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Nerve Growth Factor Binds to the 140 kd *trk* Proto-Oncogene Product and Stimulates Its Association with the *src* Homology Domain of Phospholipase C γ1

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SUMMARY: The cellular actions of nerve growth factor (NGF) involve regulation of protein phosphorylation. In PC-12 pheochromocytoma cells, exposure of [^{125}I]NGF followed by crosslinking indicates that the ligand binds to two discreet receptors, the previously described 75 kd protein, as well as the *trk* proto-oncogene product pp140$^{c\text{-}trk}$. Competition experiments reveal that of the two, pp140$^{c\text{-}trk}$ binds to NGF with higher affinity. Following exposure to NGF, pp140$^{c\text{-}trk}$ undergoes a rapid autophosphorylation on tyrosine residues, and concomitantly phosphorylates and associates with phospholipase Cγ1 (PLCγ1), through interaction with its *src* homology domains. The binding of NGF to pp140$^{c\text{-}trk}$ with high affinity, the NGF-dependent activation of its tyrosine kinase activity and the specific association with the effector molecule, PLCγ1, suggests that this is the biologically relevant signaling receptor for NGF.

Although the mechanisms by which nerve growth factor (NGF) supports the survival and differentiation of sympathetic and sensory neurons remain elusive, changes in protein phosphorylation play an important role in the cellular actions of the growth factor. NGF increases the phosphorylation of several cellular proteins (1-3), and is known to stimulate the activities of several serine/threonine kinases, including protein kinase C (4, 5), MAP kinase (6, 7), ribosomal S6 kinase (8), and others (9-13). Recent evidence suggests that one of the earliest responses to NGF is the stimulation of a protein tyrosine kinase (14), which may initiate the subsequent serine phosphorylation events (15). The cloning of a cDNA encoding a 75 kd NGF receptor predicted a protein with a relatively short cytoplasmic domain lacking a consensus tyrosine kinase sequence (16). Recent evidence indicates that the *trk* proto-oncogene product (a member of the tyrosine kinase receptor family) also binds NGF (17) and that activation of the tyrosine kinase activity of *trk* by NGF plays a significant role in NGF-dependent signal transduction. In this report, we have

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Abbreviations: PLCγ1, phospholipase Cγ1; GAP, GTPase-activating protein of *ras*; NGF, nerve growth factor; EGF, epidermal growth factor; SH, *src* homology.

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examined the interaction of pp140$^{c\text{-}trk}$ with the potential effector proteins PLCγ1 and the GTPase activating protein of *ras* (GAP).

MATERIALS AND METHODS

Immunoprecipitation of crosslinked NGF-receptor complexes.
PC-12 cells were harvested, pelleted, and resuspended in ice-cold binding buffer (137 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4 [pH 7.4], 1.2 mM $MgSO_4$, 1 mg/ml glucose, 1 mg/ml BSA, 10 mM HEPES [pH] 7.0), containing 1 mM phenylmethlsulfonyl flouride (PMSF) to a cell concentration of 1 X 10^6 cells per ml. [^{125}I]NGF (Amersham) was added at a concentration of 1 nM, and the cells were incubated on ice for 2 hr. Disuccinimidyl suberate (DSS) in dimethylsulfoxide was added at a final concentration of 150 μM, and further incubated for 30 min at room temperature. Unreacted reagents were quenched by washing the cells twice in 10 mM Tris-saline (pH 7.4). Cells were lysed in 500 μl of HY buffer, consisting of 50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton-X 100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 100 mM NaF, 30 mM p-nitrophenol phosphate, 10 ug/ml leupeptin, and 1 mM PMSF according to Margolis et. al. (18). Lysates were centrifuged for 10 min at 10,000 x *g* and supernatants incubated for 60 min with the indicated antisera. 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 HY buffer. Immune complexes were solubilized in 25 μl of Laemmli sample buffer and loaded on 8% SDS gels (19). The dried gels were exposed to Kodak XAR film.

