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SNT, a Differentiation-Specific Target of Neurotrophic Factor-Induced Tyrosine Kinase Activity in Neurons and PC12 Cells

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To elucidate the signal transduction mechanisms used by ligands that induce differentiation and the cessation of cell division, we utilized $p13^{suc1}$-agarose, a reagent that binds $p34^{cdc2/cdk2}$. By using this reagent, we identified a 78- to 90-kDa species in PC12 pheochromocytoma cells that is rapidly phosphorylated on tyrosine following treatment with the differentiation factors nerve growth factor (NGF) and fibroblast growth factor but not by the mitogens epidermal growth factor or insulin. This species, called SNT (*suc*-associated neurotrophic factor-induced tyrosine-phosphorylated target), was also phosphorylated on tyrosine in primary rat cortical neurons treated with the neurotrophic factors neurotrophin-3, brain-derived neurotrophic factor, and fibroblast growth factor but not in those treated with epidermal growth factor. In neuronal and fibroblast cells, where NGF can also act as a mitogen, SNT was tyrosine phosphorylated to a much greater extent during NGF-induced differentiation than during NGF-induced proliferation. SNT was phosphorylated in vitro on serine, threonine, and tyrosine in $p13^{suc1}$-agarose precipitates from NGF-treated PC12 cells, indicating that this protein may be a substrate of kinase activities associated with $p13^{suc1}$-$p34^{cdc2/cdk2}$ complexes. In addition, SNT was associated predominantly with nuclear fractions following subcellular fractionation of NGF-treated PC12 cells. Finally, in PC12 cells, NGF-stimulated tyrosine phosphorylation of SNT was dependent on the levels of Trk tyrosine kinase activity and was constitutively induced by expression of $pp60^{v\text{-}src}$. However, Ras was not required for constitutive SNT tyrosine phosphorylation, suggesting that this protein functions distally to Trk and $pp60^{v\text{-}src}$ but in a pathway parallel to that of Ras. SNT is the first identified specific target of differentiation factor-induced tyrosine kinase activity in neuronal cells.

Survival and differentiation of neurons in the peripheral and central nervous systems is regulated by neurotrophic factors. These factors include the neurotrophin family, fibroblast growth factors (FGFs), and the interleukin-like factor ciliary neurotrophic factor (1, 27, 68, 72). The neurotrophin family of factors includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5 (1, 27, 68). Neurotrophins promote the differentiation and survival of overlapping populations of neurons in culture and show distinct temporal and spatial expression patterns (30, 33, 43, 46, 48, 51). Neurotrophins bind to the Trk family of receptor tyrosine kinases, which includes Trk, TrkB, and TrkC. NGF binds to Trk; BDNF, NT-3, and NT-4/5 bind to TrkB; and NT-3 binds to TrkC. NT-3 and NT-4/5 can also interact weakly with Trk (3, 34, 36, 39, 40, 45, 64, 65).

The best-characterized neurotrophic factor is NGF. NGF is required for differentiation and survival of sympathetic and some sensory neurons in the peripheral nervous system and for the cholinergic neurons of the basal forebrain (1, 59). NGF also promotes differentiation of the rat pheochromocytoma tumor cell line PC12 into cells resembling sympathetic neurons (22). The PC12 system has provided a well-characterized model for investigation of cellular differentiation induced by neurotrophic factors. Following several days of exposure of PC12 cells to NGF or FGF, long-term transcriptionally mediated events occur, including extension of neurites and acquisition of a differentiated phenotype characterized by development of electrical excitability and biosynthesis of neurotransmitters (22, 23). While considerable information is known about the morphological effects of these factors on neurons, the precise mechanism of signal transduction used by these factors is not known.

