Trk Receptors Use Redundant Signal Transduction Pathways Involving SHC and PLC-γ1 to Mediate NGF Responses

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Summary

In response to NGF, the Trk receptor tyrosine kinase forms a complex with SHC, a protein that couples receptor tyrosine kinases to p21. Complex formation between Trk and SHC, SHC tyrosine phosphorylation, and association of SHC with Grb2 were mediated by auto-phosphorylation at Y490 in Trk (NPQYFSD). To determine the role of SHC and other Trk substrates in NGF signaling, Trk receptors with mutations in Y490 and Y785 (the PLC-γ1 association site) were introduced into PC12nrr5 cells. NGF treatment of PC12nrr5 cells expressing Trk with mutations in either substrate-binding site resulted in normal neurite outgrowth and Erk1 activity and tyrosine phosphorylation. However, PC12nrr5 cells expressing Trk with mutations at both sites failed to stably extend neurites and efficiently induce Erk1 activity and tyrosine phosphorylation in response to NGF. We postulate that Trk receptors can activate Erk1 by either SHC- or PLC-γ1-dependent signaling pathways. These results suggest a model whereby Trk receptors utilize at least partially redundant signal transduction pathways to mediate NGF responses.

Introduction

Recent investigations of the mechanisms of signal transduction used by receptor tyrosine kinases (RTKs) have provided important clues as to how proliferative and differentiative signals are propagated. A striking finding has been the discovery that ligand-activated receptors form complexes with various intracellular proteins, many of which have potent growth- and differentiation-promoting activities. These proteins include regulators of phosphatidylinositol metabolism, including phospholipase C-γ1 (PLC-γ1) and phosphatidylinositol 3-kinase (PI-3 kinase); proteins involved in controlling p21 activity, such as rasGAP, Grb2, SHC, and the guanine nucleotide exchange factors; the serine/threonine kinase Raf-1; the tyrosine kinases c-Src, c-Yes, and c-Fyn; and the phosphotyrosine phosphatase Syp/SH-PTP2, and Nck (reviewed in Cantley et al., 1991; Lowenstein et al., 1992; W. Li et al., 1992; Feng et al., 1993; Vogel et al., 1993; Park and Rhee, 1992; Meisenhelder and Hunter, 1992; Pelicci et al., 1992). Complex formation in most cases is mediated by phosphotyrosine residues in specific sequence contexts on receptors and by SH2 (Src homology 2) domains on the intracellular substrates (reviewed in Cantley et al., 1991; Pawson and Gish, 1992). The recognition of particular phosphotyrosine residues on RTKs by the substrate is required for substrate tyrosine phosphorylation, activation of substrate activity, and in certain cases, growth factor-induced mitogenesis.

Most of the experiments on RTK-mediated signal transduction have examined the role of receptor substrates in mitogenic systems. Several RTKs, including the Trk/nerve growth factor (NGF) receptor, can induce cell differentiation and the cessation of cell division. NGF and the NGF family of neurotrophins regulate the survival and differentiation of neurons in the peripheral and central nervous systems (Barde, 1989; Thoenen, 1991; Hérit et al., 1992). NGF also promotes the differentiation of the rat pheochromocytoma tumor cell line, PC12, into cells resembling sympathetic neurons (Greene and Tischler, 1976). Following several days of exposure to NGF, PC12 cells undergo transcriptionally dependent acquisition of a neuronal phenotype characterized by the extension of neurites and development of electrical excitability (Greene and Tischler, 1976, 1980). Since NGF stimulation of Trk leads to differentiation of neurons while it induces proliferation of fibroblasts, critical elements of the Trk signaling pathway may vary from cell to cell, indicating the importance of studying this receptor in a neuronal context.

Although considerable information is known about the morphological effects of NGF on neurons, the precise mechanisms of signal transduction used by this factor are not known. The binding of NGF to Trk is the initial event in the neuronal differentiation of PC12 cells (Kaplan et al., 1991a; Klein et al., 1991). This interaction stimulates Trk homodimer formation and the activation of the intrinsic tyrosine kinase activity of Trk (Kaplan et al., 1991b; Klein et al., 1991; Jing et al., 1992). These events initiate a signaling cascade involving the phosphorylation of intracellular proteins on tyrosine residues (Kaplan et al., 1991b; Maher et al., 1988; Vetter et al., 1991). These signals are in turn propagated to other messengers, ultimately leading to differentiation and the cessation of growth (reviewed in Haleygou et al., 1991; Chao, 1992b). Within minutes of NGF binding, Trk interacts with three identified intracellular substrates, PLC-γ1, PI-3 kinase, and Erk1 (Vetter et al., 1991; Soltoff et al., 1992; Loeb et al., 1992). Studies utilizing dominant inhibitory ras mutants suggest that several signaling proteins act downstream of Trk and PLC-γ1, including the serine/threo-
nine kinases Erk1, Erk2, p90RSK, and the Raf-1 family of serine/threonine kinases (Thomas et al., 1992; Wood et al., 1992; Robbins et al., 1992; Troppmair et al., 1992). These experiments also indicate that p21ras functions downstream of Trk and PLC-γ1 and upstream of Raf, Erk1, Erk2, and p90RSK in the NGF signal transduction pathway. SNT, a recently identified specific target of factors with neurotrophic activity, acts upstream or in a signal transduction pathway parallel to that of p21ras (Rabin et al., 1993). In PC12 cells, SNT is phosphorylated on tyrosine within 1 min of NGF treatment, but not after exposure to the mitogen epidermal growth factor (EGF) (Rabin et al., 1993).

The SHC proteins are additional potential mediators of NGF signals. SHC proteins of 48, 53, and 68 kd have been previously reported to be phosphorylated on tyrosine in response to EGF, insulin, platelet-derived growth factor, and NGF (Pellicci et al., 1992; Pronk et al., 1993; Rozakis-Adcock et al., 1992). In addition, SHC associates with ligand-activated EGF receptors through a single SH2 domain encoded in the SHC protein (Pellicci et al., 1992). SHC also contains a glycine- and proline-rich region that is related to a domain in α1 collagen (Pellicci et al., 1992). In addition to RTKs, SHC also associates with Grb2, which couples receptors to a regulator of p21ras activity, the SOS guanine nucleotide exchange protein (Rozakis-Adcock et al., 1992; 1993; Egan et al., 1993; Li et al., 1993; Gale et al., 1993; Olivier et al., 1993; Simon et al., 1993; Lowenstein et al., 1992), and coinmunoprecipitates with SOS from lysates of v-src-transformed rat fibroblasts (Egan et al., 1993). SHC has both transforming and differentiation potential: overexpression of SHC in NIH 3T3 cells and PC12 cells induces transformation and neurite outgrowth, respectively (Pellicci et al., 1992; Rozakis-Adcock et al., 1992).