Anti-phosphotyrosine Immunoblots. PC-12 cells, NR18 cells, *trk*-transformed and control NIH3T3 cells (20) were grown in 150 mm dishes. Prior to hormonal treatment, the medium was replaced with serum-free medium and incubated for 1 hr. 10 nM NGF or 10 nM EGF were directly added to the medium and incubated for 1 min at 37°C. Cells were washed twice with 12 ml ice-cold PBS and lysed in 1 ml HY buffer. Lysates were centrifuged at 10,000 x *g* for 10 min. Supernatants were incubated for 1 hr with the anti-*trk* antiserum (21) or anti-PLCγ1 antiserum (22). 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 HY buffer. Immune complexes were solubilized in 25 μl of Laemmli sample buffer and loaded on 8% SDS gels. Transfer to nitrocellulose paper, immunoblotting with anti-phosphotyrosine antiserum and washing were performed as described (21). For blotting of tyrosine phosphorylated proteins binding to bacterially expressed SH domains of PLCγ1 and GAP, supernatants were incubated for 90 min with protein A-Sepharose beads to which immune complexes of anti-trpE-trpE-PLCγ1 or anti-trpE-trpE-GAP fusion proteins had been bound. The trpE-PLCγ1 fusion protein encompassed residues 545 to 951 of bovine PLCγ1 including both SH2 and SH3 domains of PLCγ1. The trpE-GAP fusion protein contained residues 117 to 444 of bovine GAP also encompassing both the SH2 and the SH3 domains of GAP. The beads were washed 3 times with 1 ml of HY buffer and samples resolved on 8% SDS gels. Immunoblotting with anti-phosphotyrosine antiserum was performed as described above.

RESULTS AND DISCUSSION

To substantiate that pp140$^{c\text{-}trk}$ was itself a receptor for NGF, PC-12 cells were exposed to [^{125}I]NGF (Fig. 1A). After a 2 hr incubation, NGF was crosslinked using disuccinimidyl suberate (DSS) (23). Lysates were absorbed with preimmune serum, antiserum against the 75 kd NGF receptor, antiserum against the conserved cytoplasmic domain of the *trk* oncogene, or a fusion protein containing the *src* homology (SH) domain of PLCγ1 fused to a bacterial trpE protein (24). Immunoprecipitation of the [^{125}I]NGF crosslinked cells with anti-75 kd antiserum yielded a

