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Transfection with *trk* Restores "Slow" NGF Binding, Efficient NGF Uptake, and Multiple NGF Responses to NGF-nonresponsive PC12 Cell Mutants

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NGF binds to and activates the protein tyrosine kinase gp140^{prototrk}. Expression of this receptor is required for at least some responses to NGF. Three outstanding issues are addressed in the present work. First, we determined whether expression of gp140^{prototrk} is required for all neuronal NGF responses. Second, we examined the role of gp140^{prototrk} in NGF binding and internalization. Third, we addressed the utility of NGF-nonresponsive PC12nnr5 cells for study of the NGF mechanism. In contrast to wild-type PC12 cells, PC12nnr5 cells do not express endogenous gp140^{prototrk}. We therefore asked whether they possess other defects that compromise NGF signaling pathways. To answer these questions, we transfected PC12nnr5 cells with a cDNA encoding full-length human gp140^{prototrk} and isolated cell lines permanently expressing the receptor. Introduction of *trk* rescued all of the many and varied NGF responses assessed, including enhanced protein tyrosine phosphorylation, induction of immediate-early and neural-specific genes, neurite outgrowth and regeneration, maintenance of survival in serum-free medium, and stimulation of AChE activity. In contrast to PC12nnr5 cells, the *trk*-transfected lines also bind and internalize NGF with wild-type PC12 cell characteristics. These findings indicate that gp140^{prototrk} is required for many, if not all, responses of neuronal cells to NGF and is necessary for proper NGF binding and internalization. Additionally, as no signaling defect other than the absence of *trk* expression was revealed in PC12nnr5 cells, this work supports the utility of this line for genetic dissection of the NGF mechanism of action.

[Key words: NGF, PC12 cells, PC12nnr cells, Trk, signal transduction, NGF receptor, NGF binding, NGF internalization, tyrosine kinase]

NGF mediates the differentiation and survival of discrete neuronal populations in both the CNS and PNS (Martinez et al., 1985; Levi-Montalcini, 1987; Barde, 1989; Cattaneo and McKay, 1990; Ruit et al., 1990). The rat pheochromocytoma cell line PC12 (Greene and Tischler, 1976) has been used extensively as an *in vitro* model to study NGF signaling (Levi et al., 1988). Two distinct NGF receptors, designated p75 and gp140^{prototrk} (Trk), have been identified, and cDNAs encoding these have been cloned (Chao et al., 1986; Radeke et al., 1987; Martin-Zanca et al., 1989). The discovery that NGF binds to and stimulates the tyrosine kinase activity of the gp140^{prototrk} (Bothwell, 1991; Kaplan et al., 1991a,b; Klein et al., 1991) has focused attention on its role in NGF signal transduction. Transfection experiments have demonstrated that gp140^{prototrk} mediates NGF-stimulated fibroblast transformation (Cordon-Cardo et al., 1991) and oocyte maturation (Nebreda et al., 1991) as well as neurite outgrowth and cell survival (Loeb et al., 1991). The latter results were achieved by transfection of mutant PC12-derived cell lines (designated PC12nnr) selected for their inability to respond to NGF (Green et al., 1986). These cells express p75 but not gp140^{prototrk} (Green and Greene, 1986; Loeb et al., 1991).

The present study addresses several outstanding issues concerning NGF receptors and PC12nnr cells. First is the question of whether expression of gp140^{prototrk} mediates all neuronal responses to NGF. Our initial study with transiently transfected cells permitted only a few responses to be assessed (Loeb et al., 1991). The *trk* family is diverse (Bothwell, 1991), and multiple forms of some family members have been detected (Klein et al., 1990; Middlemas et al., 1991). It is not inconceivable that *trk* isoforms each mediate only a subset of responses, and that activation of multiple different Trk receptors is required for complete responsiveness. Furthermore, although the role of p75 is not entirely clear, it has been postulated that this receptor alone can mediate certain responses to NGF (Seilheimer and Schachner, 1987; Represa et al., 1991; Saad et al., 1991).

A second issue concerns the role of gp140^{prototrk} in NGF binding and uptake. Two classes of NGF binding sites have been detected on PC12 and other NGF-responsive cells (Sutter et al., 1979; Landreth and Shooter, 1980; Meakin and Shooter, 1992). There is disagreement regarding the definition of these classes, but it is clear that one class is distinguished by a "slow" rate of NGF dissociation, while the other undergoes "fast" dissociation (Schechter and Bothwell, 1981). PC12nnr cells appear to express predominantly "fast" receptors, suggesting that they lack an essential component for "slow" binding (Green et al., 1986). When expressed in fibroblasts, gp140^{prototrk} appears to show properties of "slow" NGF receptors (Meakin et al., 1992), suggesting that introduction of this molecule into the neuronal background of PC12nnr cells would restore "slow" NGF binding. In addition to surface binding, another important component of the NGF mechanism appears to be uptake and retrograde

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transport of the factor by neurons (Calissano and Shelanski, 1980; Levi et al., 1980; Korsching and Thoenen, 1983a; Palmetier et al., 1984; Johnson et al., 1987). PC12 cells efficiently internalize NGF, while PC12nnr cells show greatly reduced NGF uptake (Green et al., 1986). These observations raise the possibility that gp140^{prototrk} may be essential for efficient NGF internalization.

A third issue is the utility of PC12nnr cells for studying the mechanism of NGF signaling. If the only defect in the NGF signaling pathway in PC12nnr cells is the absence of Trk expression, then they may be used genetically to dissect functional domains of this receptor in a neuronal background. However, if the cells contain additional mutations, such analysis would be compromised.

To address these questions, we have established PC12nnr cell lines that have been permanently transfected with human gp140^{prototrk}. These lines have been screened for a wide variety of NGF responses and signaling pathways and for their abilities to bind and internalize NGF.

Materials and Methods

Cells and growth factors. PC12 cells were grown on collagen-coated tissue culture dishes in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum as previously described (Greene and Tischler, 1976). NGF was purified from male mouse submaxillary glands as previously described (Mobley et al., 1976). For assay of neurite outgrowth, cultures were maintained in RPMI 1640 medium with 1% heat-inactivated horse serum and 100 ng/ml NGF. Regeneration assays were performed as described (Greene, 1977). For each culture condition, 300 randomly chosen individual cells were scored under phase-contrast optics. A neurite was defined as a cellular process greater than two cell body diameters in length. Cells assayed for survival in serum-free medium were cultured in RPMI 1640 medium without supplement.

Transfection and selection of permanently transfected clones. PC12nnr5 cells were transfected with pDM115 (Kaplan et al., 1991a) by electroporation as previously described (Greene et al., 1991). Briefly, cells from a confluent 150 mm dish were washed free of serum and electroporated with 40 μg of plasmid DNA using a Bethesda Research Labs (Gaithersburg, MD) Cell-Porator electroporation apparatus. After a 30 min recovery on ice, the cells were plated on two 100 mm dishes and incubated with complete medium. Selection was initiated 7 d after transfection by including 500 μg/ml geneticin in the culture medium. After approximately 4–6 weeks, surviving colonies were picked, expanded, and screened for responsiveness to NGF.

Immunoprecipitation, Western blotting, and kinase assays. Routinely, immunoprecipitations were performed using confluent 100 mm dishes of cells. Cultures were treated with 100 ng/ml NGF for 5 min unless otherwise stated. Cultures were harvested in lysis buffer (LB) as previously described (Loeb et al., 1992) and extracted on ice for at least 5 min. After centrifugation at 190,000 × *g* for 15 min at 4°C, supernatants were normalized for protein concentration by a dye-binding assay (Bethesda Research Labs, Gaithersburg, MD) using bovine serum albumin (BSA) as a standard. Lysates were immunoprecipitated with anti-Trk antiserum 203 (Hempstead et al., 1992) at 1:200 for 2 hr at 4°C. Protein A-Sepharose (Pharmacia, Piscataway, NJ) prepared according to Upstate Biotechnology (Lake Placid, NY) was added for a further hour. Pellets were collected by centrifugation and washed with TNTG (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, and 5% glycerol), boiled in Laemmli sample buffer, and subjected to SDS-PAGE (Laemmli, 1970).

