The TrkB receptor protein-tyrosine kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. In response to brain-derived neurotrophic factor and neurotrophin-3 treatment, TrkB expressed exogenously in Rat-2 cells is rapidly phosphorylated on tyrosine residues. At least 2 regions of TrkB contain phosphorylated tyrosines. The major sites of autophosphorylation are in the region containing Tyr-670, Tyr-674, and Tyr-675, which lies in the kinase domain and corresponds by sequence homology to the Tyr-416 autophosphorylation site in p60src. Tyr-785, which lies just to the COOH-terminal side of the kinase domain in a relatively short tail characteristic of the Trk family of protein-tyrosine kinase receptors, is also phosphorylated in response to neurotrophin-3 treatment. The sequence around Tyr-785 fits a consensus sequence for binding phospholipase C-γ1. The simplest interpretation of these results is that, in response to neurotrophin binding, at least two and perhaps all three of the tyrosines in the Tyr-670/674/675 region are autophosphorylated independently, and Tyr-785 is autophosphorylated in vivo. Following activation of TrkB, phospholipase C-γ1 is phosphorylated on Tyr-783, Tyr-771, and Tyr-1254. Phospholipase C-γ1 also forms a complex with TrkB in response to neurotrophin-3 treatment, consistent with the possibility that one of the TrkB autophosphorylation sites provides a binding site for the phospholipase C-γ1 SH2 domains, as is the case for other receptor protein-tyrosine kinases. We conclude that phospholipase C-γ1 is directly phosphorylated by TrkB. Since phosphorylation of Tyr-783 and Tyr-1254 results in inactivation of phospholipase C-γ1, we predict that neurotrophin-3 leads to activation of phospholipase C-γ1 following binding to TrkB in Rat-2 cells.

TrkB was the second member discovered in the growing Trk family of receptor protein-tyrosine kinases (PTKs), which so far includes Trk (Martin-Zanca et al., 1989), TrkB (Klein et al., 1989; Middlemas et al., 1991), and TrkC (Lamballe et al., 1991). The Trk family is defined by strong similarities in both the extracellular (35–50% amino acid sequence similarity) and intracellular domains (75% amino acid sequence similarity). The Trk family is expressed almost exclusively in cells of the nervous system (Klein et al., 1989; Lamballe et al., 1991; Martin-Zanca et al., 1990; Middlemas et al., 1991). The pattern of expression and the sequence similarities in the extracellular regions of the Trk family receptors implies that they might bind a family of related cytokines that would regulate neuronal function. This was borne out by the demonstration that TrkB is the receptor for NGF (Hempstead et al., 1991; Kaplan et al., 1991a, 1991b; Klein et al., 1991a). Since NGF is the prototypic member of the neurotrophin family, this in turn suggested that TrkB and TrkC might bind other neurotrophins. It was subsequently shown that BDNF, NT-3 (Glaes et al., 1991; Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991), and NT-4NT-5 (Berkemeier et al., 1991; Klein et al., 1992) are ligands for TrkB, whereas NGF is not. NT-3 is also a ligand for TrkC (Lamballe et al., 1991). Although it remains to be determined which neurotrophins are physiological ligands for TrkB, BDNF is the prime candidate (see below).