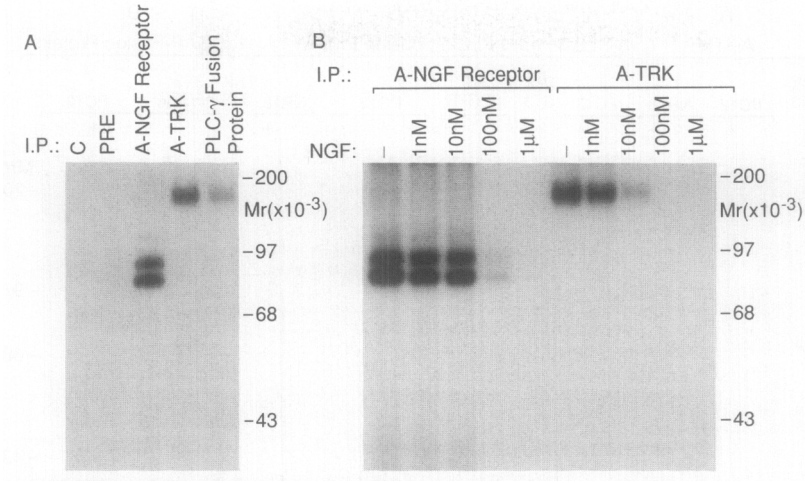

Fig. 1. **Immunoprecipitation of crosslinked NGF-receptor complexes.**
A) Lysates from [^{125}I]NGF-labeled and DSS-crosslinked PC-12 cells were precipitated with the indicated antisera. Lane 1: no antiserum. Lane 2: preimmune rabbit serum. Lane 3: rabbit anti-NGF receptor (75 kd) serum which was raised by immunizing rabbits with a trpE-NGF receptor bacterial fusion protein containing residues 260 to 399 of human NGF receptor (34). Lane 4: rabbit anti-*trk* serum (21). Lane 5: immune complex of anti-trpE-trpE-PLCγ1 fusion protein which encompassed residues 545 to 951 of bovine PLCγ1 including both SH2 and SH3 domains of PLCγ1. In (B) unlabeled NGF at concentrations of 1 nM to 1 μM was added to cells for 5 min before the initial 2 hr binding reaction of [^{125}I]NGF. Lysates from [^{125}I]NGF-labeled and DSS-crosslinked PC-12 cells were immunoprecipitated with rabbit anti-NGF receptor (75 kd) serum and rabbit anti-*trk* serum, as indicated.

doublet with Mrs of approximately 80 and 95 k, but no other bands. Precipitation with anti-*trk* antiserum, or with the PLCγ1 fusion protein revealed a single labeled band with Mr of approximately 160 kd. The 75 kd protein was not detected in these immunoprecipitates. These data suggest that NGF binds to two discreet species of proteins in PC-12 cells, and that one of these, pp140$^{c\text{-}trk}$, binds to the SH regions of PLCγ1.

To compare the relative affinities of binding of these two proteins, cells were exposed to [^{125}I]NGF in the presence of increasing concentrations of unlabeled NGF, followed by crosslinking and immunoprecipitation (Fig. 1B). Concentrations of 10-100 nM NGF were required to displace [^{125}I]NGF from the 75 kd protein. In contrast, binding of [^{125}I]NGF to pp140$^{c\text{-}trk}$ was displaced with 1-10 nM unlabeled NGF. While these results do not represent equilibrium binding experiments, and thus do not yield meaningful dissociation constants, they strongly suggest that the binding of NGF to pp140$^{c\text{-}trk}$ is by at least an order of magnitude, of higher affinity than the binding to p75.

A receptor is defined by its ability to transmit a relevant signal in the cell. PC-12 cells or PC-12 mutant cells (NR18) that do not respond to NGF (20) were exposed to NGF, lysed and subject to immunoprecipitation with the anti-*trk* antiserum (Fig. 2A). Tyrosine phosphorylation was detected by SDS PAGE followed by immunoblotting (22). In confirmation of a recent report (17), NGF stimulated the tyrosine phosphorylation of pp140$^{c\text{-}trk}$ in the responding PC-12 cells, but not in the nonresponding mutant cell line, whereas epidermal growth factor (EGF) had no effect (not shown).