Immunoprecipitates used for kinase assays were not boiled. Instead this material was incubated in kinase buffer (50 mM Tris, pH 7.5, 10 mM $MnCl_2$, 5 mM $MgCl_2$, 5 μM ATP, 5 μCi [γ-^{32}P]ATP, and 15 μg myelin basic protein [MBP]) for 10 min at 37°C. Reactions were stopped by adding 4× sample buffer (Laemmli, 1970) and boiling. Results were quantified by subjecting the reaction mixture to SDS-PAGE and autoradiography.

Material for Western blotting without prior immunoprecipitation was prepared from untreated or NGF-treated cell cultures harvested in LB without detergent. After homogenization in a glass homogenizer, cellular material was centrifuged as described above, and the supernatant mixed with 4× sample buffer (Laemmli, 1970), boiled, and subjected to SDS-PAGE.

Protein was transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) by electroblotting (Loeb et al., 1992). Nonspecific binding sites on nitrocellulose membranes were blocked with 3% BSA in PBS for 2 hr at room temperature. Membranes were then incubated with the appropriate antiserum diluted as indicated in the text in 3% BSA in PBS overnight at 4°C. Membranes were washed with 0.2% NP-40 in PBS for 1 hr, and immunoreactive bands were revealed with ^{125}I-Protein A (ICN, Irvine, CA) and autoradiography using Kodak X-AR film. Results were quantified by scanning densitometry using an Apple OneScan driven by OPTOTO (Light Source, Inc., Greenbrae, CA) and IMAGE (version 1.43, National Institutes of Health) software run on an Apple Quadra model 700 computer.

Isolation of RNA and Northern blotting. RNA was prepared from cultured cells by the method of Chomczynski and Sacchi (1987). RNA was separated by electrophoresis through 1% agarose gels as previously described (Loeb et al., 1991). After transfer to nitrocellulose, blots were incubated with probes labeled with [α-^{32}P]dCTP using a random primed labeling kit (Boehringer Mannheim, Indianapolis, IN). RNA species were then detected by autoradiography. Results were quantified by scanning densitometry as described above.

NGF labeling and binding studies. NGF was labeled by lactoperoxidase according to the protocol of Vale and Shooter (1985). Unincorporated iodine was separated from labeled NGF using a disposable gel filtration column (Speedy Column, Pierce, Rockford, IL) followed by centrifugation through a Centriflo CF50 filter (Amicon, Danvers, MA) as suggested by Vale and Shooter (1985). NGF was labeled to a specific activity of 14 cpm/pg and used within 2 weeks of labeling.

Dissociation studies were performed on confluent 35 mm dishes of cells. Cells were incubated with 5.55 ng/ml (~210 pM) NGF in RPMI 1640 medium with 1% heat-inactivated horse serum for 3 min at 37°C. After transfer to ice, cultures were washed rapidly six times with ice-cold PBS and incubated with 1 μg/ml unlabeled NGF in HEPES (*N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid])-buffered Krebs–Ringer solution (pH 7.4) with 1% horse serum. At various times cultures were assayed for released (soluble) ^{125}I-NGF, surface-bound (as determined by acid stripping; Bernd and Greene, 1984) ^{125}I-NGF, and internalized ^{125}I-NGF (that which was not removed by acid treatment). Cells were acid stripped for 5 min on ice by incubation with 0.2 *N* acetic acid containing 0.5 M NaCl. This protocol has been shown to completely remove surface-bound NGF (Bernd and Greene, 1984).

NGF uptake was measured by incubating 35 mm dishes of cells with 5.55 ng/ml ^{125}I-NGF in RPMI 1640 with 1% horse serum for various times at 37°C. Cells were transferred to ice and rapidly washed six times with ice-cold PBS, and surface and internalized ^{125}I-NGF were determined as described above. All results were quantified using a Clinigamma 1272 gamma counter (Pharmacia–LKB, Gaithersburg, MD).

Acetylcholinesterase assay. Acetylcholinesterase activity was determined as previously described (Greene and Rukenstein, 1981).

Results

Expression of gp140^{prototrk} in stably transfected PC12nnr5 cells. PC12nnr5 cells (Green et al., 1986) were transfected by electroporation with a plasmid (pDM115; Kaplan et al., 1991a) containing the full-length cDNA encoding human gp140^{prototrk} and the gene for neomycin resistance, both under the control of a retroviral LTR. After selection with geneticin, colonies were isolated, expanded, and examined for *trk* expression. Since previous work showed that transient expression of *trk* renders PC12nnr5 cells capable of NGF-promoted neurite outgrowth (Loeb et al., 1991), this response was used as an initial screen. Of 30 colonies isolated, nine (30%) expressed *trk* as judged by their ability to grow neurites in response to NGF. Of these, the three with morphologies most closely resembling that of PC12nnr5 and PC12 cells (in the absence and presence of NGF; designated nnr5T8, nnr5T9, and nnr5T14) were chosen for further evaluation (Fig. 1). One line derived from the transfection that was resistant to geneticin but did not grow neurites (designated nnr5T1) was carried as a control.

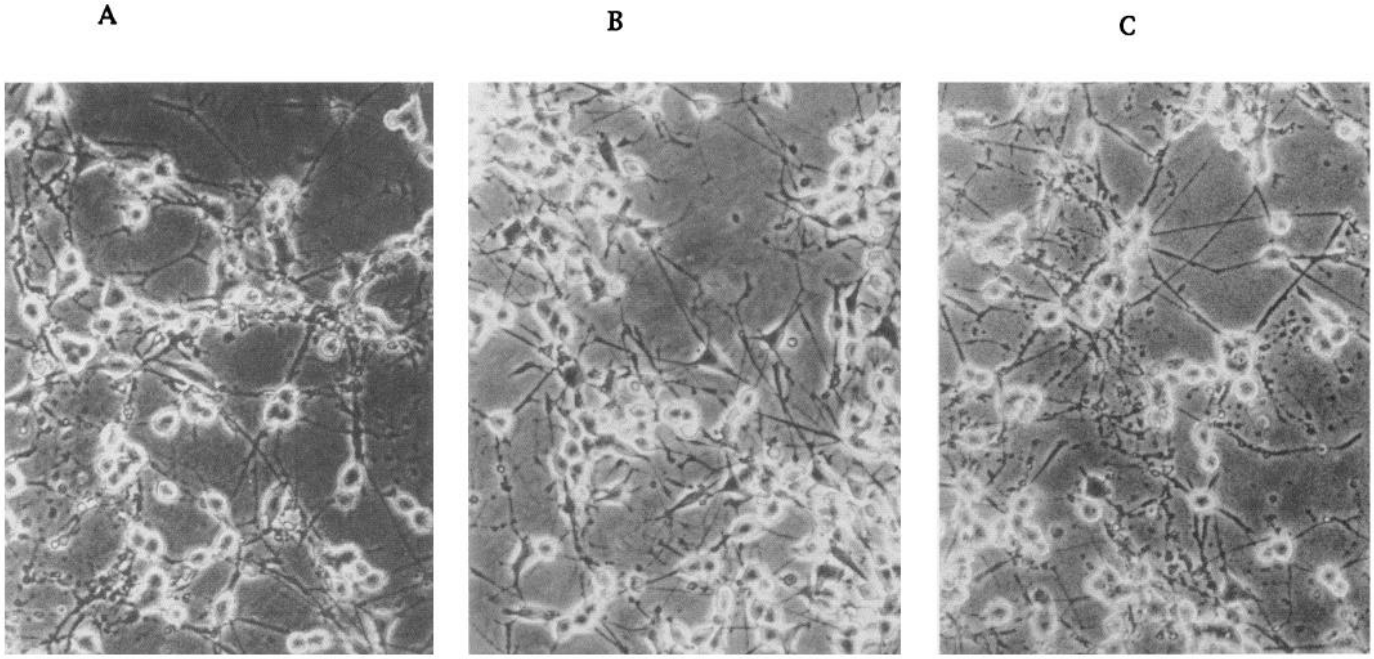

Figure 1. Morphological response of *trk*-transfected PC12nnr5 cell lines to NGF: nnr5T8 (*A*), nnr5T9 (*B*), and nnr5T14 (*C*) cells treated for 7 d with 100 ng/ml NGF. Photographs were taken under phase-contrast optics.

