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Nerve growth factor induces the association of a 130-Kd phosphoprotein with its receptor in PC-12 pheochromocytoma cells

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To explore the molecular mechanisms of nerve growth factor (NGF) action, we have attempted to identify proteins that immunoprecipitate with the NGF receptor. An anti-NGF receptor antibody was developed that immunoprecipitated the 75-Kd receptor in PC-12 cells. In [^{35}S]methionine-labeled cells lysed with nonionic detergent, immunoprecipitation with this antireceptor antisera specifically brought down several associated proteins, although prior treatment of cells with NGF produced no apparent change in the distribution of these proteins. However, in vitro phosphorylation assays of the immunoprecipitated complex revealed the presence of a serine kinase that phosphorylated two predominant substrates with Mrs of 60 and 130 Kd. Prior treatment of cells produced no change in the appearance of the 60-Kd phosphoprotein, but NGF did stimulate the appearance of the 130-Kd protein. This effect was observed with as little as 0.1 nM NGF and was maximal at 5 min, but declined thereafter. Prior treatment of cells with NGF did not increase the phosphorylation of enolase added exogenously to the immunoprecipitates, suggesting that this action of NGF may have reflected the hormone-dependent association of the 130-Kd protein with the receptor, rather than activation of a receptor-associated kinase. Thus the association of the NGF 75-Kd receptor with a 130-Kd protein may be involved in signal transduction for the growth factor, although the role of this receptor in the NGF-dependent tyrosine phosphorylation remains unclear.

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Introduction

Nerve growth factor (NGF) promotes the survival, growth, and development of a number of sympathetic and sensory neurons. Although the precise biochemical events involved in NGF action remain unknown, numerous studies have indicated that protein phosphorylation plays a central role in the actions of this factor. NGF-dependent increases in the phosphorylation of several cellular proteins in the pheochromocytoma cell line PC-12 have been reported, including ribosomal S6 kinase (Matsuda and Guroff, 1987), tyrosine hydroxylase (Halegoua and Patrick, 1980; Lee *et al.,* 1985), microtubule-associated protein (MAP) (Landreth and Rieser, 1985; Aletta *et al.,* 1988; Vulliet *et al.,* 1989), and others (Nakanishi and Guroff, 1985; Hama *et al.,* 1986; Rowland *et al.,* 1987; Heasly and Johnson, 1989a,b). A number of NGF-responsive protein kinases have been identified, including the recently identified MAP kinase (Miyasaka *et al.,* 1990a,b), a serine/threonine kinase that is acutely activated by the growth factor.

In an attempt to elucidate the mechanism whereby MAP kinase is activated by NGF, we recently observed that NGF rapidly stimulates the tyrosine phosphorylation of two predominant proteins in PC-12 cells (Miyasaka *et al.,* 1991). This tyrosine phosphorylation pattern was similar but not identical to that produced by exposure of cells to epidermal growth factor (EGF). However, in contrast to the EGF receptor, the sequence of a previously described 75-kd NGF receptor, predicted by cDNA cloning (Johnson *et al.,* 1986; Radeke *et al.,* 1987), indicated a relatively short cytoplasmic region without a tyrosine kinase domain. Taken together, these results suggested that if the cellular effects of NGF are mediated through the previously identified receptor, it is likely that this receptor undergoes a noncovalent interaction with a separate tyrosine kinase. These findings led us to explore the NGF-dependent association of its receptor with other cellular proteins.