Interest in the therapeutic potential of the neurotrophins for the treatment of neurodegenerative disorders has been heightened by experiments demonstrating that BDNF increases the survival of cultured septal cholinergic (Alderson et al., 1990) and dopaminergic neurons (Hyman et al., 1991). Studies using several experimental paradigms involving seizures induced in rats by hippocampal kindling showed that there is an increase in BDNF mRNA in specific regions of the hippocampus after seizures (Dugich-Djordjevic et al., 1992; Ernfors et al., 1991). These studies were extended to show that seizures induced by hippocampal kindling in the rat lead to a rapid increase of both trkB mRNA and TrkB proteins in the dentate gyrus (Merio et al., 1992) in parallel with the increase in BDNF mRNA. These results imply that BDNF may be a physiological ligand for the TrkB receptor and suggest a role for BDNF in neuronal protection via an autocrine or paracrine mechanism. In another experimental model, an increase in the expression of TrkB is observed in the adult rat spinal cord following injury, which suggests a role for TrkB in axonal sprouting and glial interactions (Frisén et al., 1992). This coupled with the fact that BDNF can rescue spinal motoneurons from axotomy-induced cell death in neonatal rats (Sendtner et al., 1992; Yan et al., 1992) and from naturally occurring cell death in chick development (Oppenheim et al., 1992) raises the possibility that TrkB is the receptor involved in the neurotrophic effects of BDNF in motoneurons. Striking additional evidence for the role of TrkB in motoneurons is provided by mice with a mutation produced by a targeted disruption of the trkb gene. Mice which are homozygous for the mutation lack functional TrkB receptor and exhibit a decrease in the number of motoneurons in addition to several other nervous system lesions (Klein et al., 1993).
The finding that the Trk family are the receptors for the neurotrophin family has prompted a renewed focus on the molecular mechanisms of neuronal cellular signal transduction. Like other receptor PTKs, the Trk family are activated following ligand binding by dimerization (Jing et al., 1992). Activation leads to autophosphorylation (Berkemeier et al., 1991; Kaplan et al., 1991b; Klein et al., 1991a, 1991b; Lamballe et al., 1991; Midlands, 1993; Soppet et al., 1991; Squinto et al., 1991), and phosphorylation of a set of cytoplasmic substrates (Maher, 1988). Little is known about the substrates of the Trk family PTKs, but there has been considerable progress in identifying substrates for other receptor PTKs (Anderson et al., 1990; Pawson and Gish, 1992). To determine whether any of these substrates plays a role in TrkB-mediated signal transduction in neurons, we have begun investigating whether such proteins may be substrates for the TrkB receptor PTK. Many growth factors induce turnover of PI, through the activation of PI-specific phospholipase C's, which generate the second messengers diacylglycerol and inositol triphosphate from phosphatidylinositol 4,5-bisphosphate. This activated PI kinase C elicits effects as the result of its direct phosphorylation by their respective receptor PTKs (Margolis et al., 1989; Meisenhelder et al., 1989). However, PLC-γ is not activated by all PTK receptors. For instance, CSF-1 does not stimulate PI turnover, nor is PLC-γ a substrate for the CSF-1 receptor (Downing et al., 1989). With regard to the Trk family, it has been shown that NGF treatment of PC12 cells induces PI turnover (Altin and Bradshaw, 1990; Chan et al., 1989; Contreras and Guroff, 1987), and rapid phosphorylation of PLC-γ on tyrosines (Kim et al., 1991b; Vetter et al., 1991). In this study we investigated neurotrophin-induced activation and auto-phosphorylation of TrkB and identified the major autophosphorylation sites. We also showed that PLC-γ is a substrate for the TrkB receptor and that PLC-γ forms a complex with the activated TrkB receptor.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antibodies—**TrkB-expressing cell lines were made by infection of Rat-2 fibroblast cells, which do not normally express TrkB, with a virus encoded by a retroviral vector, pNLN-SLX CMV (Scharffmann et al., 1991), into which we subcloned the entire coding sequence for the rat TrkB receptor (Midlands, 1993). TrkB expression is under the control of a cytomegalovirus promoter which gives a high level of constitutive expression. The vector also contains neo^R^ to provide a selectable marker. After G418 selection, clonal cell lines were analyzed for TrkB expression by immunoblotting of whole cell lysates resolved by SDS-PAGE. Additional analysis indicated that the RST 15 cell line expressed TrkB, which was phosphorylated on tyrosine in response to BDNF and NT-3 treatment, indicating that the exogenously expressed TrkB receptors were functional. Polyclonal antibodies were raised in rabbits against the entire extracellular region of TrkB (residues 21-417 according to the numbering in Midlands et al. (1991) expressed in and purified from Escherichia coli (Midlands, 1993)). Monoclonal antibodies raised against PLC-γ were generously provided by Sue Goo Both (National Institutes of Health, Bethesda, MD) (Ryu et al., 1987; Suh et al., 1998). Polyclonal anti-phosphotyrosine antibodies were provided by Mark Kamps and Bart Sefton (The Salk Institute, La Jolla, CA). Purified recombinant NT-3 and BDNF were provided by Arnon Rosenthal and Mark Kamps and Bart Sefton (The Salk Institute, La Jolla, CA). RST 15 cells were cultured in and treated with neurotrophins in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum unless otherwise stated. The line was maintained under G418 selection until being split for an experiment. PDGF (the c-Sis chain dimer) was from Advanced (Thousand Oaks, CA), and EGF was from United States Biochemical Corp.

**Labeling Cells and Immunoprecipitations of PLC-γ and TrkB—**Subconfluent RST 15 cells (10-cm dishes) were labeled for 16 h in 10% dialyzed fetal calf serum in DMEM containing [32P]orthophosphate (ICN, Costa Mesa, CA) at a concentration of 1 mCi/ml. The cells were then harvested with either 100 ng/ml at 15, 50 ng/ml at 5 ng/ml EGF for 5 min, washed twice with cold TD (Tris-buffered saline lacking MgCl₂ and CaCl₂), and then lysed in 1 ml of RIPA buffer (Harlow and Lane, 1988). One 10-cm dish was used for each phosphotryptic map. Slightly modified conditions were used for the PLC-γ immunoprecipitation in that cells were cultured in 6-cm dishes until they reached confluence, and then they were incubated for the last 24 h in 0.5% calf serum in DMEM before a 15-h incubation in 2.5 ml of DMEM containing 1 mg/ml of F-1-4, 0.25 mg/ml F-7-2, and 2 mCi/ml [32P]orthophosphate. Following treatments as above, cells were lysed in 0.5 ml of RIPA buffer. Two 6-cm dishes were used for each phosphotryptic map. PLC-γ was immunoprecipitated with a mixture of antibodies containing a final concentration of 0.5 µg of B-6-4, 0.25 µg F-1-4, and 0.25 µg F-7-2 in 1 ml of RIPA buffer, as described in Meisenhelder and Hunter (Meisenhelder and Hunter, 1991). TrkB was immunoprecipitated using a final concentration of 5 µl of anti-TrkB serum 5050 in 1 ml of RIPA buffer. The lysates were incubated for 2 h on ice, followed by incubation with 25 µl of protein A-Sepharose beads (Repligen, Cambridge, MA) with rotation at 4 °C. The beads/immuno complexes were pelleted in a microcentrifuge then resuspended in 1 ml of RIPA buffer. Such washes were repeated three times after which remaining RIPA buffer was removed from the beads by aspiration through a 26-gauge needle. The samples were then taken up in 30 µl of 2 x SDS-PAGE sample buffer. After SDS-PAGE, the gels were dried and autoradiographed. PLC-γ and TrkB bands were excised for either tryp- tokinase digestion or phosphoamino acid analysis, which were then performed according to the detailed protocols reviewed by Boyle et al. (1991). PLC-γ samples were treated with performic acid to oxidize cysteines. The peptide pattern was similar for TrkB with or without oxidation. TrkB was oxidized in the experiment in Fig. 1 and not in the subsequent phosphotryptic peptide mapping experiments. The tryptic phosphopeptides were resolved on cellulose thin layer plates by electrophoresis in pH 1.9 buffer for 20 min at 1 kV in the first dimension, followed by chromatography in phosphochromobu buffer in the second dimension (Boyle et al., 1991).