To confirm expression of the human *trk* gene and gp140^{prototrk} in the geneticin-resistant cells, Northern and Western blot analyses were performed. Northern blot analysis using a *trk*-specific probe (Kaplan et al., 1991a) revealed that—like PC12 cells—nnr5T8, nnr5T9, and nnr5T14 cells express *trk* mRNA (Fig. 2*A*). The *trk* mRNA expressed by the transfected cell lines migrates more slowly than the corresponding mRNA from PC12 cells, consistent with expression of the exogenous gene and not with reactivation of endogenous *trk* (Fig. 2*A*). PC12nnr5 and nnr5T1 cells do not express detectable *trk* message, consistent with their inability to grow neurites in response to NGF (Fig. 2*A*). All three *trk*-expressing transfectants overexpress the transgene relative to wild-type PC12 cells. Densitometric analysis of the Northern blot shown in Figure 2*A* indicated that the range of *trk* overexpression varies from about 10-(nnr5T14) to 20-fold (nnr5T9). Similar results were obtained in additional Northern blot experiments.

To detect gp140^{prototrk} expression, PC12, nnr5T8, nnr5T9, and nnr5T14 cells were lysed in a Triton X-100–containing buffer, and the solubilized material was immunoprecipitated with anti-Trk antiserum 203 (Hempstead et al., 1992). SDS-PAGE of the immunoprecipitates, followed by Western blotting with the same antiserum, revealed that all four lines express gp140^{prototrk} (Fig. 2*B*), and that, consistent with the Northern blot results, the transfected lines overexpress the protein relative to PC12 cells. No gp140^{prototrk} expression was detected in nnr5T1 or PC12nnr5 immunoprecipitates (data not shown). Densitometric analysis of the Western blot shown in Figure 2*B* indicated that the range of gp140^{prototrk} overexpression varied from about 6- (nnr5T9) to 10-fold (nnr5T8). In addition, immunoprecipitation of lysates from untreated and NGF-treated (100 ng/ml for 5 min) PC12, nnr5T8, and nnr5T14 cells with anti-Trk antiserum 203 followed by SDS-PAGE and Western blotting with anti-phosphotyrosine revealed an NGF-dependent tyrosine phosphorylation of gp140^{prototrk} in each cell line (Fig. 2*C*). Similar results were obtained with nnr5T9 cells (data not shown). In contrast to the other lines, nnr5T8 cells exhibit a low level of tyrosine-phosphorylated gp140^{prototrk} in the absence of NGF (Fig. 2*C*). This presumably reflects ligand-independent receptor activation caused by the overexpression of this protein. However, even though nnr5T8 cells contain a basal level of tyrosine-phosphorylated (and presumably active) gp140^{prototrk}, NGF is still required for these cells to elaborate neurites.

Expression of gp140^{prototrk} rescues wild-type NGF binding and uptake characteristics. To test the effect of Trk expression on binding and uptake of NGF by PC12nnr5 transfectants, the rate of dissociation of radiolabeled NGF from the cell surface and the rate of factor internalization were examined. For dissociation studies, PC12, PC12nnr5, nnr5T1, and nnr5T14 cells were incubated with 5.55 ng/ml (~210 pM) iodinated NGF for 3 min at 37°C to allow cell surface binding with minimal internalization. Cells were then shifted to 0°C, the labeled NGF rapidly washed away, and the medium replaced with one containing 1 μg/ml unlabeled NGF. As described in Materials and Methods, labeled NGF in the medium, on the cell surface, and inside the cell was measured at various times after washout and chase. During the 3 min binding period, the cell lines bound different amounts of labeled NGF. While PC12nnr5 and nnr5T1 cells bound approximately 60–90 fmol NGF/mg cell protein this time, PC12 and nnr5T14 cells bound approximately threefold more. This is consistent with the expression of more receptors on the surface of gp140^{prototrk}-expressing cells than on the nonexpressing cells. Previous work also demonstrated fewer binding sites on the surface of PC12nnr5 cells as compared with parental PC12 cells (Green et al., 1986). In agreement with published results (Schechter and Bothwell, 1981; Eveleth and Bradshaw, 1988), the cell lines exhibited biphasic NGF dissociation kinetics. PC12 and nnr5T14 cells had relatively small amounts of rapidly dissociating NGF (losing 10–20% of the total surface-bound NGF after 10 min) while the PC12nnr5 and nnr5T1 cells