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Results

Because the 75-kd NGF receptor itself does not contain any protein kinase activity, we sought to identify receptor-associated proteins that mediated the effect of the hormone on cellular protein phosphorylation. PC-12 cells were metabolically labeled with [^{35}S]methionine/cysteine, followed by lysis in sodium dodecyl sulfate (SDS) (Figure 1A). The proteins were then subjected to immunoprecipitation with preimmune or polyclonal anti-NGF receptor antiserum and to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography. The NGF receptor was detected, exhibiting an apparent Mr of 75 Kd. To identify proteins associated with the receptor, [^{35}S]-labeled cells were lysed with the buffer containing the nonionic detergent 3-[(3-cholamidopropyl)dimethyl]-Z-hydroxy-1-propane sulfonate (CHAPS) in lieu of SDS (Figure 1B). Immunoprecipitation of these lysates with anti-receptor antisera yielded numerous additional proteins, including four predominant proteins with apparent Mr 150, 130, 41, and 40 Kd. Pretreatment of cells with NGF, EGF, or phorbol esters for up to 10 min produced no apparent change in the pattern of [^{35}S]-labeled proteins coimmunoprecipitating with the receptor (data not shown).

To explore the possibility that a protein kinase was present in the antireceptor immunoprecipitates, an in vitro phosphorylation experiment was performed. Cells were treated with or without 10 nM NGF for 5 min. Cells were lysed and proteins were immunoprecipitated with preimmune or antireceptor antiserum. The resulting immunoprecipitates were then incubated with [γ-^{32}P]ATP, $MnCl_2$, and $MgCl_2$, and the phosphorylation of endogenous proteins was evaluated by SDS PAGE followed by autoradiography. Two phosphorylated proteins were detected with apparent Mrs of 130 and 60 Kd that were specifically immunoprecipitated with anti-NGF receptor serum (Figure 2). Interestingly, the appearance of only the 130-Kd phosphorylated protein was increased by pretreatment of cells with NGF. The 60-Kd band was detected in both NGF-treated and -untreated cells but was unchanged by NGF treatment. The 75-Kd NGF receptor found in [^{35}S]-labeled cells was not detected by this in vitro phosphorylation assay. The effect of various detergents on the appearance of the 130-Kd phosphorylation substrates in the antireceptor immunoprecipitate was also evaluated. The NGF-sensitivity of the appearance of the 130-Kd protein was detected in immunoprecipitates from cells solu-

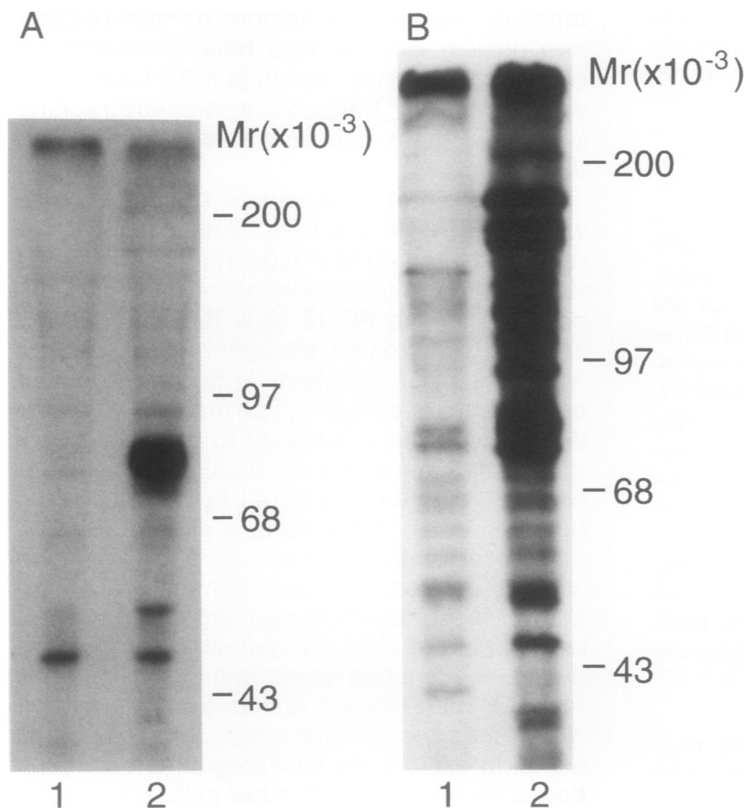

Figure 1. **Immunoprecipitation of NGF receptor from PC-12 cells.** Cells were labeled with [^{35}S]methionine/cysteine (200 μCi/ml) for 12 h in methionine/cysteine-free RPMI medium with 10% dialyzed fetal bovine serum and 5% dialyzed horse serum. Cells were lysed with SDS (A) or lysis buffer containing CHAPS (B), and lysates were immunoprecipitated with preimmune serum (lanes 1 and 3) or polyclonal anti-NGF receptor serum (lanes 2 and 4) before analysis on 7.2% SDS gels.