**Synthesis of Phosphopeptides for Comigration Experiments—**Two peptides matching the sequence of rat TrkB residues 666-676 and 781-790 were synthesized using Fmoc (N-(9-fluorenyl)methoxycarbonyl chemistry with a Synergy automated peptide synthesizer according to the protocols provided by the manufacturer (Applied Biosystems, Foster City, CA). The sequence of peptide Salk 1 was derived from GSTYDRFQAT. The sequence of peptide Salk 7 was derived from ASPYVLDDLG (Salk 7-81-790). The peptides were phosphorylated in vitro by baculovirus-expressed p60src immunoprecipitated with anti-Src monoclonal antibody 327 and protein A-Sepharose beads provided by Martin Broome (The Salk Institute, La Jolla, CA). The peptides were dissolved in water at 1 mg/ml. The c-Src-Sepharose beads were taken up in 50 µl of 10 mW PIPES, pH 7.0, and 1 µm diethiotheitol and pelleted in a microcentrifuge and then suspended in 15 µl of kinase buffer (10 mW PIPES, pH 7.0, 5 µm MnCl₂, and 1 µm diethiotheitol) with 1 µl of peptide solution and 1 µl of [γ-32P]ATP (10 µCi) (3000 Ci/mmol, Amerham Corp.) and incubated for 5 min at 30 °C. The peptide was then immersed in the presence of thin layer cellulose plates by electrophoresis at pH 1.9 for 20 min at 1 kV (Boyle et al., 1991). A control reaction lacking peptide was performed and run beside the peptide samples to facilitate identification of the peptide components from the kinase reaction. The crude yield of the phosphopeptides derived from Salk 1 was 145,500 cpm, and the crude yield of the phosphopeptide derived from Salk 2 was 31,500 cpm. Both peptides were further purified by a two-dimensional separation involving electrophoresis at pH 1.9 followed by chromatography in phosphochromobu buffer (Boyle et al., 1991) on thin layer cellulose plates. Phosphoamino acid analysis (Boyle et al., 1991) confirmed both peptide preparations contained phosphotyrosine, but not phosphoserine or phosphothreonine. Comigration experiments were performed with these phosphopeptides and TrkB phosphotryptic peptides derived from RST 15 cells stimulated with NT-3 as described above. The conditions for the comigration experiments were identical to those described above.

**Anti-phosphotyrosine Immunoblotting of PLC-γ—**RST 15 cells were grown to near confluence in 10-cm dishes and then treated with 50 ng/ml NT-3 or PDGF for 5 min at 37 °C. Following two washes with TD at 4 °C, cells were lysed in 1 ml of RIPA buffer. Immunoprecipitations were carried out by incubation of the lysates with either 5 µl of rabbit polyclonal anti-TrkB serum 5050 for 2 or 1 µg of anti-PLC-γ monoclonal antibody F-7-2 for 1 h followed by incubation with 3 µg of goat anti-mouse IgG (Cappel, Durham, NC) for 1 h. The lysates were then incubated with 20 µl of protein A-Sepharose beads with rotation at 1 h at 4 °C. The beads were pelleted by centrifugation for 30 s in a microcentrifuge and resuspended in 2 µl of 2 x SDS-PAGE sample buffer prior to SDS-PAGE. Prestained molecular weight mark-
ers (Amersham Corp.) were used. After resolution on a 7.5% SDS-PAGE gel, the proteins were transferred to Immobilon-P (Millipore) using a semidyry rapid blotter (Aercylitech, Irvine, CA) according to the protocol of Kyhse-Anderson (Kyhse-Anderson, 1984). Anti-phosphotryptic immunoblotting was performed using a rabbit polyclonal serum raised against poly-Ala/Gly/phosphotyrosine (Kamps, 1991).

**Immunoprecipitation of PLC-yl-TrkB Complexes and Immunoblotting**—Confluent 10-cm dishes of RST 15 cells were stimulated for 5 min with 50 ng/ml NT-3 followed by two washes with TD buffer at 4 °C. The cells were then lysed in either 1 ml of Nonidet P-40 buffer (337 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 1 mM CaCl₂, 1% Trasylol, and 800 μM sodium vanadate) or RIPA buffer. PLC-yl or TrkB were immunoprecipitated, resolved by electrophoresis, and transferred to Immobilon-P as described above. Immunoblot analysis to detect PLC-yl was performed as follows. The membrane was blocked for 1 h at 22 °C in 2% bovine serum albumin in TBS (10 mM Tris-HCl, pH 8.0, and 150 mM NaCl). The blot was washed three times in TBST (TBS with 0.05% Tween 20) with shaking and then incubated with an anti-PLC-yl monoclonal antibody mixture (F-7-2, F-1-4, D-7-3, and E-9-4) in TBST for 4 h, followed by three further washes in TBST prior to autoradiography. Immunoblot analysis for TrkB was performed using a rabbit polyclonal antisera raised against the extracellular region of rat TrkB, serum 5049 (Middlemas, 1993).

**RESULTS**

**TrkB Autophosphorylation**—In the absence of a suitable neuron-derived cell line expressing the full-length TrkB to study neurotrophin-induced TrkB autophosphorylation and signaling, we made use of RST 15 cells, which are a clone of Rat-2 fibroblasts expressing exogenous rat TrkB. RST 15 cells were made by infecting Rat-2 cells with a retroviral vector containing the entire coding region of full-length rat TrkB under the control of a cytomegalovirus promoter and a selectable marker gene, neoβ (Middlemas, 1993). Although it will ultimately be necessary to study TrkB-induced phosphorylation in a neuronal cell, the use of a TrkB-expressing fibroblast line to study TrkB-induced phosphorylation events appears justified, since NT-3 and BDNF treatment of such cells induces a number of responses including immediate early gene expression, DNA synthesis, and cell growth and transformation (Cordon-Cardo et al., 1991; Glass et al., 1991; Klein et al., 1991a, 1992).