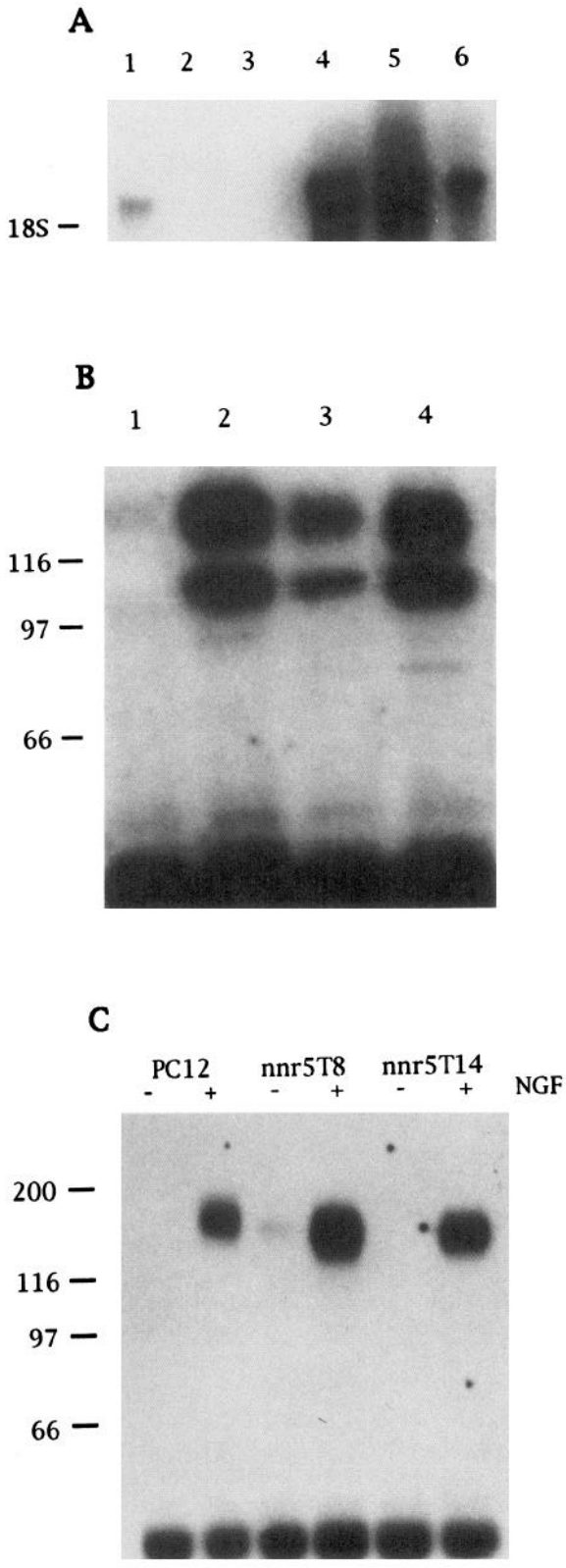

Figure 2. Expression of *trk* mRNA and functional gp140^{prototrk} in permanently transfected cell lines. *A*, Total cellular RNA was isolated from PC12 (*lane 1*), PC12nnr5 (*lane 2*), nnr5T1 (*lane 3*), nnr5T8 (*lane 4*), nnr5T9 (*lane 5*), and nnr5T14 (*lane 6*) cells and subjected to Northern blot analysis (20 μg/lane) with a *trk*-specific probe (pDM97; Kaplan et al., 1991a). Equal loading of RNA in each lane was verified by ethidium bromide staining. The position of the 18S rRNA is indicated. *B*, Trk protein was immunoprecipitated from lysates of PC12 (*lane 1*), nnr5T8 (*lane 2*), nnr5T9 (*lane 3*), and nnr5T14 (*lane 4*) cells with anti-Trk antiserum 203. After SDS-PAGE, proteins were subjected to Western blotting analysis with the same antiserum. The relative migration of molecular weight standards ($M_r \times 10^{-3}$) is indicated. The *lower band* of each doublet in *lanes 2–4* is partially glycosylated gp140^{prototrk} (Martin-Zanca et al., 1989). *C*, PC12, nnr5T8, and nnr5T14 cells, treated with (+) or without (−) 100 ng/ml NGF for 5 min, were lysed and subjected to immunoprecipitation with anti-Trk antiserum 203. After SDS-PAGE, proteins were subjected to Western blotting analysis with anti-phosphotyrosine. Relative migration of molecular weight standards ($M_r \times 10^{-3}$) is indicated.

lost from 45–65% of their surface-bound NGF in the same time period (Fig. 3*A*).

Substrate-attached PC12 cells show a marked time-dependent intracellular accumulation of NGF, reflected by an increasing ratio of internal to surface-bound factor. In contrast, PC12nnr5 cells do not show a similar accumulation or high internal-to-surface (I/S) ratio (Green et al., 1986). To examine the role of gp140^{prototrk} expression in this phenomenon, internalization of NGF by PC12, PC12nnr5, nnr5T1, and nnr5T14 cells was examined. After incubation for 3 or 60 min at 37°C with 5.55 ng/ml (~210 pM) radiolabeled NGF, cell surface and internalized NGF was measured. PC12nnr5 and nnr5T1 cells had an I/S ratio of approximately 1 after 60 min, while PC12 cells had a ratio of 2–3 and nnr5T14 cells had a ratio of approximately 4 (Fig. 3*B*). The amounts of ligand bound to the surfaces of the different cell lines after 60 min were approximately equivalent (Fig. 3*B*).

Expression of gp140^{prototrk} rescues rapid tyrosine phosphorylations and association with cytoplasmic serine/threonine kinases. Among the earliest responses of PC12 cells to NGF is the rapid tyrosine phosphorylation of cytoplasmic proteins (Maher, 1988, 1989). To determine if this response is rescued in PC12nnr5 cells by transfection with *trk*, PC12 and nnr5T14 cells, untreated or treated with 100 ng/ml NGF for 5 min, were homogenized in an isotonic buffer, and soluble proteins were subjected to SDS-PAGE followed by Western blotting with an anti-phosphotyrosine antibody. The major NGF-stimulated tyrosine phosphorylations are bands of 44 and 42 kDa (probably representing ERK1 and ERK2; Boulton et al., 1991) and approximately 100 kDa (Fig. 4*A*). All of these were seen in both the PC12 cell lysates and the nnr5T14 lysates. Similar results were obtained with nnr5T8 cells (data not shown). No NGF-dependent tyrosine phosphorylation was seen with PC12nnr5 cells (data not shown).

NGF induces association between gp140^{prototrk} and at least two different serine/threonine kinases in PC12 cells, ERK1 (Loeb et al., 1992), and a non-ERK kinase activity (Volonté et al., 1993). To determine if these complexes are formed in PC12nnr5 cells transfected with *trk*, thus strengthening the hypothesis that the sole defect underlying the lack of NGF responsiveness in these cells is the absence of *trk* expression, their presence in nnr5T9 cells was assessed. Untreated cells and cells treated for 5 min with 100 ng/ml NGF were lysed in a Triton-containing buffer, and the lysates were immunoprecipitated with antiserum 43, which recognizes gp140^{prototrk} (Klein et al., 1991a). Precipitated material was subjected to Western blotting using antiserum X837, which recognizes ERK1 and ERK2 (Boulton and Cobb, 1991). An NGF-dependent association between gp140^{prototrk} and ERK1 was detected in PC12 cells and in nnr5T9 cells (Fig. 4*B*). No

such complex was detected in PC12nnr5 cells or in nnr5T1 cells (data not shown) regardless of NGF treatment.

NGF also stimulates a serine/threonine kinase activity that is associated with gp140^{prototrk}, that uses MBP as an *in vitro* substrate, but that is not ERK1 (Volonté et al., 1993). To determine if this association and activation also occur in *trk*-transfected PC12nnr5 cells, PC12, PC12nnr5, nnr5T1, and nnr5T9 cells, untreated or treated with 100 ng/ml NGF for 5 min, were lysed in a Triton-containing buffer, immunoprecipitated with anti-Trk antiserum 443 (which does not precipitate the gp140^{prototrk}/ERK1 complex; Loeb et al., 1992), and assayed for protein kinase activity using MBP as a substrate. The basal level of kinase activity toward MBP is higher in anti-Trk immunoprecipitates derived from PC12 and nnr5T9 cells than in anti-Trk immunoprecipitates from PC12nnr5 or nnr5T1 cells (Fig. 4*C*). Additionally, NGF treatment augmented this protein kinase activity in the immunoprecipitates from PC12 and nnr5T9 cells but not in the material immunoprecipitated from PC12nnr5 or nnr5T1 cells (Fig. 4*C*).

Expression of gp140^{prototrk} rescues NGF effects on gene expression and purine analog–defined signaling pathways. Through the use of purine analog protein kinase inhibitors (Zinn et al., 1988; Volonté et al., 1989), three divergent NGF signaling pathways within PC12 cells have been identified (Volonté et al., 1989; Batistatou et al., 1992). This identification is based in part on the ability of these compounds to inhibit differentially the induction of immediate-early genes by NGF (Batistatou et al., 1992). To determine if all three of these signaling pathways are functional in PC12nnr5 cells transfected with *trk,* induction of genes representative of each pathway were assessed, including c-*fos* (Greenberg et al., 1985; Milbrandt, 1986), c-*jun* (Wu et al., 1989), TIS1 (also referred to as NGF-IB; Kujubu et al., 1987; Milbrandt, 1988), and TIS8 (also referred to as NGF-IA; Kujubu et al., 1987; Milbrandt, 1987). Total cellular RNA was isolated from PC12 and nnr5T14 cells treated for various times with 100 ng/ml NGF, and Northern blot analyses using probes for these genes were performed. All four genes were induced by NGF in both PC12 and nnr5T14 cells with similar rapid and transient kinetics (an example is shown in Fig. 5*A*). Induction of these genes by NGF is not detected in PC12nnr5 cells (Altin et al., 1991). The extent of induction of the genes differed somewhat between PC12 and nnr5T14 cells, but the significance of this finding is not clear.