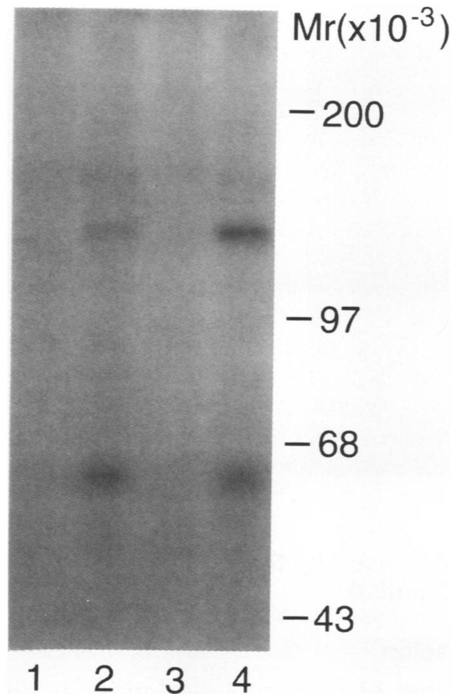

Figure 2. **In vitro phosphorylation of proteins in immunoprecipitates of NGF receptor.** PC-12 cells were pretreated with (lanes 3 and 4) or without (lanes 1 and 2) 10 nM NGF for 5 min. Detergent extracts of cells were prepared in a buffer containing 50 mM Tris HCl pH 7.4, 10 mM EDTA, 0.25% CHAPS, 150 mM NaCl and subjected to immunoprecipitation with preimmune serum (lanes 1 and 3) or anti-NGF receptor serum (lanes 2 and 4) as described in Methods. Immune precipitates were incubated with 2 μl of 20 μM [γ-^{32}P]ATP (10 μCi) for 5 min in kinase buffer at 24°C and phosphoproteins were analyzed on 7.2% SDS gel electrophoresis.

bilized with NP-40, octyl glucoside or CHAPS, and was most easily detected using CHAPS (data not shown).

To distinguish whether the effect of NGF on the appearance of the phosphorylated 130-Kd band in the immunoprecipitate was due to activation of a receptor-associated kinase or a hormone-dependent association of the 130-Kd protein with the receptor, the phosphorylation of an exogenous protein substrate was evaluated in the in vitro kinase reaction (Figure 3). Acid denatured enolase was added to the kinase assay performed with the anti-NGF receptor immunoprecipitate from cells treated with (Figure 3, lanes 1 and 3) or without (Figure 3, lanes 2 and 4) NGF. Enolase was rapidly phosphorylated in these precipitates. Phosphoamino acid analysis of the phosphorylated protein after hydrolysis yielded exclusively phosphoserine, with no detectable phosphotyrosine or phosphothreonine (data not shown). However, treatment of cells with NGF caused no increase in the ^{32}P incorporated into the enolase band (Figure 3, lanes 3 and 4). In contrast, NGF did cause an increase in the ^{32}P in the endogenous 130-Kd band. These results suggest that the increased phosphorylation of the 130-Kd protein observed in immunoprecipitates from NGF-treated cells was due to increased association of the protein with the receptor complex, rather than hormone-dependent stimulation of an endogenous, receptor-associated protein kinase.