As a means of determining the significance of Trk substrates in NGF-mediated signaling events, we have generated cell lines expressing mutant Trk receptors that lack tyrosines which are potential interaction sites for intracellular substrates. These mutants were expressed in a PC12 cell variant (PC12nnr5) that lacks expression of endogenous Trk and fails to respond to NGF (Green and Greene, 1986; Loeb et al., 1991). Expression of Trk in PC12nnr5 cells restores NGF responsiveness (Loeb and Greene, 1993).

In contrast to several other RTKs, Trk contains only 2 tyrosines in the cytoplasmic region of the receptor outside of the kinase domain. These are at positions 490 and 785 of human Trk. Tyrosine 785 is a site for PLC-γ1 interactions (Obermeier et al., 1993; Loeb et al., 1994). We have shown that this site is an autophosphorylation site of Trk in vitro and is required for PLC-γ1 tyrosine phosphorylation in vivo (Loeb et al., 1994). However, PC12nnr5 cell lines expressing Trk receptors containing mutations in the PLC-γ1 interaction site showed no observable defects in morphological responses to NGF (Loeb et al., 1994). These results suggest that either the PLC-γ1 interaction with Trk is dispensable for many NGF responses, or other signaling proteins can functionally substitute for PLC-γ1 in PC12nnr5 cells.

In this study, we identify tyrosine 490 (Y490) as a site of Trk phosphorylation. The consequences of mutating Y490 of Trk were then examined, either alone or in combination with Y785. We show that SHC proteins associate with NGF-activated Trk receptors, and that phosphorylation at Y490 is required for both SHC tyrosine phosphorylation and receptor association. Whereas PC12nnr5 cells expressing Trk receptors containing mutations at either Y490 or Y785 showed no apparent defects in NGF-induced signaling, receptors containing mutations in both of these sites failed to exhibit stable NGF-mediated neurite outgrowth and Erk1 tyrosine phosphorylation responses. These results suggest that RTKs can utilize partially redun-
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dant signaling pathways to regulate cellular differentiation.

Results

Tyrosine Phosphorylation of SHC in PC12 Cells Treated with NGF and EGF, but Not FGF
NGF has been reported to stimulate the tyrosine phosphorylation of SHC in PC12 cells (Rozakis-Adcock et al., 1992). We further analyzed SHC tyrosine phosphorylation in PC12 cells treated with growth or differentiation factors. SHC proteins of 48, 53, and to a much lesser extent, 68 kD, were phosphorylated on tyrosine in PC12 cells treated for 5 min with NGF or the mitogen EGF, but not with basic fibroblast growth factor (FGF) (Figure 1, lanes 1–6). Although FGF stimulated the tyrosine phosphorylation of PLC-γ1, Erk1, and SNT, SHC tyrosine phosphorylation was not observed for up to 24 hr of FGF treatment (data not shown). NGF and EGF, but not FGF, also induced a shift in the electrophoretic mobility of all forms of SHC (Figure 1, lanes 7–10). SHC tyrosine phosphorylation was observed in other neurotrophin-responsive cell lines, including neurotrophin-3 (NT-3)-treated human neuroblastoma cells expressing TrkB and NGF-treated mouse NIH 3T3 cells transfected with trk (data not shown).

SHC Tyrosine Phosphorylation Requires Functional Trk and Is Prolonged When Trk Is Overexpressed
To investigate whether the level of Trk activity in PC12 cells affects SHC tyrosine phosphorylation, the phosphorylation state of SHC was examined in pc12nnr5 cells expressing kinase-inactive Trk or in PC12 cells overexpressing Trk (trk–PC12). Overexpression of Trk in PC12 cells results in a rapid acceleration of neurite outgrowth in response to NGF and the sustained tyrosine phosphorylation of Trk and Trk substrates (Hempstead et al., 1992). trk–PC12 cells express approximately 20-fold more Trk than wild-type PC12 cells (~50,000 receptors versus 3,000 receptors, respectively) (Hempstead et al., 1992). In cells expressing kinase-inactive Trk, NGF failed to induce SHC tyrosine phosphorylation (Figure 1, lanes 11 and 12). In wild-type PC12 cells, SHC tyrosine phosphorylation was attenuated after 5 min of NGF treatment (Figure 2A). In contrast, in trk–PC12 cells, SHC tyrosine phosphorylation was elevated for at least 5 hr in the presence of NGF (Figure 2B). Thus, the level of Trk tyrosine kinase activity in PC12 cells affects the appearance and kinetics of induction of SHC tyrosine phosphorylation.

SHC Associates with Ligand-Activated Trk Receptors and Other Signaling Molecules
Since a band at Trk molecular weight (140 kD) was observed when SHC immunoprecipitates from NGF-treated cells were probed with anti-phosphotyrosine (Ptyr) antibodies (Figure 1, lane 5; Figure 3A), we asked whether Trk associates with SHC in vivo. For this analysis, SHC immunoprecipitates prepared from trk–PC12 cells were probed with anti-Trk antibodies. Trk was found to coimmunoprecipitate with SHC from lysates of NGF-treated, but not mock-treated trk–PC12 cells (Figure 3B).

We next examined the SHC immune complexes for the presence of other signaling molecules. Lysates of trk–PC12 cells that were mock treated or treated with NGF for 5 min were immunoprecipitated with anti-SHC antibodies and probed with anti-PLC-γ1 (Figure 3C), anti-Grb2 (Figure 3D), or anti-rasGAP (Figure 3E) antibodies. PLC-γ1, Grb2, and rasGAP coimmunoprecipitated with SHC from lysates of NGF-treated cells. Trk, PLC-γ1, Grb2, and rasGAP did not coimmunoprecipitate with SHC from untreated cells, indicating that the association events were ligand dependent and required NGF-induced tyrosine phosphorylation of Trk or SHC.