To study neurotrophin-induced TrkB autophosphorylation, we carried out tryptic peptide mapping on 32P-labeled TrkB isolated from NT-3-treated RST 15 cells. NT-3 treatment induced the appearance of 4 new phosphotryptic peptides, peptides 1–4 (Fig. 1C), in TrkB tryptic digests (compare Fig. 1, A and B). Very similar induced phosphorylations in TrkB were obtained following BDNF treatment of RST 15 cells, but we have used NT-3 throughout this study because of availability. Phosphopeptides 1, 2, and 3 were found to be phosphorylated on only tyrosine, whereas phosphopeptides 5, 6, and 7 were phosphorylated on serine only. It is possible that phosphopeptide 4 is also phosphorylated on tyrosine, since it was induced by NT-3 treatment, but we have yet to obtain enough 32P radioactivity incorporated into TrkB for a definitive phosphoamino acid analysis of this peptide. Peptides 1 and 2 were phosphorylated to a greater extent than peptides 3 and 4 in response to neurotrophin treatment. Also, treatment of RST 15 cells with either PDGF or EGF did not produce any changes in the phosphorylation of TrkB compared to the untreated control cells (Fig. 2), thus confirming that peptides 1, 2, 3, and 4 are phosphorylated specifically in response to NT-3. There was some increase in the phosphorylation of TrkB phosphopeptides containing phosphoserine, which is presumably due to the secondary activation of protein-serine kinases by activated TrkB, as is found for other receptor PTKs.

Examination of the amino acid sequences containing tyrosines in the intracellular domain of TrkB revealed likely candidates for peptides 1, 2, and 3. Peptides 1 and 2 have a net positive charge in the electrophoretic dimension and therefore are likely to be phosphorylated on only one tyrosine. Because their mobilities are identical in the electrophoresis dimension and only slightly different in the chromatography dimension, it seemed likely that these phosphopeptides could be derived from a region of the TrkB receptor containing multiple tyrosines. Phosphorylation of a synthetic peptide corresponding to the region of p60c-src containing Tyr-90 and Tyr-92 results in a very similar doublet pattern following two-dimensional sepa-
FIG. 2. Phosphotryptic peptide maps of TrkB from RST 15 cells treated with NT-3, PDGF, and EGF. TrkB was immunoprecipitated from control or growth factor-treated 32P-labeled RST 15 cells and prepared for analysis as described in Fig. 1. TrkB tryptic digests were analyzed by a two-dimensional separation as described in the legend to Fig. 1 from cells which were untreated (A), treated for 5 min with 100 ng/ml NT-3 (B), 50 ng/ml PDGF (C), or 50 ng/ml EGF (D). The plates were exposed to presensitized XAR film with an intensifying screen for 6 days at -70 °C.

Peptides corresponding to these two tryptic peptides, Salk 1 (DYVSTDYYR) and Salk 2 (ASPVYLDDLG), were synthesized and phosphorylated in vitro by immunoprecipitated p60c-src. The mobilities of these synthetic phosphopeptides were then compared with the mobilities of TrkB phosphotryptic peptides. Phosphorylation of the peptide Salk 1 generated a doublet whose members comigrated in a two-dimensional separation with peptides 1 and 2 (Fig. 3). Furthermore, phosphopeptides 1a, 1b, 2a, and 2b, which were generated by partial amino acid hydrolysis of peptides 1 and 2 and of Salk 1, had similar mobility in the two-dimensional electrophoretic separation used for phosphoamino acid analysis (Fig. 4). This confirms that there is neurotrophin-induced phosphorylation of TrkB in the region containing Tyr-670, Tyr-674, and Tyr-675. Based on the chromatographic separation of peptides 1 and 2, it appears that at least 2 of these 3 tyrosines are phosphorylated independently, i.e., one phosphate/molecule. It is possible that all 3 tyrosines may be phosphorylated, since it seems likely that tryptic peptides phosphorylated at either Tyr-674 and Tyr-675 might have identical mobilities in this two-dimensional separation system, whereas the same peptide phosphorylated at Tyr-670 could have a different mobility. Determination of which of these three tyrosines are phosphorylated requires sequence analysis using Edman degradation and site-directed mutagenesis. There is no obvious candidate for a phosphopeptide containing two phosphates in this region, which should migrate slightly above the origin, but this possibility needs further investigation. The phosphorylated product of peptide Salk 2 had an identical mobility with peptide 3 confirming that Tyr-785 is a site of phosphorylation in response to neurotrophin treatment (Fig. 5). We conclude that one or more tyrosines in the Tyr-670/674/675 region and Tyr-785 are autophosphorylated in response to neurotrophin binding to TrkB.

Complex Formation between Activated TrkB and PLC-yl—To determine whether PLC-yl is a substrate of the TrkB receptor, growing RST 15 cells were treated with NT-3 or BDNF. PLC-yl was immunoprecipitated and examined by anti-phosphotyrosine immunoblotting for the presence of phosphorytrosine. In response to NT-3 treatment (50 ng/ml), PLC-yl was phosphorylated on tyrosine residues within 5 min (compare Fig. 6, lanes 1 and 2). As a positive control, stimulation of RST 15 cells with 50 ng/ml PDGF was shown to result in phosphorylation of PLC-yl on tyrosine (Fig. 6, lane 3). The anti-PLC-yl immunoprecipitates from NT-3-stimulated cells also contained a 145-kDa phosphotyrosine-containing protein that is probably TrkB. Stimulation with PDGF likewise resulted in the coprecipitation of a 180-kDa phosphotyrosine-containing protein that is likely to be the PDGF receptor. Whether neurotrophin treatment induces the formation of TrkB-PLC-yl complexes is addressed more specifically below.

