause the tyrosine phosphorylation of two proteins at 100 kd and 110 kd, detected by their coimmunoprecipitation with anti-p85 antiserum or association with the p85 SH2 fusion protein. The appearance of these phosphoproteins temporally correlated with stimulation of PI-3 kinase activity, although there were discrepancies regarding the extent of phosphorylation observed with NGF and EGF and their relative activations of the enzyme. It is important to note, however, that our ability to detect phosphorylation depends upon association of these proteins with the p85 protein, so the extent of phosphorylation observed here may be misleading. However, the SH2 association of pp100 and pp110 was specific for p85, since these bands were not detected in anti-PLC-γ1 or anti–GTPase activating protein (of *ras*) immunoprecipitates and were not associated with immobilized fusion proteins containing SH2 domains of these proteins (Ohmichi et al., 1991b).

It is possible that the 110 kd protein detected in these precipitates is the catalytic subunit of PI-3 kinase (Carpenter et al., 1990), although there is no direct evidence to support this speculation. Moreover, another interesting possibility is that pp100, which is phosphorylated in response to both NGF and EGF, may represent a specific p85-binding protein that mediates activation of PI-3 kinase by these factors. In this regard, pp100 may be analogous to the pp185 insulin receptor substrate 1, which contains up to ten p85 binding motifs and is likely to mediate the insulin-dependent activation of PI-3 kinase (Endermann et al., 1990; Ruderman et al., 1990).

The stimulation of PI-3 kinase activity represents another early cellular response that NGF shares with a number of mitogenic factors, notably, EGF and insulin (Saltiel and Decker, 1991). These factors produce similar, but not identical tyrosine phosphorylations (Miyasaka et al., 1991; Ohmichi et al., 1992a, 1992c) and similarly stimulate the serine/threonine phosphorylation and activation of microtubule-associated protein kinase (Miyasaka et al., 1990), S6 kinase (Matsuda and Guroff, 1987), protein kinase C (Hama et al., 1986; Heasley and Johnson, 1989a), and raf-1 kinase (Ohmichi et al., 1992b). Despite these numerous similarities in early signaling events, the downstream cellular actions of these factors in PC12 cells are quite different. Most notable is the distinction between the differentiative activity of NGF and the mitogenic activity of EGF. One possible clue for solving this apparent paradox may lie in the differential associations of these tyrosine kinase receptors with proteins containing SH2 domains. Studies in PC12 and 3T3-c-*trk* cells indicate that the NGF receptor associates with PLC-γ1 (Ohmichi et al., 1991b), does not associate with GTPase activating protein of *ras* (Ohmichi et al., 1991b), and may activate PI-3 kinase via an indirect mechanism, whereas both the EGF and insulin receptors exhibit quite different patterns of SH2 binding in these cells (Ohmichi et al., 1992c). Although the downstream events that allow cells to distinguish growth and differentiative signals remain a mystery, it is likely that the close examination of divergent signaling emerging from these receptors may yield much information.

Experimental Procedures

Materials

[γ-^{32}P]ATP (300 Ci/mol) was from New England Nuclear (Bannockburn, IL). ^{125}I-labeled protein A (30 mCi/mg) was from Amersham (Arlington Heights, IL). NGF 2.5S was from Bioproducts for Science (Indianapolis, IL). Receptor grade EGF was from Collaborative Research (Lexington, MA). Recombinant human PDGF-ββ homodimer was from Bachem (Torrance, CA). Insulin was from Eli Lilly (Indianapolis, IN). Silica gel thin-layer chromatography plates were from EM Separations (Gibbstown, NJ). Anti-phosphotyrosine monoclonal antiserum and anti-p85 polyclonal antiserum were from Upstate Biotechnology (Lake Placid, NY). All other reagents were purchased from Sigma (St. Louis, MO) and were the highest quality available. Anti-trk antiserum (Ohmichi et al., 1991a, 1991b) was developed as described previously. The pGEX plasmid expressing the amino-terminal SH2 domain of p85 in a GST fusion protein was the generous gift of Drs. B. Margolis and J. Schlessinger and contained the amino-terminal SH2 domain of the p85 subunit (Hu et al., 1992).