Identification of Trk Autophosphorylation Sites
The association of SHC with Trk is most likely mediated by an interaction of the SH2 domain of SHC with a phosphotyrosine residue on Trk. The determination of this association site requires that the sites of Trk tyrosine autoprophosphorylation be identified. Y785 has
previously been identified as an in vitro phosphorylation site on Trk (Loeb et al., 1994) responsible for PLC-γ1 association (Obermeier et al., 1993; Loeb et al., 1994). To determine the potential sites of other target interactions, the remaining in vitro autophosphorylation sites of Trk were identified. NGF induces the tyrosine kinase activity of Trk in PC12 cells or in Sf9 insect cells expressing human Trk (Kaplan et al., 1991a; Klein et al., 1991). Sf9 cells infected with a recombinant baculovirus encoding human Trk or PC12 cells overexpressing human Trk (trk-PC12) were used as a source of Trk. Trk proteins were immunoprecipitated, washed extensively with buffer containing 0.1% SDS, and autophosphorylated in the presence of [γ-32P]ATP and Mn2+. Kinase-inactive Trk (K538N) expressed in Sf9 cells failed to become phosphorylated on tyrosine using this procedure (data not shown), indicating that the immunoprecipitates did not contain insect cell tyrosine kinases capable of phosphorylating Trk. These samples were then electrophoresed on SDS-polyacrylamide gels and transferred to Immobilon filters. After autoradiography, the portions of the filter containing the 32P-labeled Trk proteins were excised, incubated with trypsin, and phosphopeptides analyzed by reverse-phase high performance liquid chromatography (HPLC). A profile of the radioactivity released from the column indicated that three major peaks were obtained with wild-type Trk (Figure 4A). A fourth peak containing the tryptic peptide containing Y785 was only weakly detected owing to the inhibition of in vitro autophosphorylation at this site by the immunoprecipitating anti-Trk antibody. Phosphoamino acid analysis of the peptides contained in the four peaks revealed that they were phosphorylated on tyrosine (data not shown). To determine the position of the phosphorylated amino acids within each tryptic fragment, the isolated peptides were subjected to automated Edman degradation in a spinning-cup sequenator (Figures 4D-4F). From this analysis, we determined that the peptide in peak 1 was phosphorylated at Y674 (amino acid 7) (Figure 4D), the peptide in peak 2 was phosphorylated at Y670, Y674, and Y675 (amino acids 3, 7, and 8) with the highest labeling at Y675 (Figure 4E), and the peptide in peak 3 was phosphorylated at Y490 (amino acid 14 of the peptide) (Figure 4F). These same peaks were detected when in vitro phosphorylated Trk from NGF-treated trk-PC12 cells was examined (Figure 4B). Ligand-activated Trk receptors are therefore capable of autophosphorylation at Y490, Y670, Y674, and Y675 in addition to the previously identified site at Y785.

To confirm that Y490 is a site of Trk autophosphorylation, Trk with phenylalanine substituted for tyrosine at position 490 (Trk Y490F) was expressed in Sf9 cells. Trk Y490F was phosphorylated in vitro in kinase assays, and the phosphorylated tryptic peptides from this receptor were analyzed by reverse-phase HPLC. Three peaks of radioactivity were obtained from Trk Y490F at the positions of tyrosine-containing peptides containing 32P-labeled Y670, Y674, Y675, and Y785 (Figure 4C). Peak 3, containing the peptide containing 32P-labeled Y490, was not present (Figure 4C). Wild-type Trk or Trk Y490F isolated from Sf9 cells phosphorylated synthetic peptides containing Y490 (or Y674 and Y675) in vitro, demonstrating that the mutation at the Y490 site does not alter Trk enzymatic activity or access to peptide substrates (data not shown). HPLC analysis of tryptic peptides derived from baculovirus-expressed Trk proteins containing mutations at Y670, Y674, and Y675 confirmed that these tyrosines are sites of in vitro phosphorylation (data not shown).

Tyrosine 490 of Trk Is Required for Association with SHC In Vitro

To determine whether Y490 mediates SHC associations, the binding of SHC to wild-type Trk and to a panel of Trk mutants was examined using in vitro system. Sf9 cells were infected with recombinant baculoviruses encoding wild-type Trk, kinase-inactive Trk (K538N), or Trks with mutations at Y490, Y670, Y674/675, Y751, or Y785. The Trk proteins were immunoprecipitated from the insect cells, washed ex-
Figure 4. Identification of Trk Autophosphorylation Sites
(A–C) In vitro phosphorylated Trk tryptic phosphopeptides were separated by reverse-phase HPLC, and fractions were collected as described (Stephens et al., 1992). The amount of 32P radioactivity in each fraction is shown. (A) Wild-type human Trk from Sf9 cells. (B) Wild-type human Trk expressed in PC12 cells. The asterisks indicate additional peaks resolved in this sample arising from unidentified phosphorylation sites in Trk. A large peak is also detected that arises from partial tryptic digestion of the peptide contained in peak 1. (C) Trk Y490F expressed in Sf9 cells. The peak containing phosphorylated Y490 (peak 3, fraction 38) is absent in the tryptic digest of Trk Y490F. The peptide in peak 4 contained phosphorylated Y785 (Loeb et al., 1994). The peak containing phosphorylated Y785 (peak 4, fraction 50) is small since the antibody used to precipitate Trk (203) masks this site from phosphorylation in vitro. (D–F) The isolated peptides in peaks 1–3 from the HPLC column shown in (A) were subjected to automated Edman degradation. (D) The peptide in peak 1, containing 32P-labeled amino acids at position 7 relative to the amino-terminal end of the peptide, encoded DIYSTDYR (667–676 of human Trk), indicating phosphorylation at Y674. (E) The peak 2 peptide, containing 32P-labeled amino acids at positions 3, 7, and 8, encoded DIYSTDYR corresponding to residues 670, 674, and 675. The highest labeling was at Y675. (F) The peptide in peak 3, containing 32P-label at amino acid position 14, encoded GSGLQCHIENPOYFDG (477–493), indicating phosphorylation at Y490. Arrows indicate trypsin cleavage sites.

tensively, and subjected to autophosphorylation in vitro to phosphorylate substrate-binding sites. Similar amounts of the Trk proteins were then incubated with lysates of mouse brain (a source of potentially interacting proteins). The immune complexes were washed extensively and subjected to immunoblotting with anti-SH2 antibody. SHC associated with wild-type Trk and all Trk mutants except Trk Y490F and kinase inactive Trk (Figure 5). A reduced association was observed with Trk Y674/675F owing to inefficient tyrosine autophosphorylation at Y490 and Y785 as a result of the mutation at Y674/675 (data not shown). The p85PI-3 kinase subunit and PLC-γ1 associated with wild-type Trk and Trk Y490F to a similar extent, indicating that Trk receptors with mutations at Y490 are capable of interacting with cellular proteins (data not shown). Analysis of the association between Trk and the other forms of SHC could not be performed in this experiment owing to coaggregation of the p48 and p53 forms of SHC with the heavy chain of the immunoprecipitating antibody.