Since it has been documented previously that phosphorylation on Tyr-783 is essential for stimulation of PLC-yl activity (Kim et al., 1991a), we further characterized the neurotrophin-dependent phosphorylation of PLC-yl by phosphotryptic peptide mapping. In addition to Tyr-783, phosphorylation of Tyr-1254 is likely to enhance the catalytic activity of PLC-yl, since mutation of Tyr-1254 to Phe decreases activation of PLC-yl upon PDGF treatment (Kim et al., 1991a). Analysis of PLC-yl phosphorylation in response to either NT-3 or PDGF treatment of quiescent RST 15 cells showed that phosphorylation of peptides a, b, and c was stimulated (Fig. 7). Peptides a and c have been identified previously as tryptic peptides phosphorylated on Tyr-783 and Tyr-1254, respectively (Kim et al., 1990; Wahl et al., 1990). Peptide b is likely to result from phosphorylation of Tyr-771. As phosphorylation on Tyr-783 of PLC-yl is essential for the activation of its phospholipase activity, it is reasonable to conclude from these experiments that NT-3 activates PLC-yl by inducing direct phosphorylation of PLC-yl on Tyr-783 and Tyr-1254 by the TrkB receptor. PLC-yl was found to be phosphorylated on the same residues following neurotrophin treatment of either quiescent or growing RST 15 cells.

Many proteins that bind to activated receptor PTKs contain regions homologous to Src defined as Src homology 2 (SH2) domains. It is these SH2 domains which bind to specific phosphotyrosines in receptor PTks. PLC-yl, which contains two SH2 domains, has been shown to form complexes with activated PDGF, EGF, and FGF receptors. We tested whether TrkB and PLC-yl might form a complex in cells stimulated with neurotrophins by performing immunoprecipitations using either anti-TrkB- or anti-PLC-yl-specific antibodies followed...
**FIG. 3.** Comigration of TrkB phosphotryptic peptides with Salk 1 synthetic phosphopeptide. TrkB was isolated from $^{32}$P-labeled RST 15 cells after treatment with NT-3 for 5 min and digested with trypsin as described in the legend to Fig. 1. The peptide Salk 1 was phosphorylated in vitro by p60-src, and the phosphorylated peptide was purified as described under "Experimental Procedures." The TrkB tryptic digest and the Salk 1 phosphopeptide were separated in two dimensions as described in the legend to Fig. 1. The top row of panels shows exposures of the entire phosphotryptic maps, whereas the bottom row shows shorter exposures of the regions of the maps containing peptides 1 and 2 to demonstrate that there were two spots corresponding to peptides 1 and 2 (see Fig. 1). A, $^{32}$P-labeled TrkB from NT-3-treated cells; B, a mixture of TrkB from NT-3 treated cells with $^{32}$P-labeled Salk 1 peptide; C, $^{32}$P-labeled Salk 1 peptide. The plates were exposed to presensitized XAR film with an intensifying screen at $-70^\circ$ C for 8 days for the tryptic maps in the top row and 4 days for the shorter exposures of peptides 1 and 2 in the bottom row.

**FIG. 4.** Phosphoamino acid analysis of TrkB phosphotryptic peptide 1 and 2 and synthetic Salk 1 phosphopeptides. TrkB was isolated from NT-3-treated $^{32}$P-labeled RST 15 cells, and a tryptic digest was prepared as described in Fig. 3. The tryptic digest was resolved in two dimensions, and phosphopeptides 1 and 2 (see Fig. 1) were scraped from the plate and eluted from the cellulose. Phosphoamino acid analysis was then performed on the individual peptides and on the mixture of phosphopeptides resulting from phosphorylation of peptide Salk 1 in vitro by p60-src. A, phosphoamino acid analysis of TrkB phosphotryptic peptide 1; B, phosphoamino acid analysis of TrkB phosphotryptic peptide 2; C, phosphoamino acid analysis of the $^{32}$P-labeled Salk 1 peptide; D, schematic indicating the positions of phosphoserine, phosphothreonine, and phosphotyrosine (S, T, Y, respectively) and the peptides 2a, 1a, 2b, and 1b, respectively. The plates were exposed to XAR film for 9 days (A and B) or to presensitized XAR film with an intensifying screen for 3 days at $-70^\circ$ C (C).

**FIG. 5.** Comigration of TrkB phosphotryptic peptides with Salk 2 synthetic phosphopeptide. TrkB was isolated from NT-3-treated $^{32}$P-labeled RST 15 cells, and a tryptic digest was prepared as described in the legend to Fig. 3. The peptide Salk 2 was phosphorylated in vitro by p60-src, and the phosphorylated peptide was purified as described under "Experimental Procedures." The TrkB tryptic digest and the Salk 2 phosphopeptide were separated in two dimensions as described in the legend to Fig. 1. A, $^{32}$P-labeled TrkB from NT-3-treated cells; B, a mixture of TrkB from NT-3-treated cells with $^{32}$P-labeled Salk 2 peptide; C, $^{32}$P-labeled Salk 2 peptide. The plates were exposed to presensitized XAR film with an intensifying screen for 6 days at $-70^\circ$ C.