Binding of NGF to the Trk receptor is the initial event in the neuronal differentiation of PC12 cells (36, 39). This interaction stimulates the intrinsic tyrosine kinase activity of Trk, initiating a signalling cascade involving the phosphorylation of intracellular proteins on tyrosine residues (37, 50). This signal is propagated to other messengers, ultimately leading to cell differentiation and cessation of cell growth (23). While the signal transduction pathways used by tyrosine kinases that regulate cell proliferation have been extensively studied, little is known about signal transduction by tyrosine kinases that mediate cell differentiation. Both PC12 cells and neurons provide ideal systems for the study of signal transduction during differentiation. During neuronal development, receptor tyrosine kinases must act in a single neuron to promote the opposing processes of cell differentiation and cell growth (9). In PC12 cells, NGF and epidermal growth factor (EGF) each activate the intrinsic tyrosine kinase activities of their receptors. However, NGF induces differentiation and cessation of cell division, while EGF stimulates proliferation without differentiation (22, 31, 58). Thus, the distinctive effects of different receptor tyrosine kinases may be studied in the same cell. Comparison of

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mitogenic and differentiative pathways should identify cellular proteins that determine the specificity of growth and differentiation factors.

Previous studies of neuronal proliferation and differentiation pathways in PC12 cells did not identify any significant differences in early signal transduction responses (10). The responses examined included increases in the transcription of a large number of early response genes (2, 21), alterations in the cytoskeleton (60), changes in ion transport (4), activation of $p21^{ras}$ (47, 54), and stimulation of the serine-threonine kinase activities of microtubule-associated protein kinase (5, 19), $p90^{rsk}$ (73), and B-Raf (53, 66a). In addition, the mitogen EGF and the neurotrophin NGF both induce activation or tyrosine phosphorylation of the Trk substrates PLC-γ1 and PI-3 kinase in PC12 cells (8, 38, 62, 71). Only serine phosphorylation of translation initiation factor eIF-4E has been demonstrated to occur in PC12 cells treated with NGF and not in those treated with EGF (18). NGF induces the appearance of several neuron-specific proteins (23), but these proteins are produced late in the differentiation process and are therefore not part of the early responses that define growth factor specificity. Thus, we postulate that one or more unidentified proteins must be regulated by Trk to promote cell differentiation.

To identify proteins that act in differentiative rather than mitogenic signal transduction pathways, we examined PC12 cells and primary neurons treated with neurotrophic factors or mitogens that stimulate cellular tyrosine kinase activity. Since the antimitogenic properties of neurotrophic factors may be mediated by proteins that interact with cell cycle regulators, we analyzed the association of tyrosine-phosphorylated proteins with the $p34^{cdc2}$ affinity reagent $p13^{suc1}$-agarose. $p34^{cdc2}$ is a serine-threonine kinase that controls entry into mitosis (52). $p34^{cdc2}$ interacts with at least two proteins, cyclin and $p13^{suc1}$, that are required for $p34^{cdc2}$ activity (6, 14, 15, 32). While both positive and negative effects on cell division have been attributed to $p13^{suc1}$, its true role is unknown (6, 16, 25, 29, 56). One of the most useful properties of $p13^{suc1}$ is its ability to bind $p34^{cdc2}$ with high affinity (6, 16). In the study described here, we used $p13^{suc1}$ affinity chromatography to identify a species of 78 to 90 kDa that is rapidly phosphorylated on tyrosine in neurons and PC12 cells treated with differentiation factors but not in those treated with mitogens. This protein is thus a specific component of differentiative rather than mitogenic signal transduction pathways in neuronal cells.