Tyrosine 490 of Trk Is Required for Association with SHC In Vivo
We next expressed wild-type and mutant Trk receptors in NIH 3T3 cells to assess whether Y490 of Trk mediates interactions with SHC in vivo. NIH 3T3 cells were chosen for these assays since much higher levels
expression plasmids encoding wild-type Trk, kinaseinactive Trk (K538N), Trk Y490F, Trk Y785F, or Trk Y490/785F and a G418 resistance marker. Following selection in G418, the cells were examined for SHC-Trk associations. Lysates of untreated and NGF-treated cells were incubated with anti-SHC antibody and the immunoprecipitates were probed with anti-Trk antibody. Trk was observed in SHC immunoprecipitates from cells expressing wild-type and Trk Y785F, but not from cells expressing Trk receptors with mutations at Y490 (Trk Y490F, Trk Y490/785F) (Figure 6A). In addition, the association of Trk and SHC was not observed in untreated cells or in NGF-treated cells expressing kinase-inactive Trk (K538N), indicating that the association event requires ligand-activated and autophosphorylated Trk. The NIH 3T3 cell lines stably transfected with wild-type and mutant Trk expressed approximately equivalent levels of SHC and Trk (Figure 6B).

Y490 of Trk Is Required for SHC Tyrosine Phosphorylation in NGF-Treated PC12m5 Cell Lines Expressing Wild-Type and Mutant Trks

To determine the role of the Y490 SHC association site in NGF-mediated neuronal differentiation, PC12m5 cells were transfected with plasmids encoding Trk Y490 or Trk with mutations at both Y490 and Y785 (PLC-γ1 association site). To examine the tyrosine phosphorylation of SHC, lysates prepared from untreated or NGF-treated cell lines expressing Trk Y490F, Trk Y785F, or Trk Y490/785F were immunoprecipitated with anti-SHC antibody, and the SHC proteins were probed with anti-Ptyr antibody. SHC was phosphorylated only in the NGF-treated samples, and the levels of SHC were similar in each case. The relative intensities of the bands were quantified by densitometry, and the results were expressed as a percentage of the intensity of the band in the wild-type Trk sample. The data showed that the tyrosine phosphorylation of SHC was significantly reduced in the NGF-treated samples expressing Trk Y490F, Trk Y785F, or Trk Y490/785F compared to the wild-type Trk sample. This suggests that the tyrosine phosphorylation of SHC is dependent on the presence of Y490 in Trk.
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**A**

p53SHC

p48SHC

**B**

p68SHC

p53SHC

p48SHC

**C**

Grb-2

**D**

Trk

**E**

Trk

Figure 7. Tyrosine Phosphorylation of SHC and Association of SHC with Grb2 in PC12nrr5 Cells Expressing Wild-Type and Mutant Trks

PLC12nrr5 cell lines expressing wild-type Trk (Δk alone T14), Trk Y490F (T490F), Trk Y785F (785.24), and Trk Y490F/785F (490/785.21) were mock treated (−) or treated with 100 ng/ml NGF (+) for 5 min. Lysates were equalized for cell protein. (A) Tyrosine phosphorylation of SHC. Lysates were immunoprecipitated with anti-SHC and probed with anti-Pyr. (B) Mobility shift of p68 SHC. Cell lysates were probed with anti-SHC. (C) Association of SHC with Grb2. Lysates were immunoprecipitated with anti-SHC and probed with anti-Grb2. (D) Trk expression in cell lysates. Lysates were immunoprecipitated with anti-Trk and probed with anti-Trk. (E) Tyrosine phosphorylation of Trk. Lysates were immunoprecipitated with anti-Trk and probed with anti-Pyr.

Tyrosine phosphorylation and association with Grb2.

To confirm that the PC12nrr5 cell lines were expressing Trk, Trk immunoprecipitates were prepared from lysates of untreated and NGF-treated cells and examined by immunoblotting with anti-Trk antibody. Each of the cell lines expressed different amounts of Trk (Figure 7D). However, when the Trk proteins were probed with anti-Pyr antibody to assess NGF-induced tyrosine autophosphorylation, the Trk Y490F, Trk Y490/785F, and PC12nrr5 cells expressing wild-type Trk exhibited similar levels of tyrosine phosphorylation. All of the cell lines, with the exception of the Trk Y490F, showed a degree of NGF-independent Trk tyrosine phosphorylation (Figure 7E). Since NGF-independent constitutive autophosphorylation of Trk has been observed in most other cell lines overexpressing Trk (Kaplan et al., 1991a; Cordon-Cardo et al., 1991; Hempstead et al., 1992), the constitutive phosphorylation observed in the cell lines derived here is most likely not a consequence of a particular Trk mutation.

Defective Erk1 Tyrosine Phosphorylation and Activity in Trk Y490F/785F, but Not in Trk Y490F

The potential involvement of SHC in the regulation of Grb2 and the SOS guanine nucleotide exchange protein suggests that proteins whose activity or tyrosine phosphorylation is dependent upon p21α activity may be affected by the SHC interaction site mutation in Trk. We assayed the activity and tyrosine phosphorylation of one such protein, Erk1 (Robbins et al., 1992; Thomas et al., 1992; Wood et al., 1992; Toppin et al., 1992), in PC12nrr5 cells expressing Trk Y490F, Trk Y490/785F, and Trk Y785F. Lysates prepared from untreated and NGF-treated cells were immunoprecipitated with anti-Erk1 antibody, and the Erk proteins were probed with anti-Pyr antibody. As previously reported, Erk1 was phosphorylated on tyrosine in NGF-treated PC12nrr5 cells expressing wild-type Trk or Trk Y785F (Trk containing a mutation at the Y785 PLC-γ1-binding site), but not in cells expressing Trk Y490F or Trk Y490/785F (Figure 7A). Similar results were obtained with two other independently derived cell lines expressing Trk Y490F or Trk Y490/785F (data not shown). Although NGF did not induce SHC tyrosine phosphorylation in the cell lines expressing Trk Y490F or Trk Y490/785F, a partial shift in electrophoretic mobility of p68 SHC was observed in SHC immunoprecipitates from these cells (Figure 7B). In addition to the lack of SHC tyrosine phosphorylation, the NGF-induced association of Grb2 with SHC was not observed in cells expressing Trk Y490F or Trk Y490/785F (Figure 7C). A constitutive level of SHC tyrosine phosphorylation and association with Grb2 was observed in the Trk Y785F cell line (785.24), possibly owing to high levels of Trk expression. Elevated basal levels of SHC tyrosine phosphorylation were not observed in a second PC12nrr5 cell line expressing Trk Y785F, 785.11 (data not shown). Thus, Y490 of Trk is required

for SHC tyrosine phosphorylation and association with Grb2.
alent in all cell lines (data not shown). These results indicate that Trk receptors containing mutations at both Y490 and Y785 are defective in mediating the tyrosine phosphorylation and activation of Erk1.

