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Expression of Human p140^{trk} Receptors in p140^{trk}-Deficient, PC12/Endothelial Cells Results in Nerve Growth Factor–Induced Signal Transduction and DNA Synthesis

Hao Jiang,[1] Vilen Movsesyan,[1] Donald W. Fink, Jr.,[2] Monika Fasler,[1] Michael Whalin,[1] Yasuhiro Katagiri,[1] Mariam Monshipouri,[1] Geneva Dickens,[1] Peter I. Lelkes,[3] Gordon Guroff,[1]* and Philip Lazarovici[1]

[1]Section on Growth Factors, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892
[2]Division of Cytokine Biology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892
[3]Laboratory of Cell Biology, Department of Medicine, University of Wisconsin, Milwaukee, Wisconsin 53201

Abstract Nerve growth factor (NGF) regulates proliferation, differentiation, and survival of sympathetic and sensory neurons through the tyrosine kinase activity of its receptor, p140^{trk}. These biological effects of NGF depend upon the signal-mediating function of p140^{trk} substrates which are likely to differ from cell to cell. To define p140^{trk} receptor substrates and the details of signalling by NGF in the hybrid cell PC12EN, we stably transfected cultures with a vector encoding a full-length human p140^{trk} cDNA sequence. Two stably transfected clones, one expressing p140^{trk} with higher affinity (PC12EN-trk3; K_d 57.4 pM, B_{max} 9.7 pmole/mg) and one expressing p140^{trk} with a lower affinity (PC12EN-trk1; K_d 392.4 pM, B_{max} 5.7 pmole/mg) were generated. Radioreceptor assays indicate that transfected p140^{trk} receptors show slow NGF-dissociation kinetics, are resistant to trypsin or Triton X-100 treatment, are specific for NGF compared to other neurotrophins, and are internalized or downregulated as are native PC12 p140^{trk} receptors. NGF stimulates p140^{trk} tyrosine phosphorylation in a dose- (0.01–10 ng/ml) and time- (5–120 min) dependent manner, and tyrosine phosphorylation was inhibited by 200–1,000 nM K-252a. NGF-induced Erk stimulation for 60 min was assessed using myelin basic protein as a substrate. NGF treatment also led to an increased phosphorylation of p70^{S6k}, SNT, and phospholipase Cγ, demonstrating that the major NGF-stimulated signalling pathways found in other cells are activated in PC12EN-trk cells. Staurosporine (5–50 nM) rapidly and dBcAMP (1 mM) more slowly, but not NGF induced morphological differentiation in PC12EN-trk cells. Rather, NGF treatment in low-serum medium stimulated a 1.3- and 2.3-fold increase in DNA synthesis measured by [^{3}H]thymidine incorporation in PC12EN-trk1 and PC12EN-trk3, respectively. These data

Abbreviations used: BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; EDTA, ethylenediamine N, N, N′, N′-tetraacetic acid; EGF, epidermal growth factor; EGTA, ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; Erk, extracellular signal-regulated kinase; G418, geneticin; HEPES, N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid; K-252a, 8R*, 9S*, 11S*-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy- 1H,8H,11H-2, 7b, 11a-triazadibenzo (a, g) cyclo-octa (cde) trindene-1-one; MBP, myelin basic protein; NGF, nerve growth factor; NT-3, neurotrophin 3; NT-4, neurotrophin 4; p75^{NGFR}, low affinity NGF receptor; p140^{trk}, high affinity NGF receptor; PBS, phosphate-buffered saline; PCEN-trk1 and -trk3, two different p140^{trk}-overexpressing PC12EN clones; PC12, pheochromocytoma cells; PC12EN, hybrid cells obtained by somatic hybridization of PC12 cells and bovine adrenal medullary endothelial cells; PC12nnr5, a pheochromocytoma clone nonresponsive to NGF; PMSF, phenylmethylsulfonyl fluoride; RT-PCR,reverse transcriptase–polymerase chain reaction; SDS, sodium dodecyl sulfate; Shc, src homologous and collagen protein; PLCγ, phospholipase C gamma; SNT, suc-associated neurotrophic factor-induced tyrosine-phosphorylated target; TBS, Tris-buffered saline; 3T3-trk, NIH3T3 fibroblast cells overexpressing human p140^{trk}; 6.24, a pheochromocytoma clone overexpressing human p140^{trk}.

Vilen Movsesyan's permanent address is Laboratory of Cell and Tissue Cultures, Institute of Biotechnology, Yerevan 375056, Armenia.

Philip Lazarovici's permanent address is Department of Pharmacology, School of Pharmacy, Faculty of Medicine, Hebrew University of Jerusalem, Jerusalem 91010, Israel.

*Correspondence to: Gordon Guroff, Section on Growth Factors, National Institute of Child Health and Human Development, National Institutes of Health, Building 49, Room 5A64, 9000 Rockville Pike, Bethesda, MD 20892.

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highlight the functionality of the transfected p140^{trk} receptors and indicate that these transfected cells may serve as a novel cellular model facilitating the study of the mitogenic properties of NGF signalling and the transducing role of the p140^{trk} receptor substrates. J. Cell. Biochem. 66:229–244.

Key words: nerve growth factor; fibroblast growth factor; K-252a; staurosporine; p140^{trk}; receptor; signal transduction; tyrosine kinase; transfection; overexpression; PC12/endothelial hybrid cells; DNA synthesis; proliferation; differentiation

Nerve growth factor (NGF) is a member of a family of neurotrophic factors called neurotrophins [Thoenen, 1991]. These peptides are crucial for the survival and differentiation of sympathetic and neural crest–derived sensory neurons [Levi-Montalcini and Angeletti, 1968] as well as for other specific neuronal cell populations in the central nervous system [Bothwell, 1991]. Additional members of the neurotrophin family include brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5) [Thoenen, 1991; Chao, 1992]. The biological activities of the neurotrophins are mediated by their binding to and activation of receptor tyrosine kinases [Chao, 1992]. These high-affinity receptors are encoded by the trk gene family [Martin-Zanca et al., 1989; Ip et al., 1993]. Binding of NGF to p140^{trk} receptors induces their dimerization [Jing et al., 1992] and a stimulation of their intrinsic tyrosine kinase activity and results in the transphosphorylation of several tyrosine residues in p140^{trk} [Kaplan and Stephens, 1994]. Tyrosine phosphorylation is required for the activation of the catalytic activity of the p140^{trk} receptor as well as for the recruitment of signalling protein substrates, such as phospholipase Cγ [Loeb et al., 1994], phosphatidylinositol 3′-kinase [Obermeier et al., 1993], Shc protein [Stephens et al., 1994], extracellular signal-regulated kinases (Erks) [Loeb et al., 1994], and the suc-associated neurotrophic factor–induced tyrosine-phosphorylated target (SNT) [Rabin et al., 1993].

Another transmembrane protein, p75^{NGFR}, binds NGF with low affinity [Chao, 1992]. Although there is evidence suggesting a role for p75^{NGFR} in the overall actions of NGF [Chao, 1992], its involvement in p140^{trk} receptor signalling is not clear [Greene and Kaplan, 1995].

Identification of the neurotrophin receptors as a family of tyrosine kinases suggested the possibility that signalling pathways activated by neurotrophins to promote neuronal differentiation and those stimulated by mitogenic growth factors to promote proliferation are analogous if not congruent [Chao, 1992]. Indeed, NGF can stimulate mitosis in certain cells [Burstein and Greene, 1982; Black et al., 1987]. In a few clones of NIH3T3 cells transfected with human p140^{trk}, NGF is a powerful mitogen [Cordon-Cardo et al., 1991]. However, upon overexpression of human p140^{trk} in PC12 cells [Hempstead et al., 1992], mutant NGF-nonresponsive PC12nnr5 cells [Loeb et al., 1991], neuroblastoma [Matsushima and Bogenmann, 1993], a hypothalamic cell line [Zhou et al., 1994], or a rat glioma cell line [Colangelo et al., 1994], NGF mediates neuronal differentiation characterized in part by promotion of neurite outgrowth. Initially, MAH cells, a neuronal progenitor cell line that expresses human p140^{trk}, respond to NGF by simultaneously proliferating and extending neurites. But after 7 days of NGF exposure, the cells stop dividing [Verdi et al., 1994].