MATERIALS AND METHODS

Cells, growth factors, and antibodies. PC12 cells (obtained from R. Kelly, University of California, San Francisco) were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated horse serum and 5% calf serum. PC12 cells stably transfected with a human *trk* cDNA under the control of a cytomegalovirus promoter and a neomycin resistance gene (trk-PC12, clone 6-24) (28) were cultured in similar conditions with addition of 200 μg of G418 per ml to the medium. A PC12 cell line expressing a temperature-sensitive Rous sarcoma virus v-*src*-encoded protein (ts:v-src3) was obtained from S. Thomas and J. Brugge (Ariad, Inc.) and maintained at 35 or 41°C as previously described (70). U7 (7) and nnr5 cells transfected with a human *trk* gene that encodes a kinase-inactive Trk protein ($PC12\text{-}trk^{KIN^-}$) (66) were grown in RPMI containing 10% heat-inactivated horse serum, 5% fetal bovine serum, and 200 μg of G418 per ml. U7 cells, obtained from L. Greene (Columbia University), were incubated for 24 h in 0, 1, or 15% serum-containing medium before treatment with NGF. These cells were maintained at low densities and used in experiments within 3 weeks of thawing from liquid nitrogen storage. PC12 cells transfected with an oncogenically activated c-H-*ras* gene (GSras1) or with a *ras* gene that encodes a dominant-negative Ras protein (GSrasDN6) under control of a dexamethasone-inducible mouse mammary tumor virus promoter (44) were obtained from S. Halegoua (State University of New York at Stony Brook) and maintained in DMEM containing 10% horse serum and 5% fetal bovine serum. GSras1 and GSrasDN6 cells were used in experiments 3 days after addition of fresh medium. Expression of Ras proteins was induced by addition of 300 nM dexamethasone for 16 h. The neuroblastoma cell line SH-SY5Y (63) was cultured in DMEM containing 10% fetal bovine serum. PC12 cells expressing mouse platelet-derived growth factor (PDGF) β receptor (26) (provided by G. Johnson, University of Colorado School of Medicine) were grown in DMEM containing 10% horse platelet-poor plasma and 5% calf platelet-poor plasma. NIH 3T3 cells transfected with a Trk cDNA (36) were grown in DMEM containing 10% calf serum. Lysates of primary rat cortical neuron cultures (from rat fetuses of embryonic age E15) treated with neurotrophic or growth factors were kindly provided by B. Knusel (University of Southern California) and prepared as previously described (41, 42).

Antiphosphotyrosine monoclonal antibody 4G10 was provided by D. Morrison (National Cancer Institute-Frederick Cancer Research and Development Center). 4G10 antibody was covalently linked to protein A-Sepharose beads (Pharmacia, Piscataway, N.J.) as previously described (24). Anti-PLC-γ1 (67) was obtained from S. G. Rhee (National Institutes of Health) or Upstate Biologicals, Inc. (Lake Placid, N.Y.). Anti-Erk-1 (11) was obtained from J. Blenis (Harvard Medical School), anti-B-Raf (61a) was from U. Rapp (National Cancer Institute), and anti-c-Jun was from Upstate Biologicals, Inc. $p13^{suc1}$ coupled to agarose was obtained from H. Piwnica-Worms (Harvard University Medical School) or Oncogene Science (Uniondale, N.Y.).

NGF was obtained from Boehringer Mannheim Biochemicals, Inc. (Indianapolis, Ind.); Upstate Biotechnology, Inc.; or Genentech (South San Francisco, Calif.). EGF, FGF, and PDGF-BB were obtained from Upstate Biotechnology, Inc., and insulin was from GIBCO-BRL, Inc. (Grand Island, N.Y.). NT-3, BDNF, and ciliary neurotrophic factor were kindly provided by Genentech.

Immunoprecipitation and immunoblotting. Cells were treated with growth factors and lysed, and the lysates were immunoprecipitated as previously described (37, 37a). After two washes with cold Tris-buffered saline, cells (10^7) were lysed in 1 ml of 1% Nonidet P-40 (NP-40) lysis buffer (20 mM Tris [pH 8.0], 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.15 U of aprotinin per ml, 20 μM leupeptin, 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 to 4 h at 4°C. Precipitates were collected with protein A-Sepharose and washed three times with NP-40 lysis buffer and once with water. The immunoprecipitates were boiled in sample buffer (2% sodium dodecyl sulfate [SDS], 100 mM dithiothreitol, 10% glycerol, 0.25% bromophenol blue) for 5 min and electrophoresed on SDS–7.5% polyacrylamide gel electrophoresis (PAGE) gels before transfer to nitrocellulose. Protein blots were probed overnight at 4°C with culture supernatant from 4G10 monoclonal antibody-producing cells

diluted 1:1 with Tris-buffered saline with a final concentration of 0.2% Tween 20. Blots were analyzed by using an ECL chemiluminescence system (Amersham Corp., Arlington Heights, Ill.).