Besides stimulating the expression of immediate-early genes, NGF induces the later expression of several additional genes in PC12 cells. Examples include the protease transin (Machida et al., 1989), the intermediate filament protein peripherin (Leonard et al., 1988), the microtubule-associated protein MAP1.2/1b/5 (Lewis et al., 1986), the cell surface NILE/L1 glycoprotein (Salton et al., 1983; Sajovic et al., 1986), and the p75 NGF receptor (Miller et al., 1991). Total RNA was isolated from PC12, PC12nnr5, nnr5T1, and nnr5T14 cells, untreated or treated with 100 ng/ml NGF for 3 or 7 d. Northern blot analysis of these samples revealed an NGF-dependent expression of transin mRNA in PC12 and nnr5T14 cells but not in PC12nnr5 or nnr5T1 cells (Fig. 5*B*). All four cell lines express basal levels of mRNA encoding NILE/L1 glycoprotein, MAP1.2/1b/5, p75, and peripherin in the absence of NGF; however, NGF enhances the expression of each of these genes in PC12 and nnr5T14 cells, but not in PC12nnr5 or nnr5T1 cells (Table 1). Another gene whose expression is augmented by NGF in PC12 cells is thymosin β4 (Leonard et al., 1987). However, unlike the other late

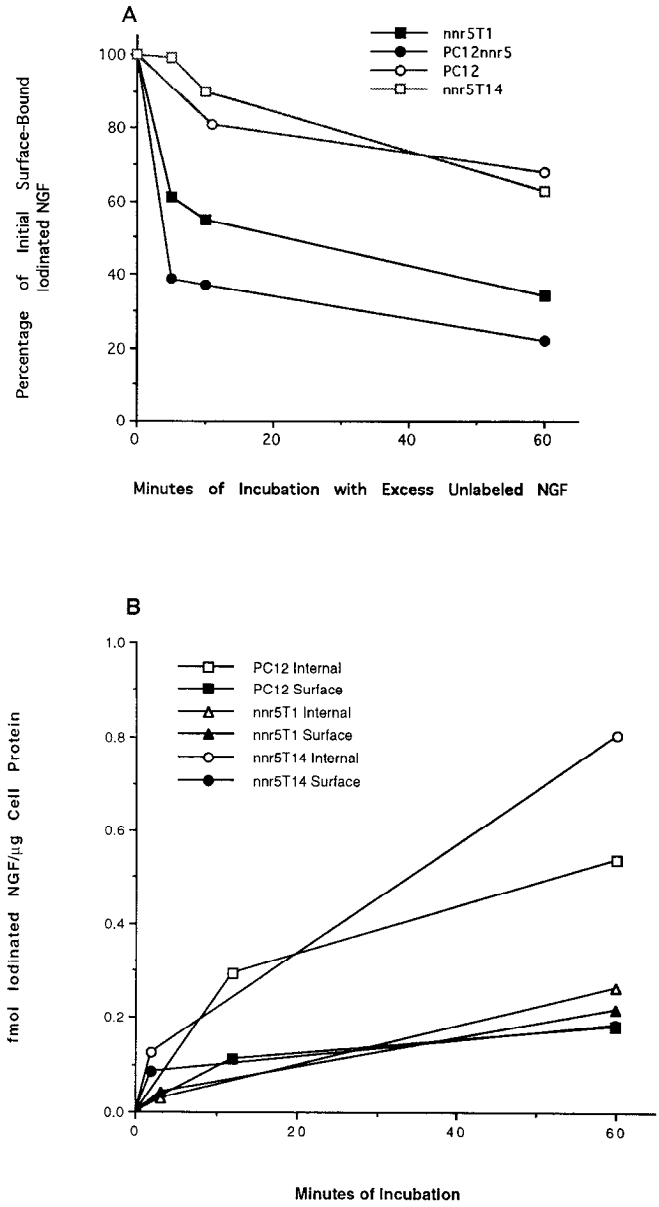

Figure 3. NGF binding, dissociation, and internalization. *A,* Relative rate of dissociation of ^{125}I-NGF from the surfaces of PC12, PC12nnr5, nnr5T1, and nnr5T14 cells, assayed as described in Materials and Methods. Briefly, replicate cultures were incubated with ~210 pM ^{125}I-NGF for 3 min at 37°C and, after washout of unbound material, were incubated on ice in the presence of 40 nM unlabeled NGF. "Acid stripping" was used to determine surface-bound NGF. Data are expressed as the amount of ^{125}I-NGF associated with the cell surface at the designated time, relative to the amount associated with the surface at the initiation of the incubation with unlabeled ligand. *B,* Amounts of ^{125}I-NGF internalized or associated with the surface of PC12, nnr5T1, and nnr5T14 cells after various times of incubation. Determination of internalized and surface-bound NGF was made as described in Materials and Methods. Briefly, replicate cultures were incubated with ~210 pM ^{125}I-NGF for the indicated times at 37°C, washed free of unbound material, and assessed for surface-bound and internalized ^{125}I-NGF by "acid stripping." Data represent the average of determinations on duplicate cultures. Variation between duplicates was <10%.

genes examined, thymosin β4 is not expressed in PC12nnr5 cells, or in the lines derived by transfection with *trk,* regardless of the presence or absence of NGF (Fig. 5*C*).