This dual response has also been observed upon NGF treatment of other clones of NIH3T3 cells transfected with human p140^{trk} [Decker, 1995]. Also, NGF is a potent inducer of proliferation and differentiation for adult rat chromaffin cells [Tischler et al., 1993]. Therefore, it appears that whether or not NGF induces proliferation or differentiation depends upon the type and capability of the cell in which p140^{trk} receptor signalling is elicited. Defining the pharmacological and molecular basis of this phenomenon is critical for a complete understanding of the mechanism of NGF action. Two possibilities merit consideration. First, the signals leading to these different responses are indistinguishable, and the different cellular responses to NGF reflect the distinct genetic and cellular programs that are inherent in the transfected cells. Alternatively, the substrates of the p140^{trk} receptor might differ between cell types and, therefore, p140^{trk} pathways signalling cell proliferation are different in key respects from those leading to differentiation. These possibilities have not yet been thoroughly addressed, due principally to the absence of a fully capable neuronal cell line model that lacks p140^{trk} and

$p75^{NGFR}$ receptors permitting systematic evaluation of this issue.

To identify $p140^{trk}$ receptor substrates potentially involved in NGF proliferative signalling, we have taken advantage of a novel cell model (PC12EN cells) obtained by somatic hybridization of rat adrenal medullary pheochromocytoma (PC12) and bovine adrenal medullary endothelial (BAME) cells [Rasouly et al., 1996]. PC12EN cells display numerous neuronal characteristics: they express neuronal glycolipids, synthesize and secrete catecholamines, and respond to differentiative agents with morphological change [Rasouly et al., 1996]. These cells are very robust, proliferate rapidly, and lack $p140^{trk}$ and receptors for epidermal growth factor (EGF). They also have exceedingly low levels of $p75^{NGFR}$ receptors, but the expression of physiological levels of fibroblast growth factor (FGF) receptors is maintained [Rasouly et al., 1996].

The present study demonstrates that transfection of the human $p140^{trk}$ cDNA into PC12EN cells reconstitutes functional, NGF-mediated pathways leading to the activation of the Erks, PLCγ, S6 kinase, and SNT and an increase in DNA synthesis suggestive of a proliferative effect. Thus, proliferation is induced in a cell fully capable of morphological differentiation and with a complete repertoire of NGF-stimulated signal transduction pathways. We anticipate that PC12EN hybrids transfected with human $p140^{trk}$ will provide a new cellular model for identifying $p140^{trk}$ substrates and determining their involvement in NGF-induced proliferative signal transduction pathways.

METHODS

Materials

Mouse NGF, bovine basic FGF, and rat collagen type II were purchased from Collaborative Biochemicals (Bedford, MA). BDNF, NT-3, and NT-4 were a kind gift from Promega Co. (Madison, WI). K-252a and staurosporine were kindly provided by Dr. Y. Matsuda (Kyowa Hakko Kogyo, Ltd., Tokyo, Japan). 8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphate (8-CPT-cAMP), a cell-permeable analogue of cAMP, was purchased from Biomol Research Lab, Inc. (Plymouth Meeting, PA). [^{125}I]NGF (specific activity: 81.4 µCi/µg) and [^{3}H]thymidine (specific activity: 85 Ci/mmol) were purchased from Dupont-NEN (Boston, MA) and Amersham (Arlington Heights, IL), respectively. The monoclonal antiphosphotyrosine antibody 4G10, the anti-PLCγ antibody, and the anticarboxyterminal $p70^{S6k}$ antibody were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The polyclonal anti-$p140^{trk}$ (C14) and anti-Erk1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The affinity-purified polyclonal anti-$p140^{trk}$ 203 antibody was the generous gift of Dr. D. Kaplan (Montreal Neurological Institute, Montreal, Canada). Anti-$p75^{NGFR}$ antibody was purchased from Promega Co.

Cell Culture

PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 7% fetal bovine serum, 7% horse serum, 100 µg/ml streptomycin, and 100 U/ml penicillin (Gibco-BRL, Grand Island, NY) [Huff et al., 1981]. PC12nnr5 cells were grown in collagen-coated tissue culture dishes in RPMI 1640 medium (Gibco-BRL) supplemented with 10% horse serum and 5% fetal bovine serum [Green et al., 1986]. 6.24 cells, a clone of PC12 cells overexpressing human $p140^{trk}$, were kindly provided by Dr. D. Kaplan and were grown under the same conditions as PC12 cells in the presence of 200 µg/ml of G418 (Gibco-BRL) [Hempstead et al., 1992]. PC12EN cells were grown under the same conditions as PC12 cells.

Transfection

The full-length human $p140^{trk}$ cDNA sequence was inserted at the EcoRI site of pBluescript KSII(+) vector (the kind gift of Dr. A. Levi, Rome, Italy). HindIII/XbaI digestion of pBluescript KSII(+)-trkA plasmid released a 2.7 kb HindII/XbaI fragment containing the full-length human $p140^{trk}$ cDNA, and this fragment was cloned into the HindIII/XbaI sites of the pRcCMV expression vector (Invitrogen, Carlsbad, CA). The resulting plasmid was used for transfection of PC12EN and NIH3T3 cells [Jiang et al., 1997] with calcium phosphate [Jiang et al., 1993]. The $p140^{trk}$-transfected cells were selected in 0.5 mg/ml of G418, subcloned by limiting dilution, and screened for $p140^{trk}$ expression by immunoblotting with anti-$p140^{trk}$ antibody [Hempstead et al., 1992]. Two subclones, PC12EN-trk1 and PC12EN-trk3, were used in the present studies and maintained under the conditions described above.

Nerve Growth Factor Binding Assay

Radioreceptor assays were carried out as previously described [Huff et al., 1981; Lazarovici et al., 1987]. The cells were grown in collagen- (200 μg/ml) and polylysine- (10 μg/ml) coated six-well plates. The monolayer was washed twice with DMEM binding medium containing 0.1% bovine serum albumin (Sigma, St. Louis, MO). The medium was replaced with 1 ml of binding medium containing 50–100 pM [^{125}I]NGF with or without 4 nM unlabeled NGF to measure total and nonspecific binding, respectively. The incubation, unless otherwise stated, was carried out at 37°C for 45 min, and then the incubation buffer was removed and the cells washed with ice-cold PBS. The monolayer was then solubilized with 1 ml of 1 N NaOH overnight at room temperature. The cell-associated radioactivity was counted in a γ counter, and a portion of the solubilized mixture was used for protein determination. All binding assays were done in quadruplicate and repeated at least three times using cells from different passages.

Nerve Growth Factor Internalization Assay

NGF internalization, measured by the acid stripping method, was carried out with [^{125}I]NGF (150 pM/culture) bound to cells either at 37°C or 4°C for 45 min. The cells were then washed and incubated for 5 min on ice with 0.2 M acetic acid in 0.5 M NaCl to separate the acetic acid wash–sensitive [^{125}I]NGF bound (cell-surface fraction) from the remaining cell-associated [^{125}I]NGF (internalized fraction). Finally, the cells were washed again with PBS and dissolved in 1 ml of 1 N NaOH. The radioactivity in the acetic acid wash (cell-surface bound) and in the NaOH (internalized) was measured.

Triton X-100 and Trypsin Treatments

Triton X-100 and trypsin treatments were performed using the sucrose cushion method as previously described [Tocco et al., 1988]. Dissociation of bound [^{125}I]NGF was measured by a cold chase method [Kasaian and Neet, 1988]. The 45 min incubation at 37°C with [^{125}I]NGF was followed by a chase with 500 nM of unlabeled NGF for 120 min at 4°C. At this time, all of the fast kinetic component had been dissociated [Meakin et al., 1992]. Internalized [^{125}I]NGF remained cell-associated, as did [^{125}I]NGF bound to slow receptors at the cell surface. Downregulation of NGF receptors by NGF was assayed as previously described [Lazarovici et al., 1987]. The cells were incubated at 37°C with 10 nM NGF for different periods of time to downregulate p140^{trk} receptors. Then they were washed once with fresh DMEM containing 7% fetal bovine serum, 7% horse serum, and 100 μg/ml of anti-NGF antiserum and twice with regular serum-containing DMEM. Thereafter, the cells were submitted to a regular binding assay using 150 pM [^{125}I]NGF for 45 min at 37°C as described above. Quantitative analysis of the binding data for the preparation of Scatchard plots was performed using a modified ligand program to determine equilibrium dissociation constants and p140^{trk} receptor density.

Immunoprecipitation and Immunoblotting

Following treatment with NGF and other compounds, the cells harvested by scraping and washed twice with ice-cold PBS and lysed for 30 min on ice in lysis buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholic acid, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin, 1 μg/ml of aprotinin) [Stephens et al., 1994]. The total cell lysates were collected by centrifugation at 10,000 rpm for 10 min. Equal amounts of cell lysates were taken for immunoprecipitation and precleared with protein A-agarose (Pierce, Rockford, IL) for 1 h at 4°C. The supernatants were collected and incubated with the appropriate antibody overnight at 4°C. Protein A-agarose was then added and incubated for 1 h at 4°C. The protein A-agarose beads were collected, washed three times with lysis buffer and once with 25 mM Tris-HCl, pH 6.8, and then resuspended in 4× SDS sample buffer. The supernatant was collected and applied to 8% SDS polyacrylamide gels. The proteins were detected using the appropriate specific antibody and an ECL (Amersham) or Tropix (Tropix, Bedford, MA) enhanced chemiluminesence protein detection kit according to the manufacturer's instructions.