Cell Culture

NIH 3T3 cells were transfected with the plasmid pCO12-*trk* containing the full-length human *trk* cDNA (generous gift of Dr. Luis Parada) (3T3-c-*trk*) (Ohmichi et al., 1992b) in the pCO12 vector as described previously (Velu et al., 1987). NIH 3T3 cells were transfected with the normal human EGFR (3T3/hEGFR) (Decker et al., 1990). PC12 cells, 3T3-c-*trk* cells, NIH/hEGFR cells, and Swiss-3T3 cells were grown in Dulbecco's modified Eagle's medium containing 4500 mg per liter of D-glucose and 10% fetal bovine serum. Rat fibroblasts expressing 300,000 normal human insulin receptors (HIRc), which were the generous gift of Dr. Donald A. McClain (Maegawa et al., 1988; McClain et al., 1988), were grown in Dulbecco's modified Eagle's/F12 medium with 10% fetal bovine serum and 100 nM methotrexate.

Assay of PI-3 Kinase Activity

Cells were grown in 150 mm dishes. Growth factors were directly added to the medium and incubated for the indicated times at 37°C. After hormonal treatment, the medium was removed, and cells were washed twice with 12 ml of ice-cold phosphate-buffered saline before the addition of 1 ml of Nonidet P-40 buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 100 μM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 10,000 × g for 10 min. The resulting supernatants were incubated for 1 hr with the indicated antisera. Following the incubation, protein A-Sepharose beads were added for 30 mim with mixing, and immune complexes bound to the beads were washed three times with Nonidet P-40 buffer, once with phosphate-buffered saline, once with 0.5 M LiCl/0.1 M Tris (pH 7.5), once with distilled water, and once with 0.1 M NaCl/0.1 mM EDTA/10 mM Tris (pH 7.5). The PI-3 kinase assay was performed by adding to the immunoprecipitate 50 μl of PI kinase buffer (10 mM Tris [pH 7.5], 0.2 mM EGTA, 100 mM NaCl), 0.5 mg/ml PI, 20 mM $MgCl_2$, and 10 μM [γ-^{32}P]ATP (10 μCi per assay), followed by the addition of 100 μl of chloroform, methanol, HCl (100:200:2), and then 100 μl of chloroform and 100 μl of water were added. The resulting organic was dried, resuspended in 50 μl of chloroform, methanol (1:1), and spotted on a silica gel thin-layer chromatography plate that was developed in chloroform, methanol, water, ammonium hydroxide (43:38:7:5). ^{32}P-labeled PI-3 phosphate was visualized by autoradiography and compared with an iodine-stained standard.

Anti-Phosphotyrosine Immunoblots

Cells were grown in 150 mm dishes. Before hormonal treatment, the medium was replaced with serum-free medium and incubated for 1 hr. Unless otherwise indicated, 100 nM NGF, 10 nM EGF, 10 nM PDGF, or 100 nM insulin was directly added to the medium and incubated for 1 min at 37°C. After hormonal treatment, the medium was removed, and cells were washed twice with 12 ml of ice-cold phosphate-buffered saline before the addition of 1 ml of HNTG buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 μM sodium pyrophosphate, 200 μM sodium orthovanadate, 100 mM NaF, 30 mM p-nitrophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethyl sulfonyl fluoride) (Margolis et al., 1990a). Lysates were centrifuged at 10,000 × g for 10 min. Supernatants were incubated for 1 hr with the indicated antisera. Following the incubation, protein A-Sepharose beads were added for 30 min with mixing, and immune complexes bound to the beads were washed three times with 1 ml of HNTG buffer. Immune complexes were solubilized in 25 μl of Laemmli sample buffer (Laemmli et al., 1970) and electrophoresed on 8% SDS polyacryamide gels. Transfer to nitrocellulose paper, immunoblotting with anti-phosphotyrosine antiserum, and washing were performed as described (Decker et al., 1990). For blotting of tyrosine-phosphorylated proteins binding to the bacterially expressed SH domain of p85, supernatants were incubated for 90 min with glutathione-Sepharose beads, to which the GST-p85 fusion protein had been bound. The beads were washed three times with 1 ml of HNTG buffer, and samples were resolved on 8% SDS gels.

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