by immunoblotting with anti-PLC-γ1 or anti-TrkB specific antibodies, respectively. NT-3 treatment resulted in the formation of TrkB-PLC-γ1 complexes, which were detected by immunoprecipitation with anti-TrkB antibodies from cell lysates made in buffer containing only a non-ionic detergent (Fig. 8, lanes 1 and 2). The complex was also immunoprecipitated under harsher cell lysis conditions (RIPA buffer) (Fig. 8, lanes 3 and 4). The converse experiment demonstrates that a TrkB-PLC-γ1 complex could be immunoprecipitated with anti-PLC-γ1 antibodies and detected with anti-TrkB antibodies by immunoblot analysis (Fig. 8, lanes 6 and 7). A TrkB-PLC-γ1 complex was not immunoprecipitated from the untreated control cells. These results taken together establish that NT-3 induces the formation of a TrkB-PLC-γ1 complex.
DISCUSSION

In this study, we have established that the major sites of ligand-induced autophosphorylation in the TrkB receptor PTK catalytic domain lie in the region homologous to Tyr-416 in c-Src, which is its major autophosphorylation site. At least two and perhaps all three of the tyrosines in this region of TrkB, Tyr-670, Tyr-674, and Tyr-675, are phosphorylated in response to NT-3. The amino acid sequences of the Trk family catalytic domains are most closely related to those of the insulin receptor family amongst the receptor PTKs, even though there is no sequence homology between the extracellular regions of these two families, nor between the insulin-related growth factors and the neurotrophins. Tyr-670, Tyr-674, and Tyr-675 of TrkB are homologous to Tyr-1158, Tyr-1162, and Tyr-1163 of the insulin receptor, which are known to be major insulin-induced autophosphorylation sites (Tornqvist et al., 1988; White et al., 1988). In both the insulin and Trk family receptors, this region contains a characteristic sequence motif containing 3 tyrosines, a single tyrosine followed by two vicinal tyrosines 3 amino acids toward the COOH terminus (XXXXXY). In the case of the insulin receptor, all three tyrosines are phosphorylated in response to insulin in an ordered fashion, with 1162 being phosphorylated first followed by 1158 and then 1163 (Dickens and Tavare, 1992; Levine et al., 1991; Tavare et al., 1991; Tornqvist et al., 1988; White et al., 1988). Occupancy of these phosphorylation sites correlates with increased kinase activity, and the autophosphorylated receptor becomes ligand independent (Flores-Riveros et al., 1987; Rosen et al., 1983; White et al., 1988). Analysis of these phosphorylation sites by mutagenesis indicates that Tyr-1162 and Tyr-1163 are the most critical for biological activity (Wilden et al., 1992a, 1992b; Yonezawa and Roth, 1991; Zhang et al., 1991). Mutation of both Tyr-1162 and Tyr-1163 to Phe leads to decreased catalytic activity in response to insulin, whereas mutation of Tyr-1158 has little effect (Zhang et al., 1991). A triple mutant is even more severely affected (Murakami and Rosen, 1991; Rafaeloff et al., 1991; Wilden et al., 1992a). In general, the signaling responses of the mutant receptors, including increased glucose uptake, glycogen synthesis, DNA synthesis, and phosphorylation of substrates, such as IRS-1, correlates with the level of receptor kinase activity. The single mutants have variable effects, but even a Tyr-1158 mutant is largely defective in some cell types (Wilden et al., 1990). Some responses appear to be less sensitive to these mutations, and in some reports even the triple mutant receptor has been found to have residual insulin-stimulated signaling activity and to be able to induce limited substrate phosphorylation (Rafaeloff et al., 1991).

All three of the major TrkB autophosphorylation site tyrosines are conserved in Trk and TrkC. Tyr-503 and Tyr-504 in the Trk oncoprotein, which are equivalent to Tyr-674 and Tyr-675 in TrkB, have been mutated to phenylalanine individually, and both mutants show a significant decrease in in vitro kinase activity and in transforming activity (Mitra, 1991). Although these tyrosines have not been shown to be autophosphorylation sites in either the full length Trk receptor or the Trk oncoprotein, it seems likely that this is the case, especially since the major autophosphorylation site of the related hepatocyte growth factor (HGF) receptor PTK is the tyrosine equivalent to 675 (Ferracini et al., 1991). The phenotype of the Trk oncoprotein mutants indicates that phosphorylation at these tyrosines potentiates Trk function (Mitra, 1991). We have not yet determined whether TrkB becomes multiply phosphorylated in this region in response to neurotrophin binding and, if it does, whether this occurs in an ordered fashion as is the case with the insulin receptor. More importantly, it remains to be determined whether autophosphorylation of this domain is a requirement for activation of the TrkB receptor kinase activity and whether these sites of autophosphorylation are required for the responses of neurons. Point mutants of these tyrosines should prove useful in dissection of the cellular signal transduction pathways activated by the neurotrophins.

An understanding of the responses of cells to the activation of the TrkB receptor will require a knowledge of the substrates of the TrkB receptor kinase. Over the past few years it has become clear that many of the substrates for receptor PTKs associate with the activated, autophosphorylated receptors via a conserved domain of about 100 amino acids, termed SH2 domain, originally noted in the v-Src and v-Fps oncoproteins (Anderson et al., 1990; Koch et al., 1991; Pawson, 1988; Pawson and Gish, 1992). SH2 domain binding occurs with high affinity in a sequence specific fashion to phosphotyrosine residues, with the 3 amino acids to the COOH-terminal side of the phosphotyrosine providing key recognition elements (Fantl et al., 1992; Songyang et al., 1993). For instance, the preferred binding site for the p85 subunit of PI3-kinase is Tyr(P)-X-X-Met (Songyang et al., 1993). Because autophosphorylation sites commonly occur in the least conserved parts of the cytoplasmic domain, this means that each receptor can in principle bind to a distinct set of SH2 domain-containing proteins. Mutational analysis of receptor autophosphorylation sites supports the idea that these sites bind individual SH2 domain-containing proteins and that association is critical for the phosphorylation and activation of such substrates. Some phosphorylation site mutant receptors show defects in specific signaling responses (Fantl et al., 1992).