**Tyrosine Phosphorylation of PLC-γ1 and SNT in PC12nrr5 Cells Expressing Trk Y490F and Trk Y490/785F**

We next analyzed the tyrosine phosphorylation of two other targets of NGF-induced tyrosine phosphorylation in PC12 cells, PLC-γ1 and SNT. PLC-γ1 was phosphorylated on tyrosine to a similar extent in PC12nrr5 cells expressing Trk Y490F and wild-type Trk (Figure 8C). PLC-γ1 was not phosphorylated on tyrosine in NGF-treated Trk Y785F and Trk Y490/785F cells, since Y785 in Trk mediates the receptor association and tyrosine phosphorylation of PLC-γ1 (Obermeier et al., 1993; Loeb et al., 1994). SNT was phosphorylated on tyrosine to similar extents in PC12nrr5 cells expressing Trk Y490F, Trk Y490/785F, Trk Y785F, and wild-type Trk (Figure 8D). Thus, whereas Trk Y490/785F is defective in mediating the tyrosine phosphorylation of SHC, PLC-γ1, and Erk1, this receptor is competent in inducing SNT tyrosine phosphorylation. In addition, since tyrosine phosphorylation of PLC-γ1 and SNT requires the activity of Trk, but not p21<sup>ras</sup> (Thomas et al., 1992; Wood et al., 1992; Rabin et al., 1993), the tyrosine phosphorylation of proteins that are thought to function upstream or in signal transduction pathways parallel to that of p21<sup>ras</sup> are not affected by mutations of the SHC association site in Trk.

**Trk Y490F and Trk Y490/785F Mediate Normal NGF Binding and Uptake**

Trk Y490 is within a motif (NPXY) that may be involved in ligand internalization by the low density lipoprotein (LDL) and insulin receptors (Chen et al., 1990; Backer et al., 1992). We therefore compared the binding and internalization of NGF in PC12nrr5 cells expressing either no Trk, wild-type Trk, Trk Y490F, Trk Y785F, or Trk Y490/785F. Cultures were incubated with 6 ng/ml [125I]NGF for 3 min at 37°C to permit cell surface binding with minimal internalization, and transferred to ice. The unbound [125I]NGF was removed, and 1 μg/ml unlabeled NGF was added. At various times, surface-bound and released [125I]NGF were quantified. As reported (Loeb and Greene, 1993), cells without Trk released about half the bound [125I]NGF within 10 min. In contrast, like cells expressing wild-type Trk (Loeb and Greene, 1993) and Trk Y785F (Loeb et al., 1994), cells with Trk Y490F or Trk Y490/785F released only 10%–20% of the labeled ligand in the same period (data not shown). Thus, mutation of Trk Y490 alone or in combination with Y785 does not appear to alter the ratio of "fast" and "slow" NGF receptors.

We also examined intracellular accumulation of NGF by the various cell lines listed above. Cultures were incubated with 6 ng/ml [125I]NGF at 37°C for 60
min, and the amounts of surface-bound and internalized NGF were determined. For cells without Trk, the ratio of internalized to surface NGF (I/S ratio) was approximately 1 (Green and Greene, 1986; Loeb and Greene, 1993), whereas for cultures expressing either wild-type Trk or Trk Y78SF, this ratio was 3–4 (Green and Greene, 1986; Loeb and Greene, 1993; Loeb et al., 1994). Cells expressing Trk Y490F or Trk Y490/785F also displayed an I/S ratio of approximately 3.5 (data not shown). Therefore, Trk Y490F and Trk Y490/785F appear unaltered in their abilities to mediate efficient NGF internalization and binding.

**NGF Mediates Neurite Outgrowth in Cells Expressing Trk Y490F, but Not in Cells Expressing TrkY490/785F**

Having shown that some elements of NGF signaling are altered in cells expressing Trk Y490 or Trk Y490/785F, we next determined the effects of these mutations on NGF-mediated neurite outgrowth. Cultures of PC12nr5 cells expressing wild-type Trk (nr5 5T14), Trk Y490F (490.6), Trk Y78SF (785.11, 785.24), or Trk Y490/785F (490/785.14, 490/785.21) were treated with NGF and assessed for extent of neurite outgrowth. As previously reported, PC12nr5 cells expressing wild-type Trk or Trk Y78SF responded to NGF with neurite outgrowth similar to that seen in PC12 cells (Figure 9; Loeb and Greene, 1993; Loeb et al., 1994). Similarly, cells expressing Trk Y490F also exhibited wild-type neurite outgrowth responses to NGF, despite the absence of SHC tyrosine phosphorylation in these cells (Figure 9). However, three PC12nr5 cell lines expressing Trk Y490/785F were consistently defective in NGF-promoted neurite outgrowth (Figure 9 and data not shown). Although these cells exhibited several normal responses to NGF, including flattening, increases in size, and extension of short cytoplasmic processes, they did not form long, stable neurites. Less than 20% of the cells expressing Trk Y490/785F extended neurites to a length of more than 2 cell body diameters, and these neurites were maintained for less than 24 hr (Figure 9). Therefore, al-
though mutations at either Y785 or Y490 do not interfere with Trk-mediated neurite formation, mutations at both sites seriously compromise this response. The levels of Trk in the cells lines used for the neurite outgrowth assays were approximately equivalent to those in the wild-type Trk-expressing cells, and all of the cells expressing mutant Trks exhibited higher receptor protein expression than the cells expressing wild-type Trk (Figure 10).

Discussion

Neurotrophins are thought to be important in mediating both the development of the embryonic nervous system and the survival of neurons in the adult (Barde, 1989; Thoenen, 1991; Hefti et al., 1992). Several of the initial signaling events immediately induced by neurotrophin treatment of cells have been identified (Halegoua et al., 1991; Chao, 1992a, 1992b). However, the importance of these events with respect to their requirement for differentiation and survival of neurons remains unresolved. To begin to assess the roles of the components of the NGF signal transduction pathway, we have identified tyrosine phosphorylated residues of Trk that interact with proteins which have potential roles in signal transduction. By constructing mutants of Trk at these residues, thereby eliminating subsets of receptor–substrate interactions, we can assess the contributions of each substrate interaction site to the biochemical and morphological changes elicited by NGF.