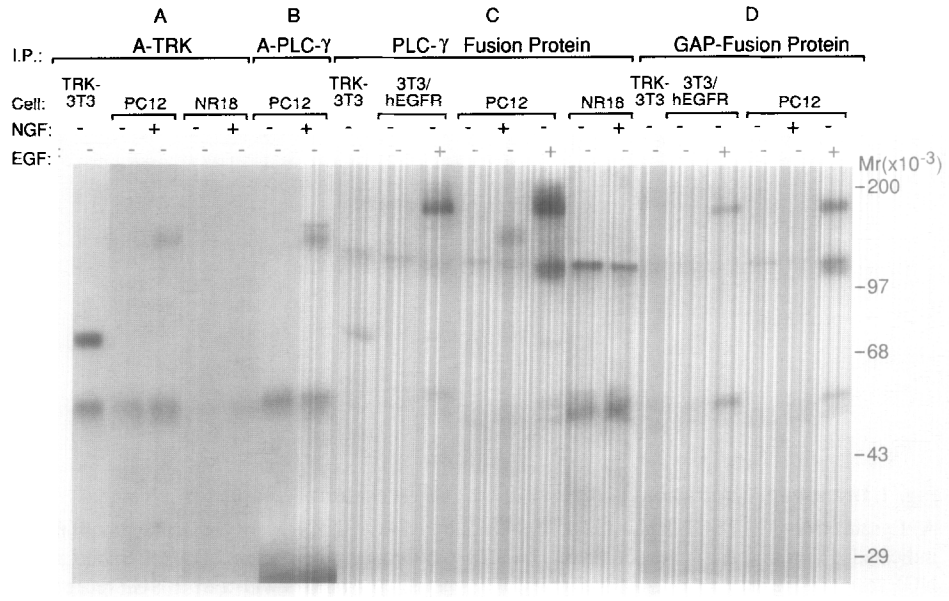

Fig. 2. **NGF induces the tyrosine phosphorylation of pp140$^{c\text{-}trk}$ and its subsequent interaction with the PLCγ1 SH region.**
PC-12 cells, NR18 cells, *trk*-transformed and control NIH3T3 cells were grown in 150 mm dishes. 10 nM NGF or 10 nM EGF were directly added to the medium and incubated for 1 min at 37°C. Lysates were immunoprecipitated with anti-*trk* antiserum (A) and anti-PLCγ1 antiserum (B) before analysis of immunoblotting with anti-phosphotyrosine antiserum as described. TrpE-PLCγ1 (C) and TrpE-GAP (D) fusion proteins immobilized with anti-TrpE antibodies were mixed with lysates and analyzed for associated proteins by antiphosphotyrosine immunoblotting.

Following autophosphorylation on tyrosine residues, many receptors bind to signaling proteins that contain SH2 domains (22, 24-26). NGF has been reported to stimulate the tyrosine phosphorylation of PLCγ1 (which contains 2 SH2 regions) (24), and we have recently observed (21) that the p70 *trk* oncogene product binds specifically to the SH domain of PLCγ1. To determine whether NGF stimulated the association of pp140$^{c\text{-}trk}$ with PLCγ1, we precipitated cells treated with or without NGF with anti-PLCγ1 antiserum (Fig. 2B). In NGF treated cells, two tyrosine phosphorylated proteins were detected in these immunoprecipitates, the 148 kd PLCγ1 and the 140 kd *trk* proto-oncogene product. We also examined the effect of NGF on the binding of pp140$^{c\text{-}trk}$ to immobilized fusion proteins containing the SH2 domains of PLCγ1 (Fig. 2C) or GAP (Fig. 2D). As a positive control, EGF was shown to stimulate the association of its tyrosine phosphorylated receptor with both PLCγ1 and GAP in 3T3 and PC-12 cells. The constitutively activated p70 *trk* oncogene product from *trk* transfected 3T3 cells associated with the PLCγ1 fusion protein. NGF stimulated the association of pp140$^{c\text{-}trk}$ with the PLCγ1 fusion protein in PC-12 cells, but not in the unresponsive NR18 cells. While EGF treatment of 3T3 or PC-12 cells caused the association of the tyrosine phosphorylated EGF receptor with an immobilized GAP fusion protein (Fig. 2C), exposure of cells to NGF was without effect. Low levels of p70^{trk} bound to the GAP fusion protein (21). Thus, the effect of NGF on the binding of pp140$^{c\text{-}trk}$ to signalling proteins appears to be specific for the SH domain of PLCγ1.

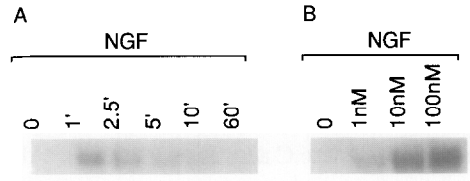

Fig. 3. **Kinetics of the NGF-induced binding of tyrosine phosphorylated pp140$^{c\text{-}trk}$ to the PLCγ1 SH domains in PC-12 cells.**
In (A), the time course of the binding of tyrosine phosphorylated pp140$^{c\text{-}trk}$ to the trpE-PLCγ1 fusion proteins was evaluated. Cells were treated with 10 nM NGF for the indicated times at 37°C. In (B), the dose response of the pp140$^{c\text{-}trk}$-trpE-PLCγ1 interaction was evaluated. Cells were treated with indicated concentrations of NGF for 1 min at 37°C. In both experiments, immunoblotting with anti-phosphotyrosine antiserum was performed as described.