For $p13^{suc1}$-agarose precipitations, 20 μl of $p13^{suc1}$-agarose (1.25 mg of $p13^{suc1}$ per ml) was added to 1 ml of lysate from 10^7 cells and incubated for 2 to 4 h at 4°C. The $p13^{suc1}$-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 SDS–7.5% PAGE minigels.

In vitro protein kinase assay. $p13^{suc1}$-agarose precipitates were phosphorylated in vitro by being washed three times with 1% NP-40 lysis buffer and once with kinase buffer (30 mM Tris [pH 7.4], 10 mM $MnCl_2$) before incubation in 20 μl of kinase buffer containing 20 μCi of [γ-^{32}P]ATP for 10 min at 25°C. The kinase reaction was terminated by boiling in sample buffer and analyzed by SDS-PAGE.

Phosphoamino acid analysis. $p13^{suc1}$-agarose-associated protein(s) was labeled in vitro as described above and resolved by SDS-PAGE. The ^{32}P-labeled SNT bands were excised from the gel and eluted by rocking in 0.75 ml of 1% NP-40 lysis buffer containing 0.1% SDS overnight at 4°C. The gel fragments were pelleted by two brief centrifugations, and the supernatant containing SNT was reprecipitated either with 4G10 coupled to agarose or with $p13^{suc1}$-agarose. The precipitated proteins were fractionated on SDS–7.5% PAGE minigels, transferred to Immobilon-P (Millipore), and visualized by autoradiography. The labeled SNT bands were excised and hydrolyzed in 6 M HCl at 110°C for 90 min, and the phosphoamino acids were separated by electrophoresis on thin-layer cellulose plates as described previously (61).

Phosphatase assay. $p13^{suc1}$-agarose precipitates were treated with protein tyrosine phosphatase 1B (a gift of Nick Tonks, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) for 1 h at 30°C in 35 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2)–2 mM dithiothreitol–0.05% Triton X-100–5 mM EDTA–5% glycerol–1 mg of bovine serum albumin per ml.

Subcellular fractionation. NGF-treated PC12 cells (4×10^7) were washed twice with cold Tris-buffered saline, scraped, and pelleted by centrifugation for 5 min at 1,000 × *g* at 4°C. The cells were suspended in 1.5 ml of hypotonic buffer (20 mM Tris [pH 7.4], 10 mM KCl, 1 mM dithiothreitol, 5 mM sodium vanadate, 20 μM leupeptin, 0.15 U of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride), allowed to swell on ice for 15 min, and disrupted by 25 to 30 strokes of a Dounce homogenizer (pestle A). The nuclei were pelleted by centrifugation as described above, and the supernatant consisting of the cytosol and most of the plasma membrane was carefully removed and transferred to a fresh tube. The nuclei were washed by resuspension in 1.5 ml of the hypotonic buffer and repelleted. After transfer of the wash buffer to a fresh tube, the nuclei were suspended in 2 ml of NP-40 lysis buffer (nuclear fraction). The cytosol-plasma membrane fraction and wash fractions were combined with a concentrated solution of NP-40 lysis buffer to give a final concentration of 1× lysis buffer in 2 ml. The three fractions were incubated with rocking at 4°C for 20 min. Insoluble material was removed from the subcellular fractions by centrifugation at 10,000 × *g* for 10 min. SNT was detected by analyzing 1-ml aliquots of each fraction as described above. To monitor the effectiveness of this fractionation procedure, aliquots from the various fractions were separated by SDS-PAGE and marker proteins known to reside in different subcellular compartments were assayed by immunoblotting.