Y490 of Trk Is an Interaction Site for SHC

We show that Y490 of Trk is required for interaction with SHC. SHC and Trk coimmunoprecipitated from PC12 or NIH 3T3 cells treated with NGF, and baculovirus-expressed Trk formed complexes in vitro with SHC from brain or PC12 lysates. In addition, mutation of the Y490 site to phenylalanine prevented the ligand-induced association of SHC with Trk in vitro and resulted in a dramatic inhibition of SHC tyrosine phosphorylation in transfected PC12 cells. However, Erk1, PLC-γ1, or SNT tyrosine phosphorylation was not significantly reduced in NGF-treated cells expressing Trk Y490F, indicating that the tyrosine phosphorylation or activation of these proteins could be mediated by other sequences on Trk. Furthermore, PLC-γ1 associated with ligand-activated Trk Y490F, demonstrating that this receptor is competent to interact with other Trk substrates. It is possible that other proteins, such as PI-3 kinase or Erk1, interact with the phosphorylated Y490 site in Trk and that these interactions do not occur with a binding affinity sufficient to be observed in the immunoprecipitates. The striking defects in SHC association with Trk receptors containing mutations at this site suggest a major role for Y490 in mediating SHC–Trk complex formation.

Y785 has been shown to be an autophosphorylation site of Trk in vitro (Loeb et al., 1994). Four additional sites of Trk tyrosine phosphorylation in vitro are identified here, Y490, Y670, Y674, and Y675. The tyrosines at positions equivalent to Y670, Y674, and Y675 are conserved in all mammalian Trk family members, and the Y674 tyrosine is present in Drosophila Trk (Pulido et al., 1992). Y674 was previously suggested to be a major target of Trk autophosphorylation (Martin-Zanca et al., 1989) based upon an analysis of autophosphorylation sites by Hunter and Cooper (1985). Although we do not know whether Y674 and Y675 are sites of Trk tyrosine phosphorylation in vivo, these tyrosines are likely to be important in regulating the Trk tyrosine kinase activity. In this regard, Mitra (1991) has shown that mutation of the amino acids corresponding to Y674 and Y675 in the trk oncogene results in an inhibition of kinase activity in vitro and transformation potential. We are currently analyzing the effect of mutation of these sites on NGF-induced differentiation responses and Trk kinase activity. Y490 is in a sequence context of NPQYFD. YFSD has not been identified as a recognition site for SH2 domain-containing proteins (Songyang et al., 1993), and it is not present in the receptors that are presently known to interact with SHC, namely the Trk and EGF receptors. NPYX is present in the intracellular domains of the LDL, insulin, insulin-like growth factor-I, and EGF receptors and in polyoma middle T antigen (Chen et al., 1990; Druker et al., 1992). Mutation of the tyrosine equivalent to Y490 to cysteine in the LDL receptor inhibits ligand-mediated receptor internalization (Chen et al., 1990). The tyrosine in the NPYX motif in the insulin receptor site may also be involved in internalization of the insulin receptor (Backer et al., 1992), although another study showed that mutation of Y960 had no such effect (Kaburagi et al., 1994). However, we did not observe an inhibition of NGF internalization in PC12 cells expressing Trk Y490F. Mutation of Y960 in the insulin receptor had dramatic effects on insulin responses, including the inhibition of insulin receptor substrate-1 and 85 kD/PI-3 kinase substrate tyrosine phosphorylation, microtubule-associated protein kinase activity, 2-deoxyglucose uptake, and mitogenesis (Kaburagi et al., 1994). Our data indi-
cate that Y490F in Trk also performs an important function in substrate interactions and signaling responses.

Either the Y490 or the Y785 Site Is Required for a Subset of NGF Signaling Responses

Cells expressing Trk receptors with mutations at either Y490 or Y785 exhibited neurite outgrowth responses that were indistinguishable from those of cells expressing wild-type Trk receptors. In contrast, cells expressing Trk with mutations in both Y490 and Y785 failed to extend neurites efficiently. In addition, the small proportion of cells that extended neurites could not maintain these projections. Whereas Trk Y490/785F did not mediate the tyrosine phosphorylation of SHC, PLC-γ1, and Erk1, this receptor was capable of NGF-induced tyrosine kinase activity and internalization, change in cell shape, and SNT tyrosine phosphorylation. The induction of TIS8 mRNA was also detected in NGF-treated cells expressing Trk Y490/785F, although only very low levels of fos mRNA were observed (D. M. L. and L. A. G., unpublished data). Therefore, Trk Y490/785F is defective in only a subset of NGF-signaling responses. SHC and PLC-γ1 are candidates for mediating the responses abrogated in cells expressing Trk Y490/785F. Since tyrosine phosphorylated SHC and Grb2 were observed in untreated cells expressing Trk Y785F (Figures 7 and 8), SHC and Grb2 activity may be necessary, but not sufficient, for neurite outgrowth. Other signals in addition to SHC and Grb2 that are initiated by NGF may be required for this response.

SHC and PLC-γ1 Are Candidate Downstream Mediators of NGF Differentiation Responses Influencing Distal Targets of p21^W^ Activity

The capacity of NGF to promote wild-type neurite outgrowth by cells expressing Trk Y490F or Y785F suggests that the interaction of either SHC or PLC-γ1 and Trk is dispensable for NGF-mediated differentiation. If SHC and PLC-γ1 initiate similar signaling pathways, the loss of SHC binding to Trk Y490 may be compensated for by the interaction between PLC-γ1 with this receptor. These substrates may thus have partially redundant roles in NGF responses. Elimination of both SHC and PLC-γ1 interaction sites in Trk resulted in a defective receptor, indicating that the activity of at least one of these proteins, or of other proteins that may interact with these sites, is required for normal phenotypic responses of cells to NGF. Although mutation of the SHC or PLC-γ1 association sites alone had no observable effect on the NGF-induced differentiation of PC12 cells, it is quite possible that the loss of Trk interactions with these proteins might alter a phenotypic property not assessed here. For example, mutation of Y785 selectively eliminates NGF-promoted regulation of the expression of peripherin mRNA and protein (Loeb et al., 1994).