To determine the nature of the phosphorylation reaction detected in the immunoprecipitate, the 130-Kd protein detected in both control and NGF-treated cells was extracted from gel slices and subjected to phosphoamino acid analysis. Phosphoserine was the predominant phosphorylated amino acid detected (Figure 4). Only a trace of phosphothreonine and no phosphotyrosine were found in this protein. The increased phosphorylation of this protein in immunoprecipitates from NGF-treated cells was found exclusively on serine. The NGF-independent 60-Kd phosphoprotein also contained exclusively phosphoserine (not shown). Moreover, western blotting of these gels with antiphosphotyrosine antisera revealed no signals (data

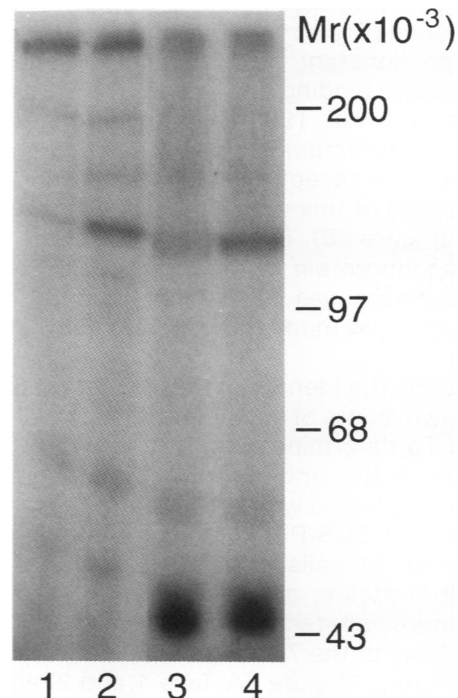

Figure 3. **Phosphorylation of enolase by immunoprecipitates of NGF receptor.** Lysates from control cells (lanes 1 and 3) or cells treated with 10 nM NGF for 5 min (lanes 2 and 4) were immunoprecipitated with anti-NGF receptor serum as described in Figure 2. An in vitro kinase reaction was performed with the immune precipitates in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 2 μg enolase. Phosphoproteins were analyzed on 7.2% SDS gel electrophoresis as described in Methods.

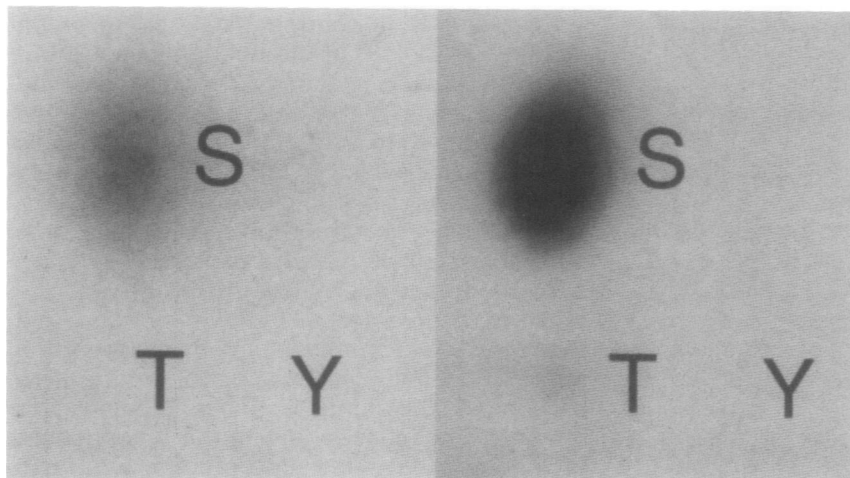

***Figure 4.* Analysis of acid stable phosphoamino acids from the 130 kd protein.** Phosphoamino acid analysis of in vitro ^{32}P-labeled 130-Kd protein from (A) control cells or from (B) cells treated with 10 nM NGF for 5 min are shown. Phosphoamino acid analysis was performed as described in Methods. S, T, and Y mark the positions of nonradioactive phosphoserine, phosphothreonine, and phosphotyrosine, respectively.

not shown), confirming the absence of a receptor-associated tyrosine kinase.