PLC-γ1 is one of the most studied receptor PTK substrates. It is activated by the EGF, PDGF, and FGF receptors. Following ligand binding, PLC-γ1 binds to specific autophosphorylation sites on these receptors via one or both of its SH2 domains and is then phosphorylated at 3 tyrosines leading to its activation (Kim et al., 1990, 1991a; Margolis et al., 1989; Meisenhelder et al., 1989; Wahl et al., 1990). PLC-γ1 binds to the COOH-terminal tail of the FGF receptor at the Tyr-766 autophosphorylation site (Mohammadi et al., 1991, 1992; Peters et al., 1992). Muta-
200 - Lanes blot was exposed to presensitized buffer treatment with growth factors for al., Lane bodies. The immunoprecipitates were made from cell lysates prepared or phosphothreonine. The plates were exposed to presensitized correspond to peptides containing %783, %771, and Tyr-1254, respectively. The open circles correspond to peptides containing phosphoserine or phosphothreonine. The plates were exposed to presensitized XAR film with an intensifying screen for 4 days at -70 °C.

Fig. 7. Phosphotryptic peptide mapping of PLC-γ1. PLC-γ1 was immunoprecipitated from ³²P-labeled quiescent RST 15 cells after treatments with growth factors as indicated. After resolution of the immunoprecipitates by SDS-PAGE, the PLC-γ1 bands were digested with trypsin, and the phosphotryptic peptides were analyzed by a two-dimensional separation on cellulose thin layer plates. Shown are the resulting maps of PLC-γ1 isolated from cells which were untreated (A), treated for 5 min with 100 ng/ml NT-3 (B), treated for 5 min with 50 ng/ml PDGF (C). D is a schematic diagram in which individual phosphotryptic peptides are identified by letters. Peptides labeled a, b, and c are likely to correspond to peptides containing Tyr-783, Tyr-771, and Tyr-1254, respectively. The open circles correspond to peptides containing phosphoserine or phosphothreonine.

Fig. 8. Immunoblot analysis of TrkB/PLC-γ1 complexes. TrkB/PLC-γ1 complexes were immunoprecipitated from RST 15 cells after treatment with growth factors for 5 min and lysed as indicated below. Lanes 1-4 show anti-TrkB immunoprecipitates immunoblotted with anti-PLC-γ1 monoclonal antibodies, whereas lanes 5-7 show anti-PLC-γ1 immunoprecipitates immunoblotted with anti-TrkB polyclonal antibodies. The immunoprecipitates were made from cell lysates prepared as follows: untreated cells lysed in Nonidet P-40 buffer (lanes 1 and 6), cells treated with 50 ng/ml NT-3 prior to their lysis in Nonidet P-40 buffer (lanes 2 and 7), untreated cells lysed in RIPA buffer (lane 3), and cells treated with 50 ng/ml NT-3, then lysed in RIPA buffer (lane 4). Lane 5 contains total cell proteins from NIH3T3 cells treated with 50 ng/ml PDGF, then lysed in SDS-PAGE sample buffer. The anti-PLC-γ1 blot was exposed to presensitized XAR film for 1 day with a screen. The anti-TrkB blot was exposed to XAR film for 3 days without a screen at -70 °C.

The prime candidate for the PLC-γ1 binding site in TrkB is the Tyr-785 autophosphorylation site. Tyr-785 is the only identified TrkB autophosphorylation site, other than the major autophosphorylation sites, and no SH2 domain-containing proteins have been found to bind to the insulin receptor or other receptor PTKs via these sites. Moreover, Tyr-785 is in a position to the COOH-terminal side of the catalytic domain similar to those of the PLC-γ1 binding sites in the other three receptor PTKs where PLC-γ1 binding sites have been identified (Mammamdi et al., 1991; Ronnstrand et al., 1992). Preliminary evidence indicates that the equivalent tyrosine in Trk, Tyr-753, is required for PLC-γ1 binding. Furthermore, studies of a chimeric receptor containing the EGF receptor extracellular region and the Trk transmembrane and kinase domain have provided evidence that the homologous COOH-terminal tyrosine in Trk (identified as Tyr-785 of Trk) is required for phosphorylation of PLC-γ and formation of a Trk-PLC-γ complex (Obermeier et al., 1993). Deletion of the COOH-terminal tail of the chimeric Trk abrogated phosphorylation of PLC-γ. Also, a point mutant of this Tyr to Phe resulted in decreased phosphorylation of PLC-γ and blocked formation of a Trk-PLC-γ complex in response to ligand stimulation. Table 1 shows a sequence alignment of the known PLC-γ1 binding sites, the putative binding site in TrkB, and the experimentally defined consensus sequences for the bind-

3 M. Barbacid, personal communication.
several growth factor receptor regulation of Ras activity. However, in preliminary experiments that activated v-Ras induces neurite outgrowth in PC12 cells. It was found that Shc overexpression in PC12 by mutation of Tyr-785 to phenylalanine. (Buday and Downward, 1993; Egan et al., 1992), which suggests a possible role for Shc in the activation of Ras by Shc. The Ras activation pathway might involve the SH2 and SH3 domains of Grb2 (Lowenstein et al., 1990; Thomas et al., 1992), which suggests that Ras activation by Shc may also have a role in regulation of Ras activity. It has been shown that c-Src is required for NGF-induced Trk signaling (Kremer et al., 1991), even in preliminary studies we have failed to detect association of c-Src with TrkB, even through c-Src does associate with other activated receptor PTKs such as the PDGF receptor (Kypa et al., 1990). We are currently testing whether other known receptor PTK substrates such as GTPase-activating protein (GAP), Shc, or Nck associate with activated TrkB, and whether Ras, Raf, and MAPKs are activated in response to TrkB activation as they are upon activation of Trk by NGF (Thomas et al., 1992; Wood et al., 1992).