Modulation of AChE activity by NGF is rescued in PC12nnr5

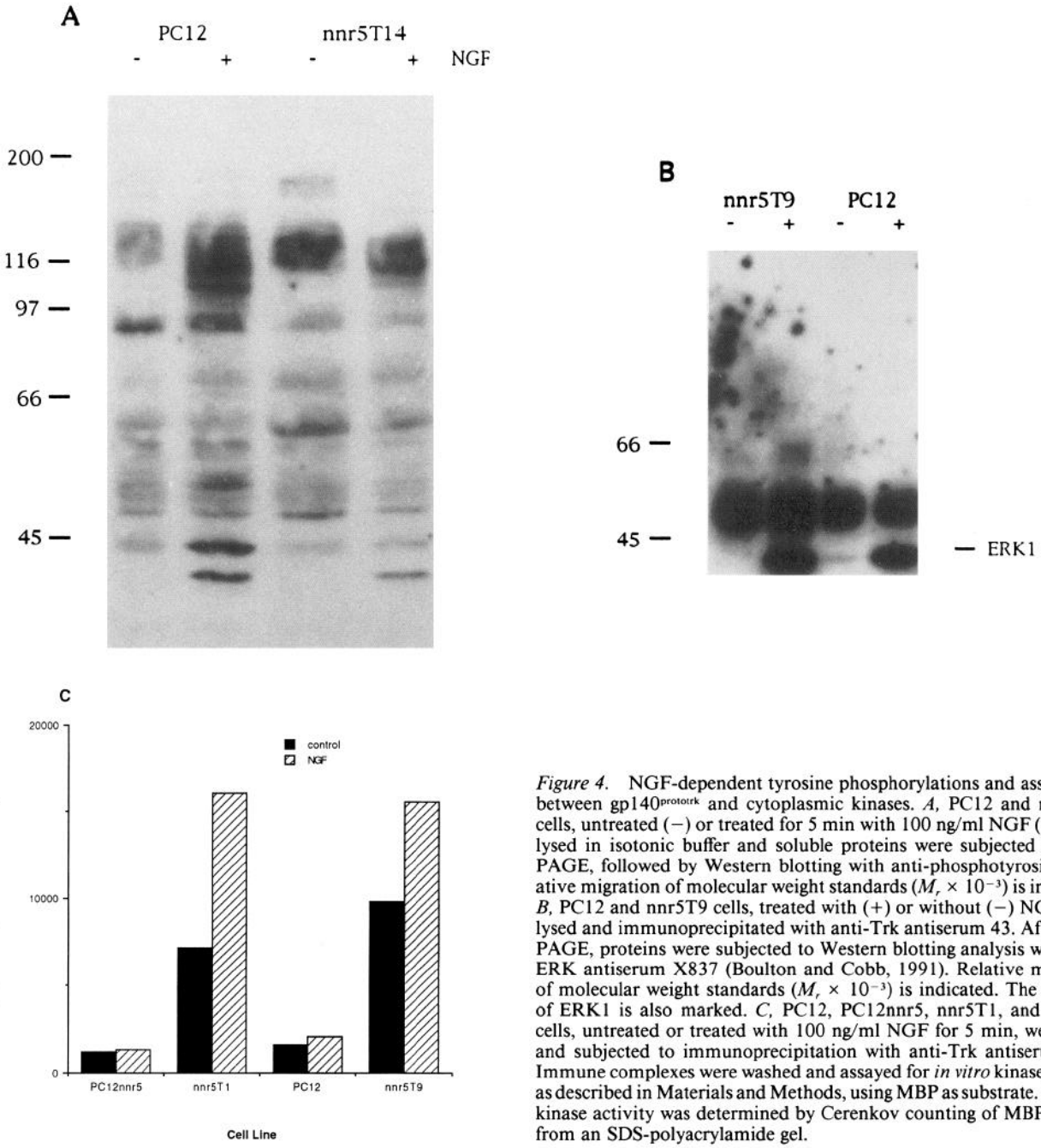

Figure 4. NGF-dependent tyrosine phosphorylations and association between gp140^{prototrk} and cytoplasmic kinases. *A,* PC12 and nnr5T14 cells, untreated (−) or treated for 5 min with 100 ng/ml NGF (+), were lysed in isotonic buffer and soluble proteins were subjected to SDS-PAGE, followed by Western blotting with anti-phosphotyrosine. Relative migration of molecular weight standards ($M_r \times 10^{-3}$) is indicated. *B,* PC12 and nnr5T9 cells, treated with (+) or without (−) NGF, were lysed and immunoprecipitated with anti-Trk antiserum 43. After SDS-PAGE, proteins were subjected to Western blotting analysis with anti-ERK antiserum X837 (Boulton and Cobb, 1991). Relative migration of molecular weight standards ($M_r \times 10^{-3}$) is indicated. The position of ERK1 is also marked. *C,* PC12, PC12nnr5, nnr5T1, and nnr5T9 cells, untreated or treated with 100 ng/ml NGF for 5 min, were lysed and subjected to immunoprecipitation with anti-Trk antiserum 443. Immune complexes were washed and assayed for *in vitro* kinase activity as described in Materials and Methods, using MBP as substrate. Relative kinase activity was determined by Cerenkov counting of MBP excised from an SDS-polyacrylamide gel.

cell lines permanently expressing gp140^{prototrk}. The neuronal character of NGF-treated PC12 cells is not limited to neurite outgrowth and gene expression. This factor also modulates the expression and activity of enzymes involved in the synthesis and degradation of neurotransmitters (reviewed in Levi and Alemà, 1991). One such enzyme is AChE (Rieger et al., 1980; Greene and Rukenstein, 1981). Accordingly, AChE activity was measured in PC12, PC12nnr5, nnr5T1, and nnr5T14 cells treated with 100 ng/ml NGF for 3 d and compared to activity measured in cells untreated with NGF. While AChE activity in PC12nnr5 and nnr5T1 cells was unaffected by NGF, PC12 and nnr5T14 cells exhibited a two- to sixfold increase in AChE activity in response to this factor (Fig. 6).

Expression of gp140^{prototrk} rescues NGF-dependent neurite outgrowth and regeneration in transfected cell lines. The most obvious phenotypic effect of NGF on PC12 cells is neurite outgrowth. The initial elaboration of neurites by PC12 cells in response to NGF takes place over a time course of days (Greene and Tischler, 1976), while in contrast, the regeneration of neurites by NGF-pretreated cells occurs over a course of hours (Greene, 1977; Burstein and Greene, 1978). The initial rate of neurite outgrowth was measured in transfected cell lines. Each *trk*-expressing cell line, but not PC12nnr5 or nnr5T1 cells, exhibits NGF-dependent neurite outgrowth (Fig. 7*A*). In each case, the rate of appearance of neurites was increased in the Trk-overexpressing cell lines as compared to PC12 cells. The *trk*-transfected cell lines were also able rapidly to regenerate neurites to an extent similar to PC12 cells (compare Fig. 7*B*).

Table 1. Regulation of late gene expression by NGF

Cell line	Periph-erin	MAP1	Transin	Thymosin β4	NILE/L1	p75
PC12	2.4	3	∞	2	4.2	1.8
nnr5T14	2	3.3	∞	–	2.1	4.3

Cell cultures were treated with or without 100 ng/ml NGF for 7 d. Total RNA was isolated and separated on a 1% agarose gel with 3.7% formaldehyde. After transfer to nitrocellulose, blots were probed with sequences specific to the indicated genes. Equal loading of RNA in each lane was verified by ethidium bromide staining. Numbers indicate fold induction by NGF in 7 d. – indicates not detected. ∞ indicates that signal was detected only after NGF treatment.

gp140^{prototrk} expression restores NGF-dependent survival in serum-free medium in transfected cell lines. Another characteristic response of PC12 cells is the ability of NGF to promote their survival in serum-free medium. PC12 and PC12nnr cells die in serum-free medium, but the former are rescued by NGF (Greene, 1978; Green et al., 1986; Rukenstein et al., 1991). The capacity of the *trk*-transfected cell lines to survive in serum-free medium was therefore assessed. NGF supported the survival of PC12, nnr5T8, and nnr5T14 cells in serum-free medium but was unable to do so for PC12nnr5 or nnr5T1 cells (Fig. 8). nnr5T9 cells showed spontaneous survival in serum-free medium, so assessment of NGF effects under these conditions was not informative (data not shown).

Discussion

We have used the PC12nnr5 cell line to examine the role of gp140^{prototrk} in NGF-mediated neuronal responses and in NGF binding and uptake. PC12nnr5 cells were derived from parental PC12 cells by chemical mutagenesis and selected for lack of NGF-promoted neurite outgrowth and serum-free survival (Green et al., 1986). Work from a number of laboratories has confirmed that a variety of other NGF responses are absent from these cells, and that their capacities to bind and internalize NGF are altered (Green et al., 1986; Kasaian and Neet, 1990; Altin et al., 1991; Eveleth and Bradshaw, 1992). It has also been demonstrated that these cells do not express the gp140^{prototrk} NGF receptor (Loeb et al., 1991). Consequently, three issues were considered in this work. Is Trk expression required for all of the many neuronal responses to NGF? Does this receptor play a role in "slow" NGF binding and in NGF internalization? Do PC12nnr5 cells possess additional defects in the NGF signaling pathway? To this end we generated and characterized three PC12nnr5 lines that are permanently transfected with a cDNA encoding human gp140^{prototrk}. These lines have enabled us to examine a wide range of phenotypic responses not accessible in transiently transfected cells, and to do so in a neuronal cell type.