The concentration dependence of the effect of NGF in inducing the appearance of the 130-Kd phosphoprotein was evaluated (Figure 5A). As little as 0.1 nM NGF caused a significant increase in the appearance of this phosphoprotein. The effect was maximal at 0.5 nM NGF. This response is similar to the equilibrium dissociation constant of 0.2 nM determined by steady-state binding analysis at 37°C (Schechter and Bothwell, 1981), suggesting that NGF-receptor interaction is closely linked to the induction of the receptor-protein association. The time course of this action of NGF was also explored (Figure 5B). The appearance of the 130-Kd phosphoprotein in the antireceptor immunoprecipitates was rapidly increased by NGF, reaching a maximum at 5 min, and declining by 30 min.

Although the identity of the 130-Kd protein is not known, some of its characteristics were explored. To determine whether the 130 Kd was a glycoprotein, anti-receptor immunoprecipitates were treated with or without N-glycanase, followed by SDS-PAGE and autoradiography (Figure 6). In cells prelabeled with [^{35}S]methionine/cysteine, glycanase treatment of the immunoprecipitated proteins caused a shift in the mobility of the 75-Kd band identified as the NGF receptor (Figure 6A, lane 1 and 2) as well as the 175-Kd band identified as the EGF receptor (Figure 6A, lanes 3 and 4). In contrast, exposure of antireceptor immunoprecipitates to N-glycanase had no effect on the 130- or 60-Kd receptor-associated proteins detected in the in vitro phosphorylation assay (Figure 6B, lanes 1 and 2), although the apparent molecular weight of the 175-Kd autophosphorylated EGF receptor was decreased by the glycosidase (Figure 6B, lanes 3 and 4).

Discussion

A number of functional similarities exists between the biological actions of insulin and NGF in PC-12 cells, including maintenance of neuronal viability (Recio-Pinto *et al.*, 1980); induction of neurite formation (Bhat, 1983); stimulation of amino acid; and glucose uptake (Yankner and Shooter, 1982), c-fos induction (Greenberg *et al.*, 1988), activation of MAP kinase (Miyasaka *et al.*, 1990a), and stimulation of glycosyl-phosphatidylinositol hydrolysis (Chan *et al.*, 1989). However, significant differences exist in the basic structures of the NGF and insulin receptors. The insulin receptor has an intrinsic tyrosine kinase activity that is thought to be necessary for receptor function. On the other hand, cDNA cloning of a 75-Kd NGF receptor indicated no evidence for a tyrosine kinase domain in the cytoplasmic region (Johnson *et al.*, 1986; Radeke *et al.*, 1987). Interestingly, tyrosine phosphorylation in PC-12 cells has been detected in response to NGF (Maher, 1988; Miyasaka *et al.*, 1991). This tyrosine phosphorylation can be specifically inhibited by the kinase inhibitor staurosporine (Miyaska *et al.*, 1991), which also blocks many of the cellular actions of the hormone (Koizumi *et al.*, 1988; Chan *et al.*, 1989). These findings raised the interesting possibility that NGF induces the association of its receptor with other cellular proteins, leading to increased tyrosine kinase activity.

To explore this possibility we used a rabbit polyclonal antiserum raised against the human NGF receptor that also recognized the rat NGF receptor. A 130-Kd protein was detected in immunoprecipitates from NGF-treated cells that