The response of neuronal and fibroblastic cells to activation of the Trk family receptors is clearly different. In the former cell type neurotrophins elicit trophic and differentiative responses, whereas in the latter they stimulate growth. There are also differences in the way that individual Trk family members respond to different neurotrophins in neuronal and nonneuronal cells (Ip et al., 1993). Further understanding of the response of different cell types to neurotrophins will require a dissection of the signaling pathways that are triggered via the binding of SH2 domain-containing proteins to activated Trk family receptors, including PI turnover, PI 3 phosphorylation, and Ras activation. It will be most important to study Trk family PTK initiated signaling pathways in neuronal cells, since this is their normal physiological setting.

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REFERENCES
Alderson, R. F., Alterman, A. L., Barde, Y.-A., and Lindsay, R. M. (1990) Neuron 5, 297–306
Altin, J. G., and Bradshaw, Z. A. (1990) J. Neurochem. 54, 166–167
Anderson, D. Koch, C. A., Greer, L., Ellis, C., Moran, M. F., and Pawson, T. (1990) Science 250, 979–982
Backer, J. M., Myers, M. J., Shoelson, S. E., Chin, D. J., Sun, X. J., Miraolpe, M., Hu, P., Marquis, B., Skolnik, E. Y., Schlessinger, J., and White, M. F. (1992) EMBO J. 11, 3469–3479
Baltensperger, K. Kozens, L. M., Cherniack, A. D., Klarlund, J. K., Chawla, A., Banerjee, U., and Czech, M. P. (1993) Science 260, 1950–1952

Table I
PLC-γ1 sites

| Sequence | Tyrosine Binding SH2 | Receptor |
|----------|----------------------|----------|
| QYTLVLSM | Tyr-766 | PGF receptor<sup>a</sup> |
| QYTVIQQD | Tyr-992 | EGF receptor<sup>b</sup> |
| PVTILGSR | Tyr-1068 | EGF receptor<sup>4</sup> |
| NDYITLPL | Tyr-1021 | PDGF β receptor<sup>c</sup> |
| VLYTVQIP | Tyr-1009 | PDGF β receptor |
| VYILDGLG | Tyr-785 | TrkB receptor<sup>4</sup> |

<sup>a</sup> Mohammadi et al. (1991).
<sup>b</sup> Rotin et al. (1992).
<sup>c</sup> Ronnstrand et al. (1992).
<sup>d</sup> Middlemas et al. (1991).
<sup>4</sup> Songyang et al. (1990).

With regard to other known receptor PTK substrates, NGF also activates PI 3-kinase in PC12 cells (Ohmichi et al., 1992a; Soltoff et al., 1992). In one study, PI 3-kinase activity was immunoprecipitated with anti-Trk antibodies, which suggests it forms a complex with the activated Trk receptor (Soltoff et al., 1992). Another study directly contradicts this finding in that although NGF stimulated PI 3-kinase activity, there was no evidence for the formation of a PI 3-kinase-Trk complex (Ohmichi et al., 1992a). Tyr-719 in Trk and the equivalent tyrosine in TrkB, Tyr-751, both lie in a potential PI 3-kinase binding site. We have not yet tested whether PI 3-kinase associates with activated TrkB, but we have determined that the insulin receptor substrate IRS-1, which binds PI 3-kinase when phosphorylated (Backer et al., 1992), is not phosphorylated on tyrosine in NT-3-treated TrkB-expressing cells. However, the Trk family of receptors are closely related by amino acid sequence homology in the kinase domains to the insulin family of receptors. Insulin stimulation of cells results in the formation of a complex containing Grb2 and IRS-1 which may lead to Ras activation (Baltensperger et al., 1993; Skolnik et al., 1993). It has been reported that a 38 kDa protein associates with PI 3-kinase after treatment with NGF in both PC12 cells and TrkB-expressing NIH 3T3 cells (Ohmichi et al., 1992b), but a functional role for this protein remains to be elucidated. The ERK1/MAPK protein-kinase kinase, which is activated by NGF treatment of PC12 cells, has been found to associate with Trk following NGF treatment, but the closely-related ERK2/MAPK, which is also activated by NGF treatment, does not associate with Trk (Loeb et al., 1992). It has been shown that c-Src is required for NGF-induced Trk signaling (Kremer et al., 1991), but in preliminary studies we have failed to detect association of c-Src with TrkB, even through c-Src does associate with other activated receptor PTKs such as the PDGF receptor (Kypa et al., 1990). We are currently testing whether other known receptor PTK substrates such as GTPase-activating protein (GAP), Shc, or Nck associate with activated TrkB, and whether Ras, Raf, and MAPKs are activated in response to TrkB activation as they are upon activation of Trk by NGF (Thomas et al., 1992; Wood et al., 1992).

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REFERENCES
Alderson, R. F., Alterman, A. L., Barde, Y.-A., and Lindsay, R. M. (1990) Neuron 5, 297–306
Altin, J. G., and Bradshaw, Z. A. (1990) J. Neurochem. 54, 166–167
Anderson, D. Koch, C. A., Greer, L., Ellis, C., Moran, M. F., and Pawson, T. (1990) Science 250, 979–982
Backer, J. M., Myers, M. J., Shoelson, S. E., Chin, D. J., Sun, X. J., Miraolpe, M., Hu, P., Marquis, B., Skolnik, E. Y., Schlessinger, J., and White, M. F. (1992) EMBO J. 11, 3469–3479
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<sup>4</sup> D. S. Middlemas and T. Hunter, unpublished results.