Trk and NGF responsiveness. We examined a wide range of NGF responses that varied with respect to their dependence on macromolecular synthesis, time and duration of expression, specificity for NGF, proximity to NGF binding in the signaling pathway, and sensitivity to purine analog protein kinase inhibitors. Without exception, every response examined is absent in the PC12nnr5 cells and is rescued by introduction of a cDNA encoding gp140^{prototrk}. This strongly supports the hypothesis that expression of gp140^{prototrk} is required for all aspects of NGF

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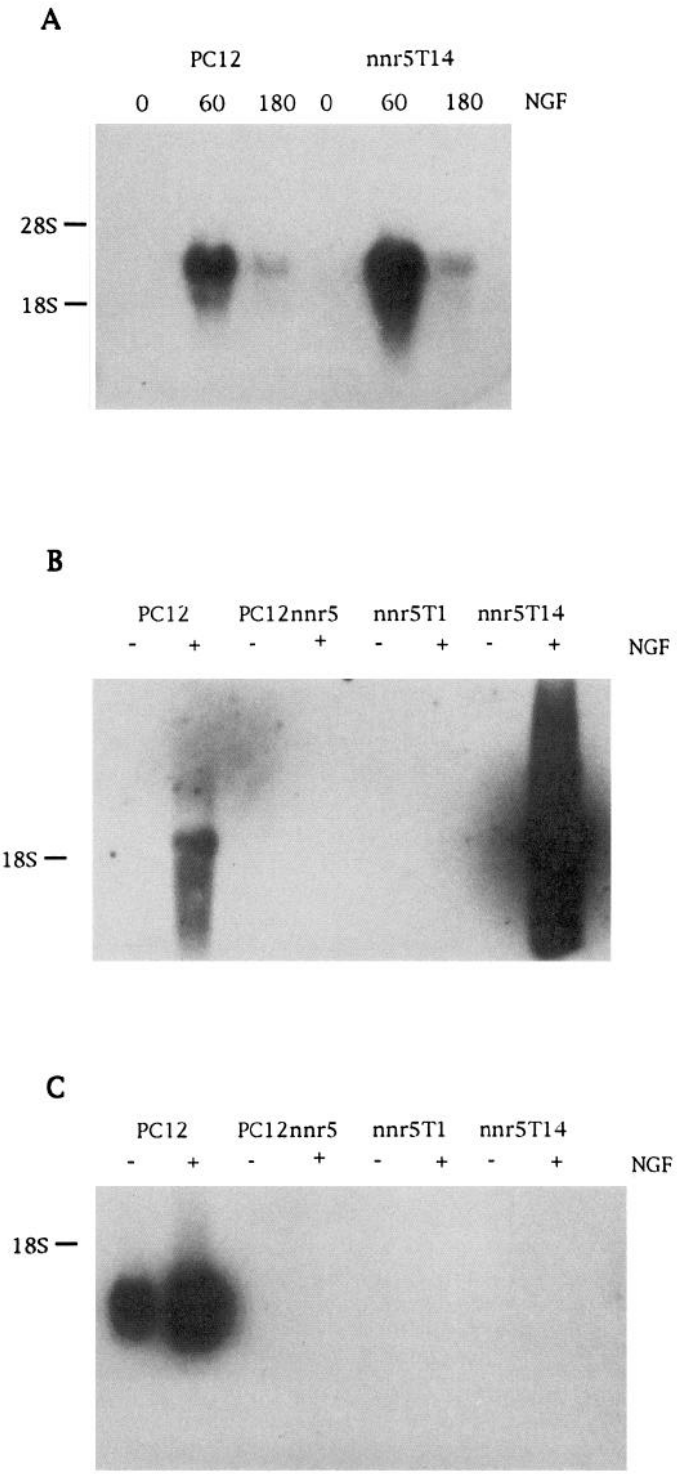

Figure 5. NGF-dependent gene expression. *A*, PC12 and nnr5T14 cells were treated for the indicated number of minutes with 100 ng/ml NGF prior to isolation of total cellular RNA. Northern blotting analysis (20 μg RNA/lane) was performed with a probe specific for c-*fos* (Curran et al., 1982). The positions of the 18S and 28S rRNAs are indicated. *B*, Total cellular RNA was isolated from PC12, PC12nnr5, nnr5T1, and nnr5T14 cells, untreated (–) or treated for 7 d with 100 ng/ml NGF (+). Northern blotting (20 μg/lane) was performed with a probe specific for transin (Matrisian et al., 1985). The position of the 18S rRNA is indicated. Blot is overexposed to emphasize the lack of signal in PC12nnr5 and nnr5T1 cells. *C*, RNA (20 μg/lane) prepared as above was subjected to Northern blotting analysis with a probe specific for thymosin β4 (Leonard et al., 1987). The position of the 18S rRNA is indicated. In each case, equal loading of RNA in each lane was verified by ethidium bromide staining.

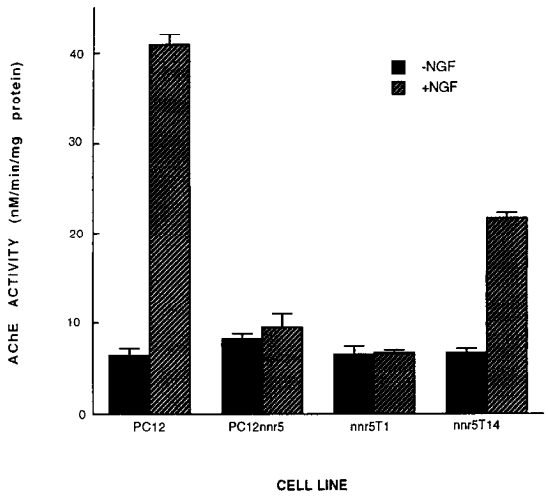

Figure 6. Induction of AChE activity. Cells were treated with or without 100 ng/ml NGF for 5 d prior to assay for AChE activity as described in Materials and Methods. Data are presented as nanomoles of ACh hydrolyzed/min/mg protein and represent the mean ± SEM (n = 3).

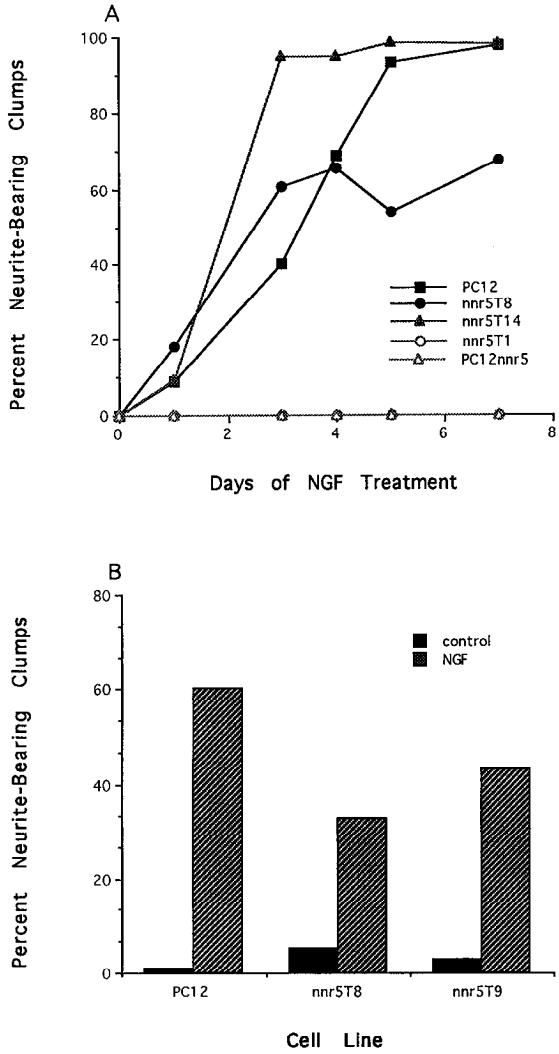

Figure 7. Neurite outgrowth and regeneration. *A*, PC12, PC12nnr5, nnr5T1, and nnr5T14 cells were treated with 100 ng/ml NGF, and the proportions of neurite-bearing cell clumps were counted each day as described in Materials and Methods. *B*, PC12, nnr5T8, and nnr5T14 cells were treated with NGF for 7 d. Cells were then triturated to remove neurites and replated in fresh medium, with or without 100 ng/ml NGF. After 18 hr, the percentage of neurite-bearing cell clumps was determined as described in Materials and Methods.

responsiveness and that all responses to NGF are mediated by this receptor (either alone or in combination with p75). Thus, it does not appear necessary to invoke additional receptors that mediate portions of the NGF response.