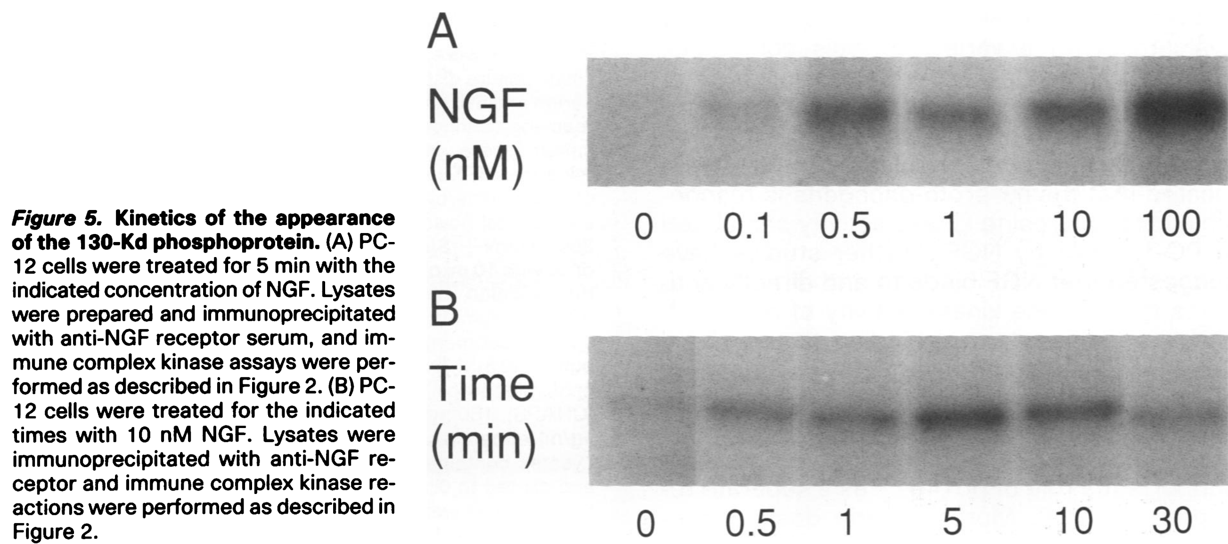

Figure 5. **Kinetics of the appearance of the 130-Kd phosphoprotein.** (A) PC-12 cells were treated for 5 min with the indicated concentration of NGF. Lysates were prepared and immunoprecipitated with anti-NGF receptor serum, and immune complex kinase assays were performed as described in Figure 2. (B) PC-12 cells were treated for the indicated times with 10 nM NGF. Lysates were immunoprecipitated with anti-NGF receptor and immune complex kinase reactions were performed as described in Figure 2.

was phosphorylated on serine. This effect of NGF was rapid (<5 min) and was observed at concentrations of <0.5 nM. The detection of this phosphoprotein appeared to be due to its NGF-dependent association with the receptor because the phosphorylation of a 60-Kd endogenous protein or exogenously added enolase was unaltered by pretreatment of cells with NGF. Additionally, phosphoamino acid analysis of this phosphoprotein revealed that the phosphorylation occurred exclusively on serine residues.

Despite the appearance of this phosphoprotein in anti-NGF receptor immunoprecipitates, there was no evidence for the association of