Examination of cells expressing Trk Y490/785F indicated that at least one protein that has been shown to function downstream of p21^W^, Erk1, failed to exhibit NGF-induced activation and tyrosine phosphorylation (Figure 7). Erk1 was phosphorylated on tyrosine to similar extents in cells expressing Trk Y490F, Trk Y785F, or wild-type Trk. In PC12 cells, this response is dependent upon the activity of p21^W^ (Thomas et al., 1992; Wood et al., 1992; Robbins et al., 1992; Tropmann et al., 1992). SHC has been shown to bind to Grb2, which functions in the regulation of p21^W^ activity (Rozakis-Adcock et al., 1992; Olivieri et al., 1993; Simon et al., 1993). We also observed the ligand-induced association of SHC with rasGAP, an effector of p21^W^ (Medema et al., 1992; Yatani et al., 1990; Duchesne et al., 1993). This then raises the question as to how Erk1 is activated in cells deficient in SHC tyrosine phosphorylation. One possible mechanism is through a PLC-γ1-dependent signaling pathway. PLC-γ1 generates the protein kinase C activator diacylglycerol (Rhee et al., 1989). Phorbol esters, which mimic diacylglycerol, can also regulate p21^W^ activity through down-regulation of GAP activity (Downward et al., 1990). Thus, in cells expressing Trk receptors that are competent to activate PLC-γ1, NGF may regulate Erk1 via an alternative mechanism of ras activation. Owing to high levels of ras-GTP in untreated PC12mnt5 cells, the relative amounts of ras-GDP and ras-GTP in cells expressing the Trk mutants could not be accurately assessed. However, we observed only a minimal shift in the electrophoretic mobility of Ras in NGF-treated PC12mnt5 cells expressing Trk Y490/785F (S. R. S. and D. R. K., unpublished data), consistent with the hypothesis that p21^W^ is defective in transmitting neurotrophin signals in these cells. Our results suggest that either SHC or PLC-γ1 can activate Erk1, perhaps through p21^W^, and that suppression of SHC and PLC-γ1 signaling pathways results in a loss in the ability to activate Erk1 activity and to stimulate neurite outgrowth responses efficiently. Substrates that may interact with other regions of Trk are therefore not sufficient to induce these responses.

Immunoprecipitates of SHC from NGF-treated trk−PC12 cells contained Trk, PLC-γ1, Grb2, and rasGAP. However, in response to NGF, Trk coimmunoprecipitated with PLC-γ1 and SHC, and not with Grb2 or rasGAP (Figure 3; Vetter et al., 1991; B.-Q. Li et al., 1992; and data not shown), indicating that SHC forms independent, stable complexes with Trk and rasGAP. These results suggest that SHC may form at least two independent coinmunoprecipitating complexes. The first of these complexes may contain NGF-activated Trk, PLC-γ1, and SHC. The second complex may contain SHC and either Grb2 or rasGAP, which are involved in regulating p21^W^ activity.

SHC was phosphorylated on tyrosine in PC12 cells treated with NGF and EGF, but not FGF. The FGF receptor does not contain an NPXY sequence, which is the putative Trk−SHC association site. FGF and NGF have previously been shown to stimulate the tyrosine phosphorylation of similar proteins in PC12 cells, including PLC-γ1, the 85 kd PI-3 kinase subunit, SNT, Erk1, and Erk2 (Kim et al., 1991; Vetter et al., 1991;
Rabin et al., 1993; Raffioni and Bradshaw, 1992; Schanen-King et al., 1991; Boulton et al., 1991; Gómez et al., 1990; Miyasaka et al., 1991). This suggests that although NGF and FGF share a subset of signaling molecules, they may also differ in the signaling mechanisms they use to promote neuronal differentiation. Although FGF can induce stable neurite formation in PC12 cells, its efficiency in doing so is substantially less than that of NGF unless conditioned medium is present (Rydel and Greene, 1987). This is consistent with the possibility that the absence of SHC tyrosine phosphorylation contributes to the poor intrinsic neurite-promoting activity of FGF and that this is ameliorated by signals provided by other factors present in conditioned medium.

Experimental Procedures

Cells, Growth Factors, Antibodies, and Peptides
PC12nrs5 and PC12 cells were grown in RPMI containing 10% heat-inactivated horse serum and 5% fetal bovine serum. PC12 cells stably transfected with a human trk cDNA (trk-PC12, clone G24) (Hempestad et al., 1992) were cultured at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated horse serum and 5% calf serum. SY5Y (SK-N-SY5Y) cells (Biedler et al., 1973) were cultured in DMEM containing 10% fetal bovine serum. SY5Y cells were treated with 1 μM trans-retinoic acid for 8 days to induce the expression of TrkB (Kaplan et al., 1993). NIH 3T3 cells (clone w2) were grown in DMEM containing 10% heat-inactivated fetal bovine serum.

The anti-Trk monoclonal antibody 4G10 was provided by D. Morrison (NCI-FCRDC). Anti-SHC (Pellicci et al., 1992), anti-CAP (Ellis et al., 1990), and anti-Trk 203 (Hempestad et al., 1992) were used as described. Anti-P-CLY (Stuhl et al., 1988) was obtained from S. G. Rhee (National Institutes of Health) or from Upstate Biologics Inc. (Lake Placid, NY). Anti-phospho-I-3 kinase subunit was from Upstate Biologics Inc. or S. Stoff and L. Cantley (Harvard Medical School), anti-CrkII from Signal Transduction Laboratories, and anti-Erk1 from J. Benes (Harvard Medical School) (Chen et al., 1992), and p13KR-arogose from V. Cleghorn and N. Michaud (NCI-FCRDC).

NGF was purified from mouse submaxillary glands as previously described (Mobley et al., 1972) or obtained from Upstate Biotechnology Inc. EGF and FGF were obtained from UBI, and NT3 was from J. Winslow and G. Burton (Genentech, Inc.).

Reverse-Phase HPLC
In vitro phosphorylated Trk proteins were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to immobilization filters. After autoradiography, the band containing ~P-labeled Trk was excised, blocked with 1.5% PVP-40 in 100 mM acetic acid for 1 hr at 37°C, washed extensively with water, and incubated in trypsin (1 μg per 50 μl) of 50 mM ammonium bicarbonate) for 24 hr at 37°C. The peptides were then analyzed by reverse-phase HPLC. Aliquots of enzymatic digests were lowered to pH 2 with 20% trifluoroacetic acid and loaded onto Waters 3.9 × 300 mm C18 column as described (Stephens et al., 1992). The column was used with an increasing gradient of acetonitrile in 0.5% aqueous trifluoroacetic acid. The stepwise gradient at a flow rate of 1 ml/min was 0–40% CH3CN over 60 min, 40% CH3CN for 10 min, 40%–60% CH3CN over 10 min, and 60% CH3CN for 10 min. One milliliter fractions were collected and Cerenkov counted for γP content in a Beckman LS 5801 scintillation counter.

Edman Degradation
Semiautomated amino-terminal sequence analysis was performed in a Beckman 890C spinning-cup sequencer as described (Stephens et al., 1992). Polybrene (2.5 mg) was applied to the spinning cup, along with 120 nmol of the dipeptide Tyr-Glu, and subjected to four cycles of Edman degradation. Equine apomorphine (9 mmol) and the γP-containing peptide in CH3CN-water were then added to the spinning cup, dried, and subjected to 20 cycles with no prewashes. Each fraction was counted for 20 min.