Our results also address the possibility that p75 functions to transduce an NGF signal independently from gp140^{prototrk}. We considerably extended the range of NGF actions previously assayed in PC12nnr5 cells, including regulation of a number of "late" genes. We confirmed that although these cells express p75 they do not respond to NGF. Because all classes of response could be rescued by introduction of a single gene, it is exceedingly unlikely these cells possess additional defects that independently compromise p75-mediated signaling. We therefore suggest that p75 alone does not mediate functional responses to NGF in PC12 cells. This is consistent with the findings of Ibáñez et al. (1992) and Drinkwater et al. (1991) that mutant NGF molecules incapable of interacting with p75 still mediate survival and neurite outgrowth in neuronal cultures.

Recently, p75 has been postulated to mediate signaling by glycosylphosphatidylinositol turnover in cochleovestibular ganglia (Represa et al., 1991). It will be of interest to determine whether this pathway exists in PC12 and PC12nnr5 cells. If such a pathway is stimulated in the absence of gp140^{prototrk}, this would indicate that this signaling mechanism is not required for the responses we considered or that it is involved in responses we did not assess.

Additional studies have suggested that p75 may mediate NGF-enhanced expression of several surface glycoproteins in glial cells that appear to lack gp140^{prototrk} (Seilheimer and Schachner, 1987; Saad et al., 1991). We found here that NGF regulation of at least one of these, the NILE/L1 glycoprotein, requires gp140^{prototrk}. Thus, there may be a fundamental difference between neuronal and glial cells with respect to NGF signaling pathways, or glial cells may indeed express an NGF receptor in addition to p75.

NGF binding and uptake. Because of the difficulties involving interpretation of experiments designed to detect "high" and "low" affinity NGF receptors in PC12 and PC12nnr cells (Green et al., 1986; Kasaian and Neet, 1990; Eveleth and Bradshaw, 1992), we chose to assess whether expression of gp140^{prototrk} would affect the dissociation rate of iodinated NGF from the cell surface. We confirmed past observations that at low concentrations of external NGF, more than 80% of the surface binding to wild-type PC12 cells appeared to be to "slow" receptors. In contrast, 45–65% of surface-bound NGF was rapidly released from the surface of PC12nnr5 cells. Transfection with *trk* reversed this situation such that most of the binding appeared to be to "slow" receptors. These observations indicate that gp140^{prototrk} restores normal levels of "slow" receptor binding and thus that this molecule plays an important role in "slow" receptor expression. This is consistent with recent findings that gp140^{prototrk} expressed in fibroblasts exhibits the characteristics of "slow" NGF receptors (Meakin et al., 1992).

The significance of an apparent fraction of "slow" receptors on PC12nnr5 cells in the absence of gp140^{prototrk} is unclear. This does not appear to be due to internalized NGF; past work has demonstrated that the acid-stripping technique we used here

successfully distinguishes externally bound from internalized NGF (Bernd and Greene, 1984). One possibility is that p75 can generate at least some "slow" receptor binding when expressed in the absence of gp140^{prototrk}. Kahle and Hertel (1992) demonstrated incomplete dissociation of NGF from the surfaces of glial cells expressing only p75 when the initial binding was performed at 37°C, as was ours. An alternative is that a portion of the p75-bound NGF is in a "sequestered" state that is not truly internalized, but that exchanges slowly with free NGF in the medium (Eveleth and Bradshaw, 1988). In either case, the presence of gp140^{prototrk} appears to affect the proportion of bound NGF that undergoes this "slow" dissociation.

A second parameter we analyzed is internalization of NGF. Neurons appear to take up NGF with high efficiency and to transport it to their cell bodies (Yankner and Shooter, 1979; Korsching and Thoenen, 1983a; Palmetier et al., 1984). Interference with this transport abolishes the actions of target-derived NGF (Chen et al., 1977; Johnson, 1978). This has led to the suggestion that internalization and retrograde transport are important components of the NGF mechanism (Korsching and Thoenen, 1983b). PC12 cells show efficient uptake of low concentrations of the factor such that by 1 hr of incubation, the I/S NGF ratio is 3–4 (Green et al., 1986; present results). In contrast, PC12nnr5 cells take up NGF with relatively low efficiency such that the I/S ratio after 1 hr is approximately 1 (Green et al., 1986; present results). We observed that reintroduction of gp140^{prototrk} yielded wild-type uptake characteristics. This indicates that the Trk receptor plays a necessary role in efficient NGF internalization. Our data do not, however, distinguish whether p75, though not sufficient, is also required for such uptake.

PC12nnr5 cells as models for analyzing functional domains of Trk receptors. If the only aspect of the NGF signaling mechanism that is aberrant in PC12nnr5 cells is the absence of *trk* expression, then this cell system should provide a powerful tool for analyzing functional domains of gp140^{prototrk} and other Trk receptors. Evaluation of this issue was among the reasons we tested our PC12nnr5 transfectants for a wide variety of responses and signaling pathways as well as for NGF dissociation and internalization. Our findings support the adequacy of this cell system for functional studies of *trk* domains.

One difference detected here between PC12 cells and PC12nnr5-derived lines is the absence of thymosin β4 expression in the latter. This polypeptide binds G-actin, preventing microfilament assembly (Weber et al., 1992), and overexpression of thymosin β4 has been shown to regulate actin filament dynamics in living cells (Sanders et al., 1992). Because actin dynamics are likely to be involved in process outgrowth (Lankford and Letourneau, 1989; Sobue and Kanda, 1989; Paves et al., 1990), and because thymosin β4 expression is regulated by NGF (Leonard et al., 1987), a functional role for this protein in neuronal differentiation might be envisioned. However, despite their lack of thymosin β4 expression, nnr5T14 cells extend neurites in response to NGF with no obvious difference from those in wild-type PC12 cells. Thus, it appears that thymosin β4 expression is not required for neurite outgrowth. We cannot exclude the possibility that another protein, perhaps the closely related thymosin β10 (Lin and Morrison-Bogorad, 1991), substitutes for thymosin β4 in these cells.

In summary, our findings indicate that expression of gp140^{prototrk} is required for many, if not all, responses of neuronal cells to NGF, that gp140^{prototrk} plays a role in "slow" NGF binding and efficient internalization of the factor, and that the PC12nnr5 cell line is a valid model for functional studies of Trk neurotrophin receptors.

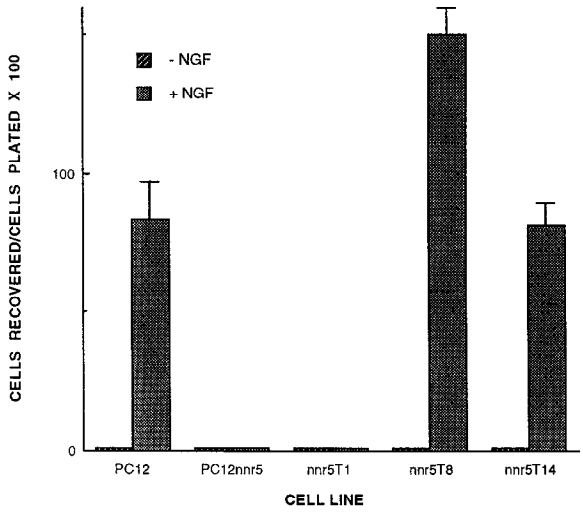

Figure 8. Survival in serum-free medium. PC12, PC12nnr5, nnr5T1, nnr5T8, and nnr5T14 cells were washed free of serum and incubated in the presence or absence of 50 ng/ml NGF for 7 d. After this period, the number of surviving cells was determined in each culture. Results are expressed relative to number of cells initially plated (10^5) and represent means ± SEM (n = 3).

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