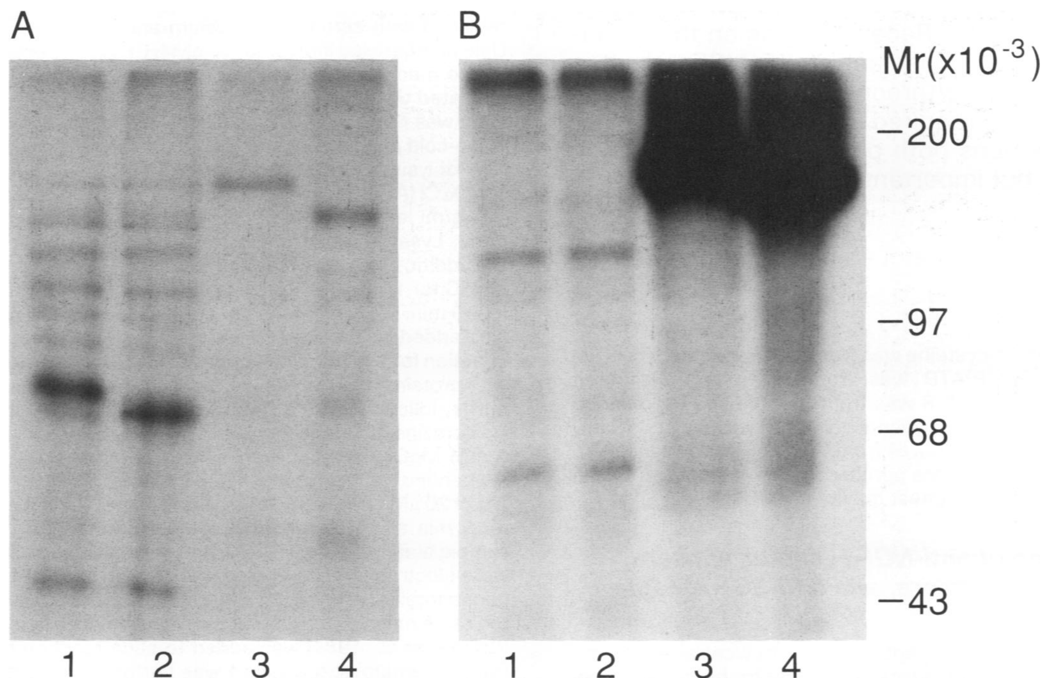

Figure 6. **Effects of N-glycanase on anti-receptor immunoprecipitates.** Detergent extracts from PC-12 cells labeled with [^{35}S]methionine/cysteine for 12 h (A) and unlabeled PC-12 cells (B) were prepared in a lysis buffer containing 50 mM Tris HCl pH 7.4, 10 mM EDTA, 0.25% CHAPS, 150 mM NaCl. Lysates were immunoprecipitated with anti-NGF receptor serum (lanes 1 and 2) and anti-EGF receptor serum (lanes 3 and 4). Anti-receptor immunoprecipitates were treated with (lanes 2 and 4) or without (lanes 1 and 3) N-glycanase, followed by 7.2% SDS-PAGE and autoradiography. In B, immune precipitates were prepared from normal growing cells and used for immune complex kinase assays. Immune complexes were treated with N-Glycanase or vehicle as described above.

the receptor with any tyrosine kinase activity evaluated with a variety of lysis conditions. These data raised the perplexing question of whether or not the 75-Kd receptor mediates the rapid stimulation of a tyrosine kinase in PC-12 cells. A recent report (Kaplan *et al.*, 1991) indicated that the *trk* proto-oncogene is responsible for the tyrosine kinase activity stimulated in PC-12 cells by NGF. Further studies have suggested that NGF binds to and directly activates the tyrosine kinase activity of $pp140^{c\text{-}trk}$, perhaps independent of binding to the 75-Kd receptor (Hempstead *et al.*, 1991; Ohmichi *et al.*, 1991). Our inability to detect any tyrosine kinase activity coprecipitating with the 75-Kd protein, including that of the *trk* proto-oncogene supports the role of $pp140^{c\text{-}trk}$ as a separate receptor for NGF. Moreover, the dose dependence of both tyrosine phosphorylation of pp40 and pp42 by NGF (Miyasaka *et al.*, 1991), as well as activation of the autophosphorylation of the $pp140^{c\text{-}trk}$ (Hempstead *et al.*, 1991; Kaplan *et al.*, 1991; M. Ohmichi *et al.*, 1991), occurs over a time course and concentration corresponding to the binding of NGF to $pp140^{c\text{-}trk}$. Thus, it is possible that the role of the 75-Kd protein in the cellular actions of NGF include an associated serine kinase and its 130-Kd substrate, but do not include activation of tyrosine phosphorylation. Recent studies on the stimulation of MAP kinase activity by NGF indicates that both serine/threonine and tyrosine phosphorylation is required (Miyasaka *et al.*, 1990b, 1991). Perhaps both p75 and $pp140^{c\text{-}trk}$ play a separate but important role in signaling.

Methods

Materials

[^{35}S]methionine/cysteine was from ICN Radiochemicals (Irvine, CA). [γ-^{32}P]ATP was from Amersham (Arlington Heights, IL) NGF 2.5 S was from Bioproducts for Science (Indianapolis, IN). N-Glycanase was from Genzyme (Cambridge, MA) and RPMI was from GIBCO (Grand Island, NY). All other reagents were purchased from Sigma (St. Louis, MO) and were the highest quality available.