The time course of the NGF-dependent association of pp140$^{c\text{-}trk}$ with the PLCγ1 fusion protein was evaluated (Fig. 3A). Addition to PC-12 cells of 10 nM NGF caused the maximal association of pp140$^{c\text{-}trk}$ with the fusion protein within 1 min of exposure. The binding rapidly declined thereafter, and was barely detectable after 10 minutes. The apparent reversal of this effect was even faster than the deactivation of MAP kinase (6, 7) or pp40/42 tyrosine phosphorylation (15), suggesting that autophosphorylation and association of pp140$^{c\text{-}trk}$ with PLCγ1 are likely to be closely regulated events. The dose dependence of this response was also evaluated (Fig. 3B). Increasing concentrations of NGF were added to PC-12 cells for 1 min, followed by lysis and evaluation of association of tyrosine phosphorylated pp140$^{c\text{-}trk}$ to the PLCγ1 fusion protein, as described above. pp140$^{c\text{-}trk}$-PLCγ1 binding was observed with as little as 1 nM NGF, and was dose dependent up to 100 nM.

These results strongly suggest that pp140$^{c\text{-}trk}$ is the important receptor for NGF. This protein meets all the criteria for a hormone receptor; it exhibits saturable binding with physiologically relevant concentrations of the ligand and mediates an important biological response, tyrosine phosphorylation and interaction with a signalling protein that is thought to be involved in receptor function. The physical separation and the large difference in apparent affinity for the interaction of NGF with pp140$^{c\text{-}trk}$ and the 75 kd protein raise questions about the roles of these two proteins in signal transduction for the growth factor. It has been suggested that these proteins together produce a high affinity binding phenotype that is responsible for insuring responsiveness to physiological levels of the hormone (27-29). However, in addition to the differences in affinities for NGF, separate immunoprecipitations from PC-12 cells that clearly express both binding proteins revealed no evidence for their coprecipitation. Moreover, NGF induces the association of only pp140$^{c\text{-}trk}$ but not p75 with PLCγ1. In this regard, a recent report (30) indicates that only the high affinity binding site is necessary for the biological activity of NGF. In fact, expression of pp140$^{c\text{-}trk}$ in Xenopus oocytes produced a cell in which seminal vesicle breakdown and cdc2 kinase activation was stimulated by NGF (31). Taken together, these data imply that pp140$^{c\text{-}trk}$ and p75 may exhibit distinct but related functions. Recent studies (6, 7) on the stimulation of MAP kinase activity by NGF indicate that both serine/threonine and tyrosine phosphorylation events are required for activation of the enzyme. Perhaps both proteins play a

What is the significance of the pp140$^{c\text{-}trk}$-PLCγ1 interaction? Although PLCγ1 undergoes tyrosine phosphorylation in response to NGF (32, Fig. 2), we have failed to demonstrate an effect of the hormone on inositol lipid hydrolysis or calcium mobilization, although there is diacylglycerol production and protein kinase C activation (4, 5, 33). Although the lack of a polyphosphoinositide turnover response to NGF may be due to other abnormalities peculiar to the PC-12 tumor cell line, an interesting possibility is that the SH domain of PLCγ1, or another protein, plays a separate regulatory role for pp140$^{c\text{-}trk}$, perhaps modulating the interaction of this receptor with other signalling proteins, or tyrosine phosphatases. The likelihood of these and other possibilities requires further investigation.

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