Construction of Recombinant Baculoviral Vectors and Expression of Recombinant Trk Mutants in SF9, PC12nrs5, and NIH 3T3 Cells
Site-directed mutagenesis was performed as previously described (Kunkel et al., 1987) using a human trk cDNA clone (Marin-Zanca et al., 1989) and the appropriate oligonucleotides to introduce the desired base changes. The amino acid residues are numbered from the methionine at the initiation site. Fragments containing the entire Trk Y490F, Trk Y670F, Trk Y674/675F, or Trk Y751F coding sequences were isolated from cDNAs and inserted into the pAcCo transfer vector. Wild-type AcNPV DNA and each of the recombinant vectors were cotransfected into SF9 cells, and recombinant baculoviruses were isolated by plaque purification as previously described (Summers and Smith, 1987). Routinely, for recombinant protein production, 5 × 10^7 SF9 cells were infected with the desired virus at a multiplicity of infection of 5–10 and lysed at 48 hr postinfection. SF9 cells were assayed for the expression of the recombinant proteins by immunoblot analysis. Recombinant baculoviruses expressing wild-type human Trk, Trk K538N, and Trk Y758F were generated as previously described (Loeb et al., 1994).

To generate PC12nrs5 cell lines expressing mutant Trks, cells were transfected by electroporation with Trk Y490F and Trk Y490/758F cDNA under the control of a cytomegalovirus promoter in vector pBRV as described (Loeb et al., 1992). G418-resistant cells were selected, colony cloned, and expanded, as previously described (Greene et al., 1991). Northern blotting was used to screen clones for expression of trk mRNA. In each case, the message corresponded in electrophoretic mobility to transfected human Trk, rather than endogenous rat Trk. At least three independent cell lines were isolated expressing each Trk mutant. Various PC12- and PC12nrs5-derived lines were assessed for NGF-promoted neurite outgrowth as previously described (Greene et al., 1991).

To generate NIH 3T3 cell lines expressing mutant Trks, cells (clone w2) were transfected with plasmids encoding wild-type and mutant Trks in the retrovirus vector pLNCX5 (Miller and Rosman, 1989). Transfected cells were selected in G418, and the resulting population of cells was assayed for Trk expression and association with SHC.

Immunoprecipitation and Immunoblotting
Cells were treated with growth factors and lysed, and the lysates were immunoprecipitated as previously described (Kaplan et al., 1990, 1991a). After two washes with cold Tris-buffered saline, cells (1 × 10^6) were lysed in 1 ml of 1% Nonidet P-40 (NP-40) lysis buffer (20 mM Tris [pH 8.0], 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride, 0.1% Ileumatpinin, 20 mM leupetin, 1 mM sodium vanadate) at 4°C for 20 min. Insoluble material was removed by centrifugation at 4°C for 10 min at 10,000 g. Immunoprecipitations were performed for 2–4 hr at 4°C. Precipitates were collected with protein A-Sepharose, then washed three times with NP-40 lysis buffer and once with water. The immunoprecipitates were boiled in sample buffer (2% SDS, 100 mM dithiothreitol, 1% glycerol, 0.25% bromophenol blue) for 5 min and electrophoresed on SDS-polyacrylamide gels before transfer to nitrocellulose. Protein blots were probed overnight at 4°C with antibody. Blots were analyzed using an ECL chemiluminescence system (Amersham Corp., Arlington Heights, IL) and horseradish peroxidase–coupled secondary antibody from Boehringer Mannheim Biochemicals.

The in vitro association assay using baculovirus-produced proteins was performed as described (Morton et al., 1989; Kaplan et al., 1990). SF9 cells (2 × 10^6) were infected, harvested, and lysed in NP-40 lysis buffer. Trk proteins were immunoprecipitated with anti-Trk 203, and the immunoprecipitates were washed once with lysis buffer containing 1% deoxycholate and 0.1% SDS (RIPA), twice with 0.5 M NaCl, 50 mM Tris (pH 7.4), and once with...
Trk Receptors Use Redundant Signaling Pathways

10 mM Tris (pH 7.4). Complexes were incubated with 50 mM Tris (pH 7.4), 5 mM MnCl₂, 5 mM ATP, 200 μM orthovanadate. Trk proteins were then washed twice with NP-40 lysis buffer and were resuspended in 1.3 ml of lysis prepared from one-fifth of an adult mouse brain or from 1 x 10⁷ PC12 cells lysed in 1% NP-40 lysis buffer. The immunoprecipitated complexes were incubated with the cell lysate for 3 h at 4°C and then washed three times with NP-40 lysis buffer and once with 10 mM Tris (pH 7.4) before immunoblot analysis.

For SNT precipitations, 20 μl of p130crk-agarose was added to 1 ml of lysate from 1 x 10⁶ cells and incubated for 2-4 h at 4°C as described (Rabin et al., 1993). The p130crk-agarose beads were then washed three times with NP-40 lysis buffer and once with water before resuspension in 10 μl of sample buffer for electrophoresis on 7.5% SDS-polyacrylamide minigels.

In Vitro Kinase Assays

Proteins were immunoprecipitated as described above and washed three times with lysis buffer and once with kinase buffer (30 mM HEPES [pH 7.4], 10 mM MnCl₂, 5 μM ATP) in the presence or absence of exogenous peptide substrates. The precipitated complexes were then resuspended in 40 μl of kinase buffer containing 20 μg of (γ-[³²P]ATP) (3000 Ci/mmol, PBI-168, Amersham Corp.) in the presence or absence of exogenous substrates and incubated at room temperature for 7 min.

For MBP kinase assays, cells were either mock treated, or treated for 5 min at 37°C with NGF (100 ng/ml). Lysates prepared as described above were immunoprecipitated with 1 μl of antibody to Erk1 for 3 h at 4°C, and immune complexes were collected with 25 μl of a 50% solution of protein A-Sepharose. The immunoprecipitates were washed three times with lysis buffer and once with water. Kinase assays were performed in 10 μl of MBP (5 μg/10 μl, Sigma), 40 μl of kinase buffer (20 mM Tris [pH 8.0], 10 mM MgCl₂, 1 mM dithiothreitol, 10 μM ATP) and 10 μl of (γ-[³²P]ATP) for 2 min at 20°C. Assays were terminated by the addition of gel loading buffer (4% SDS, 80 mM dithiothreitol, 10% glycerol), the samples were resolved by SDS-polyacrylamide gel electrophoresis, and the phosphoproteins were visualized by autoradiography. Results were quantitated by scanning with a Zeineth scan laser densitometer.

NGF Binding and Internalization Analysis

Preparation of [³²P]NGF and its use to assess surface binding, dissociation, and internalization were all carried out as described previously (Loeb and Greene, 1993).

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References


Note Added in Proof