Erk1 Kinase Assay

Washed protein A-agarose pellets containing immunoprecipitated Erk1 were suspended in 30 μl of kinase assay buffer (7.5 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 μM protein kinase A inhibitor peptide PKI (6-22)-amide, 225 μM ATP, 25 μCi [γ-^{32}P]ATP, 500

μg/ml of MBP as kinase substrate) and incubated for 30 min at room temperature. The reaction was terminated by the addition of 30 μl of 2× SDS sample buffer. Samples were heated for 5 min in a boiling water bath and then electrophoresed through a 4–20% Tris-glycine gradient gel (NOVEX, San Diego, CA). Following electrophoretic resolution, radiolabeled proteins were transferred to PVDF membranes. Phosphorylated MBP was visualized by exposure of the membranes to Biomax MR film (Kodak, Rochester, NY). MBP-associated radioactivity was quantitated directly from the PVDF membrane using an AMBIS (Millipore, Bedford, MA) radioanalytic imaging system.

Measurement of S6 Phosphorylation

Cell cultures were transferred to fresh DMEM medium containing 0.5% fetal bovine serum for 60 min prior to treatment with NGF (100 ng/ml). Cell-free lysates were prepared in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 2 mM EDTA, 20 mM NaF, 100 mM NaCl, 1 mM DTT, 1% Triton X-100, 1 mM Na_3VO_4, 40 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM benzamide, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 30 min on ice. The lysates were centrifuged for 15 min at 14,000*g*, and the supernatants were used for immunoprecipitation. Assays for the S6 kinase activity of $p70^{S6k}$ were performed on immune complexes prepared by addition of 4 μg of the appropriate antibody to 500 μg of lysate protein. The extracts were incubated with the antibody overnight at 4°C with shaking. Forty microliters of protein A-Sepharose was added to the mixture, and the incubation continued for 2 h. The immunoprecipitates were washed and submitted for the S6 kinase reaction by the addition of 10 μCi of [γ-^{32}P]ATP for 30 min at 30°C as previously described [Tang et al., 1996]. The mixtures were resolved on 10% SDS-PAGE.

RT-PCR Assay

To detect the presence of $p140^{trk}$ and $p75^{NGFR}$ transcripts, we isolated total RNA from PC12, PC12EN, PC12EN-trk1, and PC12EN-trk3 cells with a PERFECT RNA™ isolation kit (5′-3′, Inc., Boulder, CO) and used as a template for first strand cDNA synthesis. The first-strand cDNA synthesis reaction included 1 μg of total RNA, an oligo-dT primer, and AMV reverse transcriptase (Invitrogen) in a final volume of 20 μl. The first-strand cDNA (3 μl of the reaction mixture) was then amplified for 35 cycles of PCR with Taq polymerase (Promega) in the presence of either $p140^{trk}$ primers or $p75^{NGFR}$ primers. Both $p140^{trk}$ and $p75^{NGFR}$ primers were selected by using sequences common to both human and rat genes. The $p140^{trk}$ primers have the following sequences: sense (5′-CTG-GATCCTCACAGAGCTGGA-3′) and antisense (5′-GGTGTTTCGTCCTTCTTCTCC-3′). The $p75^{NGFR}$ primers have the following sequences: sense (5′-GAGCCGTGCAAGCCGTGCACC-3′) and antisense (5′-CCTCAGGCTCCTGGGTGC-TGG-3′). One round of PCR amplification consisted of 1 min at 94°C, 2 min at 55°C, and 1 min at 72°C. PCR products were analyzed on 3% agarose gels.

Cell Proliferation Assay

The cells (PC12EN, PC12EN-trk1, PC12EN-trk3) were cultured in 96-well plates with cell densities of about 20,000 cells per well in 200 μl of DMEM supplemented with 7.5% fetal bovine serum and 7.5% horse serum per well. The medium was changed to low-serum medium (0.5% fetal bovine serum/0.5% horse serum) the next day, and the cells were grown for an additional 24 h. Then 100 μl of low-serum medium and NGF (50 ng/ml), bFGF (20 ng/ml), or serum (7.5% fetal bovine serum/7.5% horse serum) were added. The same volume of low-serum medium was added to control wells. After 2 h of incubation, 1 μCi/well of [^{3}H]thymidine was applied. After an additional 24 h of incubation, the medium was replaced with a trypsin-EDTA solution (Gibco-BRL), and the cells were detached and transferred to 3 MM filters using a PHD cell harvester (Cambridge Technology, Inc., Watertown, MA). The filters were then washed with ice-cold 5% TCA and ethanol, and their radioactivity was measured by liquid scintillation counting. In each experiment, sister plates were cultured and treated under the same conditions with growth factors or serum except for the addition of [^{3}H]thymidine and used for the evaluation of cell number using the MTT reaction as previously described [Guo et al., 1993].

Differentiation Assay

To evaluate morphological differentiation, we plated cells at low density (10,000 cells/well) in collagen- and polylysine-coated six-well tissue culture dishes in DMEM supplemented with 0.1–14% serum. Cells were treated for up to 14

days with NGF, bFGF, or staurosporine in the presence or the absence of sodium orthovanadate and examined [Rasouly et al., 1992] and photographed by phase-contrast light microscopy in an inverted Nikon-Diaphot microscope (Nikon, Melville, NY).

RESULTS

Expression of Human p140^{trk} in PC12EN Cells

The expression vector pRcCMV-trkA containing a full-length human p140^{trk} cDNA sequence under the control of the CMV promoter was used to transfect PC12EN cells. Stable transfectant cells were selected in the presence of G418 for 2 weeks, and subclones were obtained by limiting dilution. Twelve clones were screened for the expression of human p140^{trk} by immunoblotting with anti-p140^{trk} 203 antibody. Three subclones were found to express human p140^{trk}.

[^{125}I]NGF binding assays were performed to examine the binding of NGF to these three subclones, and NGF-stimulated tyrosine phosphorylation of p140^{trk} was also examined in these clones. For comparative purposes, the parental PC12EN cells lacking p140^{trk} and PC12nnr5, a mutant NGF-nonresponsive PC12 cell line were employed. PC12 cells expressing physiological levels of p140^{trk} and two p140^{trk}-overexpressing cell lines (6.24 and 3T3-trkA WT.11) were also examined. Based on these comparisons, two of the subclones, PC12EN-trk1 and PC12EN-trk3, were used in the present studies (Fig. 1A). PC12EN-trk1 expressed low levels of binding and p140^{trk} tyrosine kinase activity, similar to those of PC12 cells. PC12EN-trk3 expressed high levels of binding and p140^{trk} tyrosine kinase activity, similar to those seen in p140^{trk}-overexpressing cell lines. The ability of these two transfectants to bind [^{125}I]NGF was further evaluated by saturation experiments in which the cell cultures were incubated with increasing concentrations of labeled NGF in the presence or absence of excess unlabeled NGF (Fig. 1B). Scatchard analysis (Fig. 1C) of these binding data indicated that PC12EN-trk1 has a K_d of 392.4 pM and a B_{max} of 5.7 pmole/mg protein (95,000 receptors per cell), while PC12EN-trk3 has a K_d of 57.4 pM and a B_{max} of 9.7 pmole/mg protein (162,000 receptors per cell). Binding analysis of PC12 cells overexpressing human p140^{trk} (6.24) under identical conditions revealed a K_d of 73.5 pM and a B_{max} of 8.5 pmole/mg protein (141,000 receptors per cell) (data not shown). Therefore, these data indicate that both PC12EN-trk1 and PC12EN-trk3 can express p140^{trk} receptors with different high affinities (and levels), as has been reported in NIH3T3-trkA cells [Klein et al., 1991].

It has previously been shown by binding assays that PC12EN cells express neither p140^{trk} nor p75^{NGFR} NGF receptors [Rasouly et al., 1996]. To confirm this observation in PC12EN-trkA transfectants, we performed RT-PCR to examine the p140^{trk} and p75^{NGFR} transcripts in both parental PC12EN cells and PC12EN-trkA transfectants. No p140^{trk} PCR products were observed using total RNA isolated from parental PC12EN cells (data not shown), confirming the lack of p140^{trk} expression in these cells [Rasouly et al., 1996]. Both PC12EN-trkA transfectants show transcripts of human p140^{trk} expression. Using the same approach, we observed that a 410 bp fragment was amplified using total RNA isolated from PC12EN, PC12EN-trk1, and PC12EN-trk3 cells, indicating the presence of p75^{NGFR} transcripts. In order to confirm the presence of p75^{NGFR} protein in these cells, we performed immunoblotting using anti-p75^{NGFR} antibody. PC12EN cells and PC12EN-trk transfectants showed the presence of p75^{NGFR} protein (data not shown). However, when we compared the levels of p75^{NGFR} with PC12 cells using the same amount of cell lysate, the p75^{NGFR} level in PC12EN, PC12EN-trk1, and PC12EN-trk3 transfectants was estimated to be 2.7%, 1.7%, and 0.9% of that in PC12 cells, respectively. This low level escaped previous detection by cross-linking [Rasouly et al., 1996].