Production of anti-NGF receptor antisera, anti-$pp60^{src}$ antisera, and anti-EGF receptor antisera

Polyclonal anti-NGF receptor antiserum was raised by immunizing rabbits with a trpE-NGF receptor bacterial fusion protein containing residues 260 to 399 of human NGF receptor (Johnson *et al.*, 1986). Anti-$pp60^{src}$ antisera was raised by immunizing rabbits with a trpE-$pp60^{src}$ fusion protein. Polyclonal antiserum raised against the human EGF receptor recognizes the kinase domain of the EGF receptor (Decker, 1984).

Metabolic labeling

PC-12 cells were grown on collagen-coated plastic 100-mm tissue culture dishes in RPMI medium with 10% fetal bovine serum and 5% horse serum. Cells were used 5–7 days after reaching confluence. For labeling studies, nearly confluent cultures were washed once with 10 ml of methionine/cysteine-free RPMI medium and incubated for 12 h in 2.5 ml of methionine/cysteine-free RPMI medium with 10% dialyzed fetal bovine serum, 5% dialyzed horse serum, and 200 μCi/ml [^{35}S]methionine/cysteine. Cells were washed once with 10 ml of ice-cold phosphate-buffered saline before the addition of 50 μl of boiling 2% SDS, 10 mM tris(hydroxymethyl)amino methane (Tris)-HCl, pH 7.4. In some experiments, cells were solubilized with 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.25% 3-[(3-cholamidopropyl) dimethyl]-2-hydroxy-1-propane sulfonate [CHAPS], 150 mM NaCl) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride. Lysates containing SDS were heated for 5 min at 100°C and diluted to 0.1% SDS with ice-cold lysis buffer without SDS. Lysates were cooled to 0°C for 5 min, followed by the addition of 40 μl of rabbit IgG agarose. After incubation at 4°C for 15 min, samples were centrifuged at 10 000 $\times g$ for 10 min. The supernatant was then incubated at 4°C for 1 h with 4 μl of preimmune rabbit serum or 4 μl of polyclonal anti-NGF receptor antiserum. Immuno-complexes were precipitated with protein A-Sepharose and washed three times with lysis buffer. Isolated proteins were analyzed by electrophoresis on 7.2% SDS-polyacrylamide gels.

Assay of protein kinase activity in immune precipitates

Before hormonal treatment of PC-12 cells, the medium was replaced with serum-free medium and incubated for 1 h. Unless otherwise indicated, 10 nM NGF was directly added to the medium and the incubation was continued for the indicated time at 37°C. After hormonal treatment, the medium was removed and cells were washed once with 10 ml of ice-cold phosphate-buffered saline before the addition of 1 ml of lysis buffer (50 mM Tris-HCl pH 7.4, 10 mM EDTA, 0.25% CHAPS, 150 mM NaCl) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride. Lysates were cooled to 0°C for 5 min, followed by the addition of 40 μl of rabbit IgG agarose. After incubation at 4°C for 15 min, samples were centrifuged at 10 000 $\times g$ for 10 min. Four microliters of anti-NGF receptor antiserum was added to the collected supernatants, followed by incubation for 1 h. Immune complexes were then precipitated with protein A-Sepharose and washed three times with lysis buffer, followed by one wash with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 1 mM $MnCl_2$, 5 mM $MgCl_2$, pH 7.4. Immune precipitates were then resuspended in 20 μl of the HEPES $MnCl_2$ $MgCl_2$ buffer, and 2 μl of 20 μM [γ-^{32}P]ATP (10 μCi) was added. After incubation for 5 min at 24°C, reactions were stopped with Laemmli sample buffer (Laemmli, 1970) and equal amounts of protein were electrophoresed on 7.2% SDS gels. Immune complex phosphorylation of rabbit muscle enolase was assayed in the same manner except that 2 μg of acid-denatured enolase (Cooper *et al.*, 1984) was added to each reaction mixture. Phosphoamino acid analysis was performed as previously described (Decker, 1984).

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