Characteristics of NGF Receptors on PC12EN Transfectants

To determine whether the transfected human p140^{trk} receptors preserve the selectivity of neurotrophin binding seen with endogenous p140^{trk} receptors [Chao, 1992], we performed a competition experiment with BDNF, NT-3, NT-4, and NGF (data not shown). At a concentration of 1.9 nM, NT-3 competed by 20% and NT-4 competed by 50% with the binding of NGF (40 pM) to PC12EN-trk3 cells, while BDNF did not compete, as previously reported [Cordon-Cardo et al., 1991]. These data support the selectivity of transfected p140^{trk} receptors for NGF.

Slow, high-affinity NGF receptors in PC12 cells are resistant to both trypsin digestion and Triton X-100 extraction [Vale and Shooter, 1983]. In order to verify these criteria, we

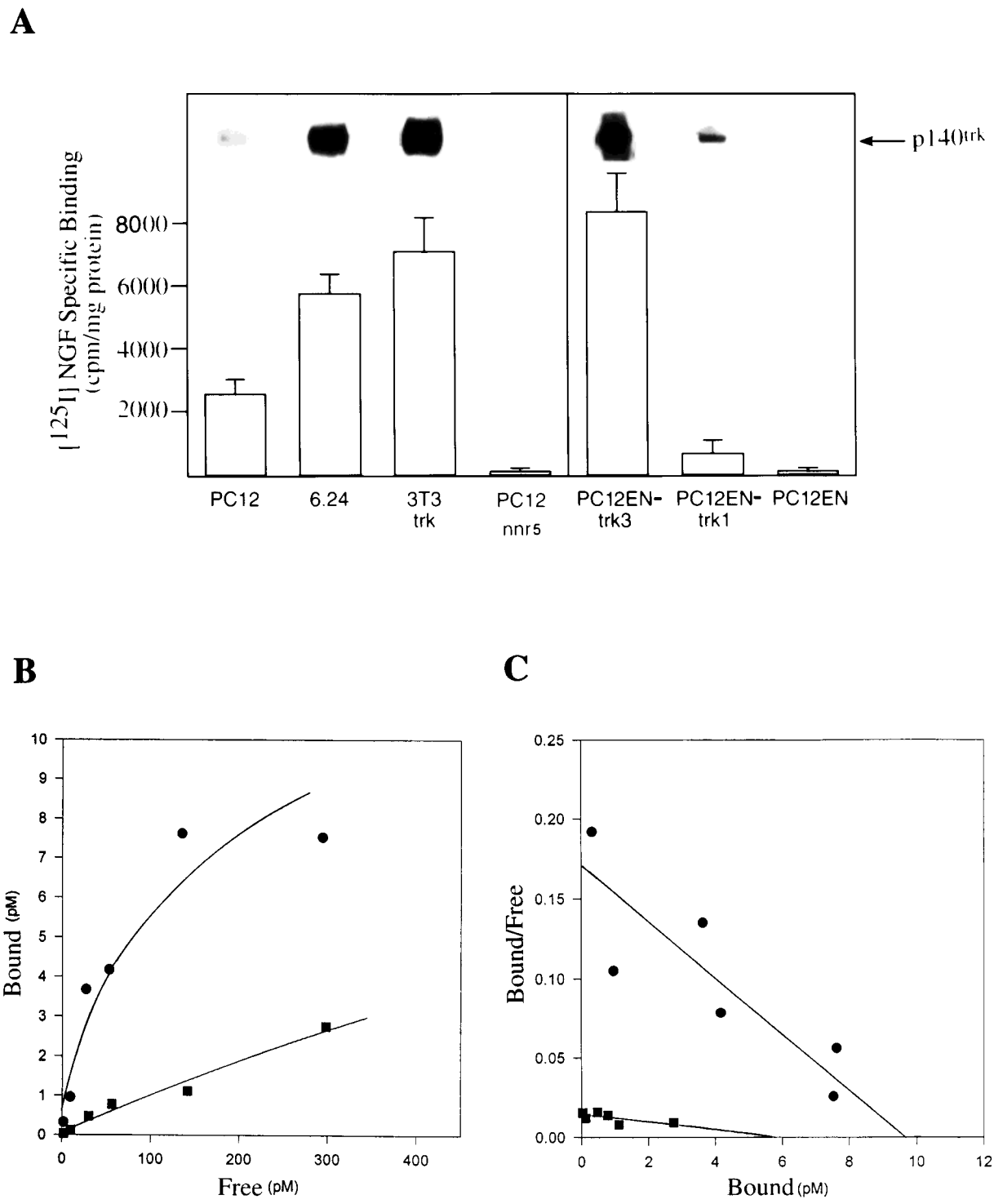

Fig. 1. Expression of human p140^{trk} in PC12EN cells and other cell lines. **A:** Specific binding of [^{125}I]NGF (100 pM) was measured by incubation for 45 min in the presence or absence of unlabeled NGF (4 nM) using confluent monolayers of different transfectants and other cell lines, as described in Methods. All data are given in counts per minute (cpm)/mg protein and are the mean ± SD from three independent experiments. The tyrosine phosphorylation of p140^{trk} was induced by stimulation with NGF at 50 ng/ml for 5 min. The cell lysates were immunoprecipitated with anti-trkA (C14) antibody and immunoblotted with antiphosphotyrosine 4G10 antibody. **B:** Saturation and (**C**) Scatchard plots of [^{125}I]NGF binding to PC12EN-trk1 and PC12EN-trk3 transfectants. Cells were incubated with increasing concentrations of [^{125}I]NGF in the presence or absence of 200 nM unlabeled NGF. Data were analyzed using the Ligand program. ■, PC12EN-trk1; ●, PC12EN-trk3.

treated PC12EN-trk3 cells with trypsin or Triton X-100 and measured the sensitivity of [^{125}I]NGF binding (data not shown). In the present study, 100% and 86% of the p140^{trk} receptors were found resistant to trypsin and Triton X-100 treatment, respectively, in PC12EN-trk1, while 86% and 77% of the p140^{trk} receptors in PC12EN-trk3 cells were found resistant to trypsin and Triton X-100 treatment, respectively. These data suggest that transfected p140^{trk} receptors show behavior similar to those in PC12 or 6.24 cells.

Binding of NGF to the p140^{trk} receptor is followed by internalization which may be quantitated by stripping the bound NGF from the cell surface receptors by a short incubation with an acid-salt solution which can distinguish the cell-surface-bound and internalized receptors [Kasaian and Neet, 1988]. The effect of temperature on [^{125}I]NGF binding to PC12EN-trkA cells is presented in Figure 2. In PC12EN-trk3 cells after 45 min at 37°C, about 60% of bound NGF was internalized, and 40% remained associated with the cell surface, compared to 27% internalized and 73% cell-surface-associated at 4°C (Fig. 2). Similar results were obtained with PC12 and 6.24 cells. These results demonstrate that internalization of transfected human p140^{trk} receptors in 6.24 and PC12EN-trkA cells was similar to that of native p140^{trk} receptors in PC12 cells.

The kinetics of dissociation of [^{125}I]NGF from PC12EN-trk1 and PC12EN-trk3 as well as from 6.24 and PC12nnr5 cells is presented in Figure 3. The results indicate a fast half-time of dissociation of [^{125}I]NGF from the fast, low-affinity, p75^{NGFR} receptors in PC12nnr5 cells and a very slow dissociation rate from the transfected p140^{trk} receptors in PC12EN-trkA and 6.24 cells. The [^{125}I]NGF remaining bound to p140^{trk}-transfected cells after 120 min of dissociation at 4°C was completely displaced when the cultures were shifted to 37°C for 10 min (data not shown), as previously reported. These data demonstrate the slow dissociation kinetics of [^{125}I]NGF from PC12EN-trkA cells, a characteristic typical of slow NGF receptors in PC12 cells and primary neurons [Meakin et al., 1992].

NGF-induced downregulation of p140^{trk} receptors in p140^{trk}-transfected cells is shown in Figure 4. At a high NGF concentration, a rapid downregulation of p140^{trk} receptors was observed. This was followed by a process of gradual recovery in 6.24 and PC12EN-trk3 cells in which

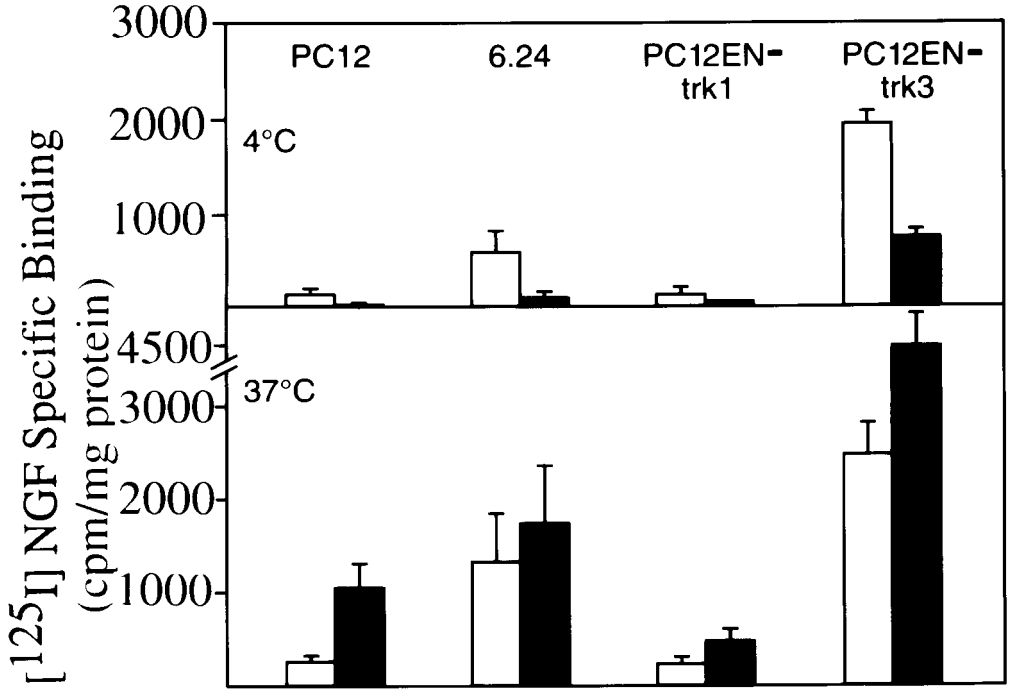

Fig. 2. The effect of temperature on binding and internalization of [^{125}I]NGF in PC12EN-trk transfectants. **A:** [^{125}I]NGF (100 pM) was incubated with different types of cells (1×10^6 cells/well) for 45 min at either 4°C or 37°C, and the specific binding was measured. At the end of 45 min incubation, cell cultures were first washed with acid-salt solution (open bars) and then solubilized in 1 N NaOH (solid bars).

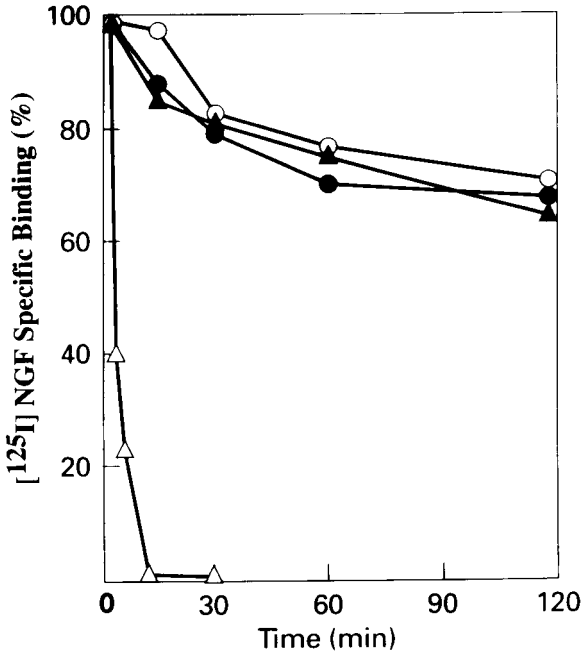

Fig. 3. Dissociation of [^{125}I]NGF from PC12EN-trk transfectants and other cell lines. The cells (2×10^6/well) were incubated at 4°C for 2 h with 500 pM [^{125}I]NGF. Dissociation was initiated by the addition of 500 nM unlabeled NGF. Nonspecific binding was measured in sister cultures containing 400 nM unlabeled NGF during the association process. ○, PC12; △, PC12nnr5; ▲, PC12EN-trk1; ●, PC12EN-trk3.

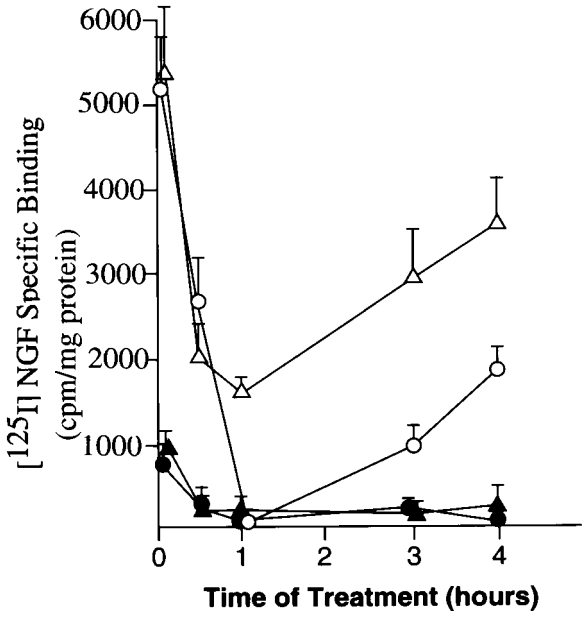

Fig. 4. Time course of the NGF-mediated downregulation of [^{125}I]NGF binding to PC12EN-trk cells and other cell lines. The cells (2×10^6 cells/well) were incubated at 37°C for the indicated time periods with NGF (5 nM) in DMEM. The cultures were washed to remove unbound NGF and incubated in 2 ml of fresh DMEM for 2 h at 37°C to internalize the remaining NGF-p140^{trk} complexes. After two acid-salt washes with DMEM, the binding of [^{125}I]NGF (150 pM) was measured under the conditions described in Methods. ▲, PC12; ●, PC12EN-trk1; △, PC12EN-trk3; ○, 6-24.

approximately 50% of control binding levels was obtained 4–5 h after the end of NGF stimulation.

NGF-Stimulated Tyrosine Phosphorylation of p140^{trk} Receptors in PC12EN Transfectants

To examine the ligand-induced tyrosine phosphorylation of transfected p140^{trk} protein, PC12EN-trk3 were treated with different concentrations of NGF (Fig. 5A) or with NGF for different periods of time (Fig. 5B). The p140^{trk} receptors were immunoprecipitated, and tyrosine phosphorylation was measured by immunoblotting with a specific monoclonal antiphosphotyrosine antibody (4G10). The NGF-induced dose response of receptor tyrosine phosphorylation in Figure 5A is consistent with the saturation curve presented in Figure 1B. In PC12EN-trk3, NGF induced p140^{trk} tyrosine phosphorylation to a maximum within 5 min, and the activity remained elevated for at least 2 h (Fig. 5B).

K-252a, a microbial alkaloid, is a selective inhibitor of the NGF-induced tyrosine protein kinase activity of p140^{trk} receptors [Koizumi et al., 1988; Berg et al., 1992; Ohmichi et al., 1992]. We explored the sensitivity of transfected p140^{trk} receptors towards K-252a (Fig. 5C). In PC12EN-trk3 cells, increasing the K-252a concentration to 500–1,000 nM resulted in a complete inhibition of NGF-induced p140^{trk} tyrosine phosphorylation (Fig. 5C). These findings provide an additional test of the similarity in the inhibition by K-252a of the NGF-induced p140^{trk} tyrosine phosphorylation in PC12EN-trk transfectants, PC12 cells, and other p140^{trk}-overexpressing cells. In order to further confirm the inhibition of p140^{trk} tyrosine phosphorylation by K-252a, we employed another protein kinase inhibitor, AG879, a tyrphostin which specifically inhibits p140^{trk} tyrosine phosphorylation [Ohmichi et al., 1993]. Pretreatment of PC12EN-trk3 cells with AG879 for 30 min at 37°C inhibited NGF-induced p140^{trk} tyrosine phosphorylation by 80% (data not shown).

NGF-Induced Signal Transduction by p140^{trk} Receptors on PC12EN-trk Transfectants

Recently, the signal transduction pathways activated by NGF have begun to be elucidated

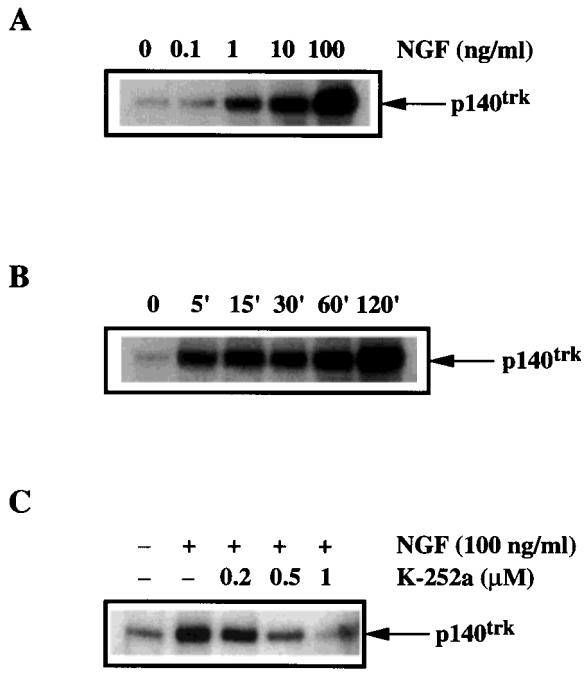

Fig. 5. NGF-stimulated p140^{trk} tyrosine phosphorylation in PC12EN-trk3 cells. **A:** Dose-response curve. Anti-p140^{trk} antibody immunoprecipitates were prepared from the cells treated with different concentrations of NGF (0.1–100 ng/ml) for 5 min at 37°C. Proteins were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine antibody 4G10. **B:** Time course. Anti-p140^{trk} antibody immunoprecipitates were prepared from the cells treated with 10 ng/ml of NGF at 37°C for different periods of time. Proteins from PC12EN-trk3 cells were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine antibody 4G10. **C:** Effect of K-252a. Anti-p140^{trk} immunoprecipitates were prepared from lysates of PC12EN-trk3 cells treated with 50 ng/ml of NGF at 37°C for 5 min after preincubation 37°C for 60 min with 200 nM K-252a, 500 nM K-252a, and 1,000 nM K-252a. The proteins were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine 4G10 antibody.

[Kaplan and Stephens, 1994]. The best characterized of these is the Ras-dependent pathway, which is also activated by mitogens such as epidermal growth factor.

Treatment of PC12 cells with NGF promotes an activation of the Erks for at least 6 h after stimulation [Qui and Green, 1992; Traverse et al., 1992]. The protein kinase activity of Erk1 (Fig. 6A) was assessed using myelin basic protein as a substrate. The enzymatic activity was induced by NGF transiently, 3.5-fold over the control, and persisted for only about 60–120 min (Fig. 6A).

SNT is an incompletely characterized nuclear protein that becomes rapidly and persistently phosphorylated on tyrosine by a Ras, Erk-independent pathway in response to NGF or FGF in PC12 cells [Rabin et al., 1993] and to FGF in PC12EN [Rasouly et al., 1996] cells. We asked if NGF was able to induce the tyrosine phosphorylation of SNT in PC12EN-trk3 cells (Fig. 6B). Addition of NGF for 5 min to PC12EN-trk3 cells resulted in a profound increase in the tyrosine phosphorylation of SNT, persisting for up to 180 min of NGF treatment. These findings are similar to observations in other p140^{trk}-overexpressing cells [Rabin et al., 1993; Kaplan and Stephens, 1994].

Phospholipase Cγ, another substrate of the trk receptor, is also present in PC12EN-trk3 cells (Fig. 6C). Upon 10 min stimulation of the cells with either NGF (50 ng/ml) or FGF (10 ng/ml), PLCγ was phosphorylated on tyrosines.

Treatment of PC12 and PC12EN-trk3 cells but not PC12EN cells with NGF (100 ng/ml) increased S6 phosphorylation by p70^{S6k} immunoprecipitates (Fig. 6D), as serum does in 3T3 fibroblasts.

The data presented above indicate a functional reconstitution of Ras-dependent NGF-stimulated Erk and PLCγ pathways as well as the Ras-independent p70^{S6k} and SNT pathways in human p140^{trk}-transfected PC12EN-trk3 cells.

Mitogenic Activity of NGF in PC12EN-trk Transfectants

To ascertain whether NGF-induced p140^{trk} signal transduction mediates some of the known biological effects of NGF, we first examined whether this neurotrophin has mitogenic activity on PC12EN-trk transfectants compared to the parental PC12EN cells lacking p140^{trk} expression (Fig. 7). The mitogenic activity of NGF was evaluated in low-serum medium by two independent assays: [^{3}H]thymidine incorporation and MTT assay. Parental PC12EN cells did not respond to NGF (Fig. 7A), but bFGF and serum elicited significant stimulations of [^{3}H]thymidine incorporation and significant increases in cell number [Rasouly et al., 1996]. The addition of NGF to PC12EN-trk1 and PC12EN-trk3 for 24 h resulted in a 1.3- and 2.3-fold increase in DNA synthesis (Fig. 7B,C), and this was confirmed by the MTT assay (data not shown). Similar results were obtained upon longer (48 h) treatment with NGF (data not shown). The amount of [^{3}H]thymidine incorporated by these cells in response to NGF was comparable to that observed in the presence of bFGF or serum. In contrast, neither EGF [Rasouly et al., 1996] nor insulin-like growth factor

I induced significant [^{3}H]thymidine incorporation (data not shown), due, probably, to the absence of their respective receptors.

NGF induces neurite outgrowth in PC12 cells and in sympathetic and sensory neurons [Thoenen, 1991]. To assess the differentiating effect of NGF, we plated PC12EN-trk1 and PC12EN-trk3 cells at low density (5×10^3 cells/ml), and individual cells were observed for up to 7 days of NGF treatment (Fig. 8). The morphological endothelial appearance of the cells (Fig. 8A) was not altered by culturing for up to 7 days in the presence of NGF (Fig. 8B). However, upon staurosporine treatment, the cells exhibited a round phenotype after 3–10 h and progressively developed long processes which reached lengths of up to 300 μm after 4 days of treatment (Fig. 8D). The staurosporine-induced process generation was observed in parental PC12EN cells as well [Rasouly et al., 1996]. Treatment of PC12EN-trk transfectants with NGF and 50 mM sodium orthovanadate did not change the morphology of the cells, while staurosporine-induced processes appeared more stable and sprouted in the presence of orthovanadate (data not shown).

cAMP analogues promote survival and neurite outgrowth in PC12 cells [Rydel and Greene, 1988]. Overnight treatment of PC12EN-trk transfectants with 1 mM 8-CTP-cAMP induces the outgrowth of processes (Fig. 8C) which continue to elongate during the next 7 days of treatment, resulting in stable and long process outgrowth (data not shown), although not so sprouted as those induced by staurosporine. The 8-CPT-cAMP–induced process outgrowth was observed in parental PC12EN cells as well (data not shown). These data show that PC12EN-trk transfectants have the capacity for morphological differentiation but do not differentiate in response to NGF.

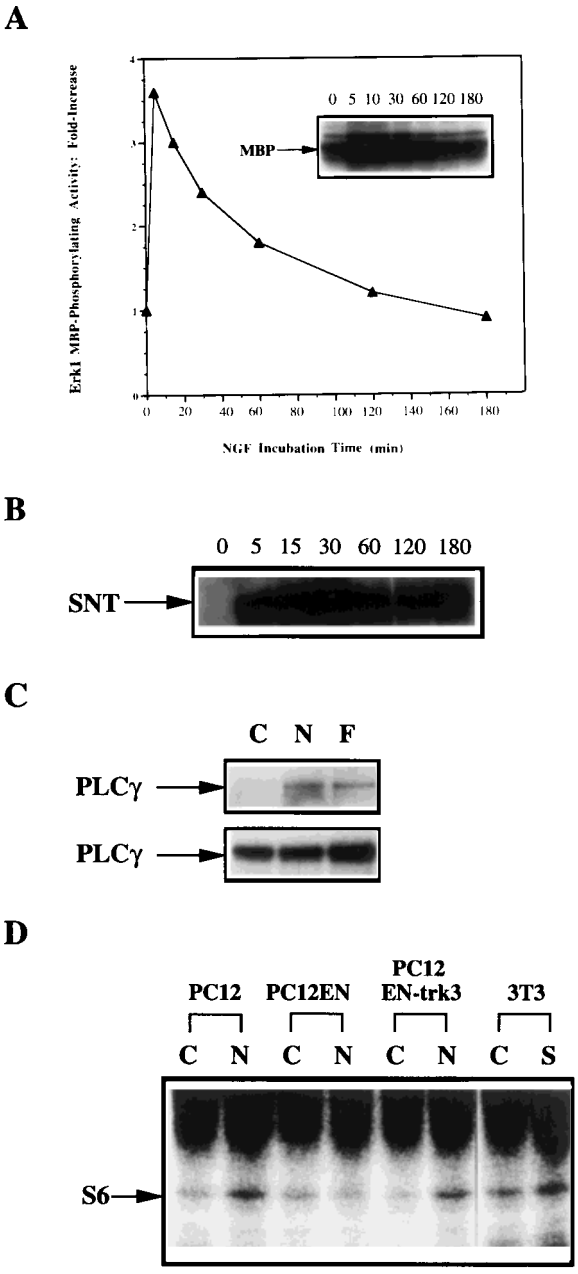

Fig. 6. Effect of NGF treatment on Erk activation and on SNT, PLCγ, and S6 phosphorylation in PC12EN-trk3 cells. **A:** Time course of NGF-induced Erk activity. p44-Erk1 was immunoprecipitated from PC12EN-trk3 cells treated with NGF at 100 ng/ml for various periods of time as indicated. The immunoprecipitates were incubated with myelin basic protein and [γ-^{32}P]ATP to assay kinase activity, as described in Methods. The fold increase in Erk activity measured by laser densitometry of the autoradiogram is presented in the inset. **B:** NGF-induced phosphorylation of SNT. The cells were treated for the indicated times with 100 ng/ml NGF; SNT proteins were precipitated with p13^{suc1}-agarose and probed with antiphosphotyrosine antibody. **C:** NGF- and FGF-induced phosphorylation of PLCγ. Cells were stimulated for 5 min with NGF (50 ng/ml). C: Control; N: NGF; F: FGF. **D:** NGF-induced phosphorylation of S6 protein by immunoprecipitates of p70^{S6k}. The cultures were treated for 30 min with NGF (100 ng/ml) or 15% serum. The lysates (500 μg protein) were immunoprecipitated with anti-p70^{S6k} antibody and submitted for the kinase assay. C: Control; N: NGF; S: Serum.

DISCUSSION

The biological effects of NGF appear to be mediated by multiple signal transduction pathways, initiated by NGF binding to both p140^{trk} and p75^{NGFR} receptors [Greene and Kaplan, 1995]. Despite the large body of data accumu-

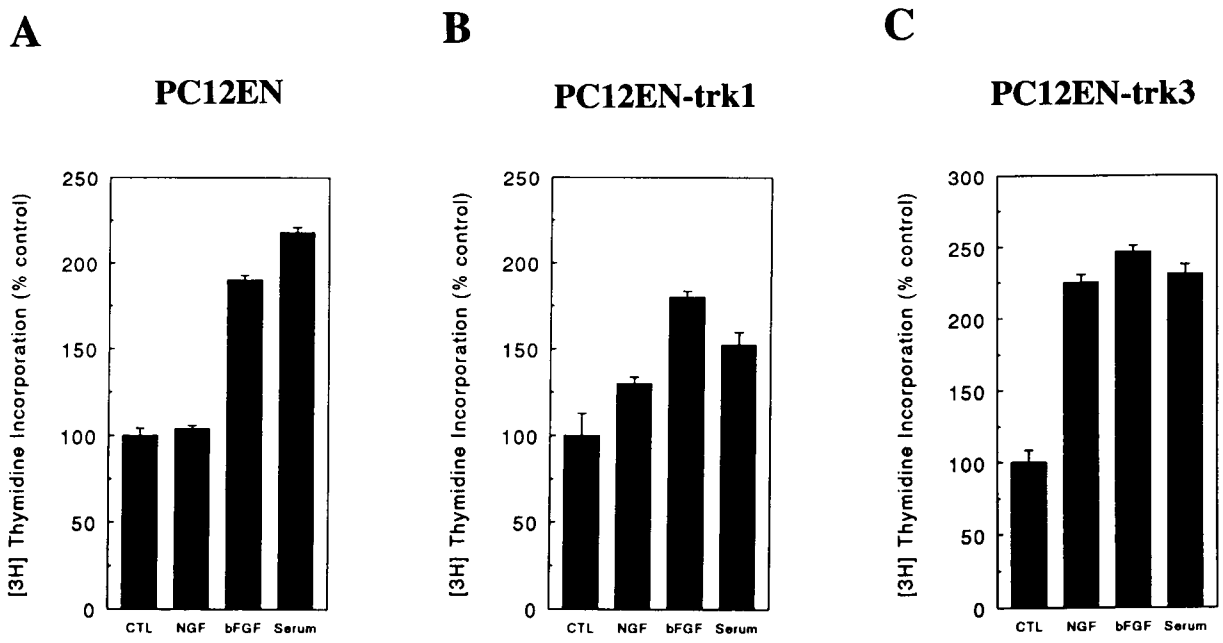

Fig. 7. NGF-induced stimulation of [^{3}H]thymidine incorporation and increase in cell number in PC12EN-trk transfectants. Parental PC12EN (**A**), PC12EN-trk1 (**B**), and PC12EN-trk3 (**C**) cells were cultured in 96-well plates and evaluated by [^{3}H]thymidine incorporation, as described in Methods. The cells were treated with 50 ng/ml of NGF, 10 ng/ml of bFGF, or 15% serum or were left untreated (CTL).

lated [Chao, 1992; Kaplan and Stephens, 1994; Greene and Kaplan, 1995], the precise roles of $p140^{trk}$ and $p75^{NGFR}$ in the signalling pathways of NGF have not been completely clarified. This is mainly due to the absence of a suitable neuronal model which lacks $p140^{trk}$ or $p75^{NGFR}$ receptors but still contains the signal transduction machinery for neuronal survival, proliferation, and differentiation. Most recent studies concerning the structure-function relationships and signal transduction by NGF receptors have been performed on either PC12, PC12-derived mutant cell lines, tumor-derived neurons, or nonneuronal cells (Table I). There are many reservations about the interpretation of results generated by transfection of $p140^{trk}$ receptors into these cell lines, because of the different levels of endogenous $p140^{trk}$ and/or $p75^{NGFR}$ receptors that are present. Therefore, it has been difficult to unambiguously define the role of the transfected $p140^{trk}$ in the biological responses generated [Greene and Kaplan, 1995]. Table I clearly emphasizes that NGF-induced biological effects differ between neuronal and nonneuronal cells and are dependent upon into which cells the $p140^{trk}$ receptors have been transfected. NGF actions range from promotion of cell survival, induction and acceleration of neurite outgrowth, and growth arrest to proliferation or cellular transformation (Table I).

Since very few neuronal cell lines are available for such studies, we attempted to transfect human $p140^{trk}$ receptors into a novel neuronal model, PC12EN hybrid cells [Rasouly et al., 1996]. After PC12EN cells were shown to be NGF-nonresponsive, due to the absence of NGF receptors, but responsive to both FGF and staurosporine [Rasouly et al., 1996], we asked whether the transfection of human $p140^{trk}$ receptors into these cells would restore NGF-induced signalling and biological effects. This cellular model is advantageous since it lacks endogenous $p140^{trk}$ and has exceedingly low levels of $p75^{NGFR}$, in contrast to the other neuronal models into which $p140^{trk}$ receptors have been transfected (Table I). We found that transfection of human $p140^{trk}$ receptor DNA into PC12EN cells generates functional, high-affinity $p140^{trk}$ receptors with coupled functional $p140^{trk}$-mediated Erk signalling. Interestingly, the major NGF-induced biological effect observed is an increase in DNA synthesis, suggesting that the expression of $p140^{trk}$ receptors confers a mitogenic effect. No evidence for neuronal differentiation was seen, even though the cells clearly retain the capacity for such differentia-

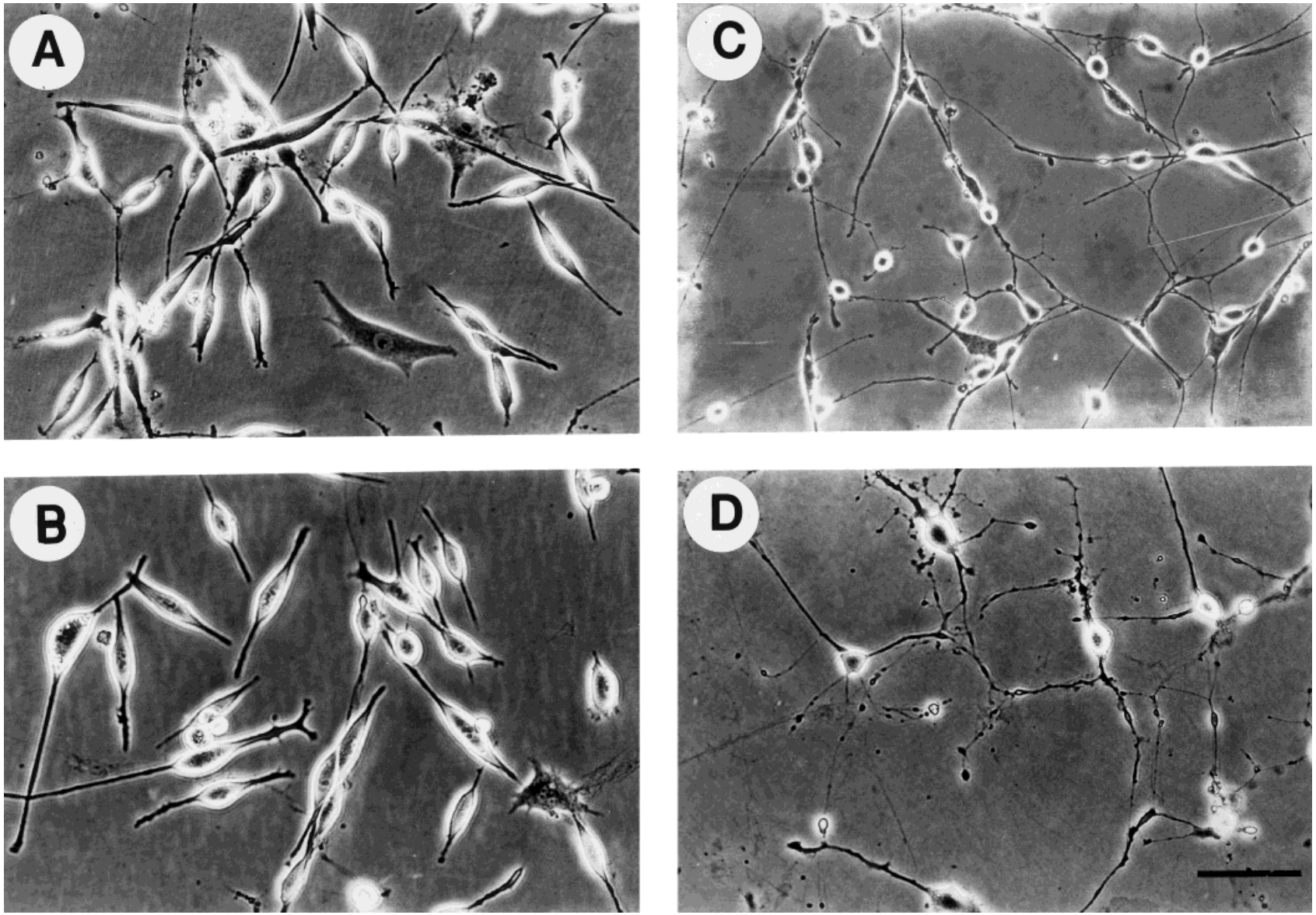

Fig. 8. Morphological responses of PC12EN-trk3 cells treated with NGF, cAMP analogue, or staurosporine. **A:** Control unstimulated cells. **B:** Treatment for 96 h with 50 ng/ml of NGF. **C:** Treatment for 18 h with 1 mM 8-CPT-cAMP. **D:** Treatment for 96 h with 50 nM staurosporine. The cells were grown on collagen- and polylysine-coated dishes. The photographs were taken by light microscopy at ×320, and the bar is 100 µm.

TABLE I. Effects of NGF on Various Cells After Transfection With p140^{trk}

Cell line	Endogenous levels[c] of p140^{trk}	Endogenous levels[c] of p75^{NGFR}	NGF-induced biological effects	Reference
Neuronal cells				
PC12[a]	High	High	Acceleration of NGF-induced neurite outgrowth	Hempstead et al., 1992
PC12nnr5[b]	Low	High	Promotion of cell survival	Loeb et al., 1994; Loeb and Greene, 1993
MAH sympathoadrenal cells[a]	Low	Low	Proliferation and neurite outgrowth, followed by mitotic arrest and maturation	Verdi et al., 1994
HTLA 230 neuroblastoma[a]	Low	Absent	Neurite outgrowth and growth arrest	Matsushima and Bogenmann, 1993
GT1 hypothalamic neurons[b]	Low	Low	Neurite outgrowth and delayed cell death	Zhou et al., 1994
C6-2B glioma cells[b]	Low	High	Increased length of process extension and increased thymidine incorporation	Colangelo et al., 1994
NIH3T3, E25 clone[a]	Absent	Absent	Strong mitogenic activity and morphological transformation	Cordon-Cardo et al., 1991
NIH3T3 clone[a]	Absent	Unknown	Growth arrest	Decker, 1995
MG 87 fibroblasts[b]	Unknown	Unknown	Increased survival	Barker et al., 1993
Pheochromocytoma/ endothelial hybrid				
PC12EN[a]	Absent	Low	Increased thymidine incorporation	Present study

[a]Human p140^{trk} cDNA.
[b]Rat p140^{trk} cDNA.
[c]Low, estimated at less than 1,000 per cell; high, estimated at more than 10,000 per cell.

tion in response to cAMP or staurosporine [Rasouly et al., 1996].

There were both similarities and differences in the NGF-induced effects on the neuronal cells listed in Table I and in the PC12EN-trk3 described in the present studies. In both cases, NGF induces rapid p140^{trk} tyrosine kinase activity which can be blocked by K-252a. However, other neuronal cells transfected with p140^{trk} initiate and maintain neurite outgrowth (Table I), which is not the case for PC12EN-trk–transfected cells. The failure of NGF to elicit neurite outgrowth in PC12EN-trk3 cells suggests that these cells lack either some element of the correct cellular environment (genetic programming) or the appropriate signal transduction machinery (certain trk substrates or regulation). The same pRcCMV-trk expression vector used in the present study was also used to stably transfect PC12nnr5 cells in our laboratory. The stable p140^{trk}-transfected PC12nnr5 clones obtained can respond to NGF stimulation with neurite outgrowth [Lazarovici et al., 1997]. The observation that PC12EN-trk3 cells do not elaborate neurites in response to NGF is surprising, since 1) the NGF-induced and p140^{trk}-mediated Ras-Erk pathway necessary for neurite outgrowth appears intact in PC12EN-trk3 cells and 2) SNT, also a candidate for a selective p140^{trk}-inducible signalling pathway, that, along with the Ras-Erk pathway, appears involved in the induction of neurite outgrowth [Greene and Kaplan, 1995], becomes rapidly phosphorylated on tyrosine in response to NGF in PC12EN-trk3 cells; the phosphorylation of SNT has been thought to be uniquely associated with cell differentiation [Rabin et al., 1993] but here appears to accompany cell division. It has been suggested that strong and prolonged activation of Erk is responsible for neurite outgrowth, while weak or transient activation such as observed in PC12EN-trk3 cells induces cell division [Qui and Green, 1992; Traverse et al., 1992]. Indeed, NGF used at a saturating concentration elicits a 2.3-fold increase in [^{3}H]thymidine incorporation. This

NGF-induced effect is similar to the mitogenic effect shown in $p140^{trk}$-transfected NIH3T3 cells and C6-2B rat glioma cells (Table I). However, in other $p140^{trk}$-transfected NIH3T3 and human neuroblastoma cells, NGF inhibits cell proliferation (Table I). These findings suggest that the cell type and not the activation of $p140^{trk}$-mediated signal transduction alone is responsible for this discrepancy.

One possible explanation for the transient activation of Erks and the failure of NGF to induce morphological differentiation in PC12EN-trk3 is the very low level of $p75^{NGFR}$ receptors expressed on these cells. Whereas $p140^{trk}$ alone can mediate most NGF responses in cultured cells independently of $p75^{NGFR}$ [Greene and Kaplan, 1995], the $p75^{NGFR}$ receptors have at least two clear effects on $p140^{trk}$ function: 1) they alter the ligand binding specificity of $p140^{trk}$ [Benedetti et al., 1993], a fact inferred from the results of this study demonstrating competition between NT-3, NT-4, and NGF, and 2) they enhance NGF-induced $p140^{trk}$ tyrosine kinase activity [Verdi et al., 1994], which might affect the strength and/or duration of Erk activity. The consequence of cotransfection of $p140^{trk}$ and $p75^{NGFR}$ in PC12EN cells on NGF-induced biological effects may provide some insights into the true role of $p75^{NGFR}$.

Consistent with $p140^{trk}$ transfection studies involving both neuronal and nonneuronal cells (Table I), we found that human $p140^{trk}$ transfection in PC12EN neuronal hybrids generates slow, high-affinity NGF receptors in the absence of endogenous $p140^{trk}$ receptors. Future work should focus on the signalling pathways, both Ras-dependent and Ras-independent, activated by $p140^{trk}$ receptors. Experimental attempts to increase the strength and/or duration of NGF-induced Erk activation, perhaps by inhibition of key phosphatases, cotransfection of $p75^{NGFR}$ receptors, identification of missing $p140^{trk}$ substrates, or transfection of mutant $p140^{trk}$ receptors into PC12EN cells, should provide important insights into the mechanisms of NGF-induced proliferation and/or differentiation.

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