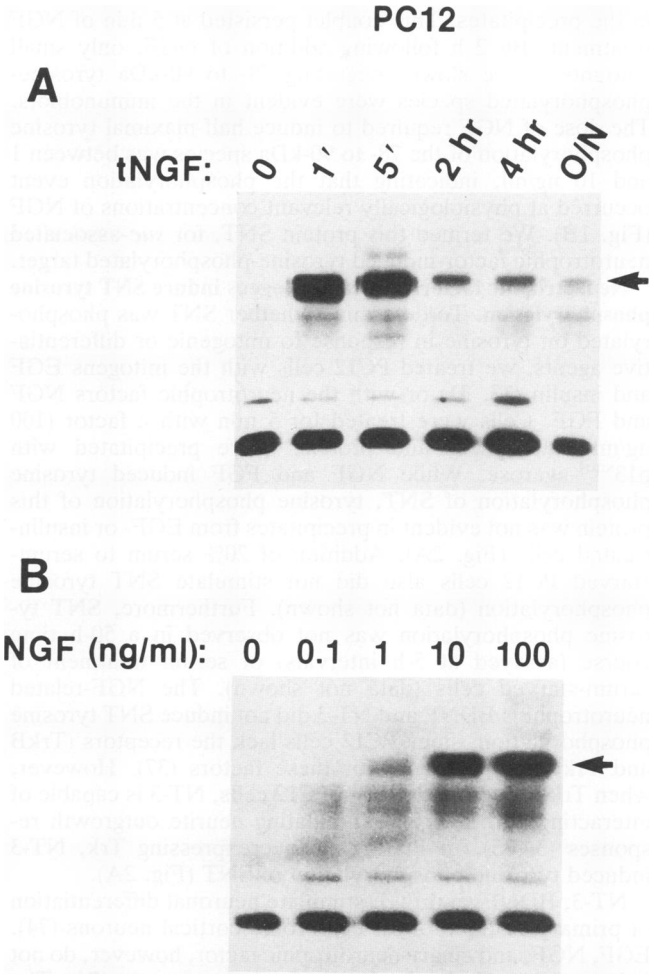

FIG. 1. Time course and dose response of SNT tyrosine phosphorylation in PC12 cells. Cells were treated with NGF at 37°C and lysed, and cell lysates were precipitated with $p13^{suc1}$-agarose. Proteins were transferred to nitrocellulose and probed with anti-Ptyr antibody. (A) Time course of SNT tyrosine phosphorylation in PC12 cells treated for 5 min with 100 ng of NGF per ml. The position of SNT is indicated by the arrows. The bands at the bottom of the gel comigrated with $p34^{cdc2}$ and $p34^{cdk2}$. O/N, overnight. (B) Dose response of SNT tyrosine phosphorylation.

RESULTS

NGF induces tyrosine phosphorylation of a 78- to 90-kDa species precipitated with $p13^{suc1}$-agarose. To investigate whether NGF addition to PC12 cells induces alterations in the tyrosine phosphorylation of proteins that associate with $p34^{cdc2}$, we used the $p34^{cdc2}$ affinity reagent $p13^{suc1}$-agarose. PC12 cells were treated with NGF and lysed, and the lysates were incubated with $p13^{suc1}$-agarose. Proteins bound to $p13^{suc1}$-agarose were probed with anti-Ptyr antibody. A band at 34 kDa was observed in the $p13^{suc1}$-agarose precipitates from untreated PC12 cells (Fig. 1A). Reprobing of the protein blots with anti-$p34^{cdc2}$ and anti-$p34^{cdk2}$ antibodies indicated that this protein migrated identically to $p34^{cdc2/cdk2}$ (data not shown). Within 1 min of NGF addition to the cells, a doublet at a molecular mass of 78 to 90 kDa was observed

in the precipitates. This doublet persisted at 5 min of NGF treatment. By 2 h following addition of NGF, only small amounts of the slower migrating 78- to 90-kDa tyrosine-phosphorylated species were evident in the immunoblots. The dose of NGF required to induce half-maximal tyrosine phosphorylation of the 78- to 90-kDa species was between 1 and 10 ng/ml, indicating that the phosphorylation event occurred at physiologically relevant concentrations of NGF (Fig. 1B). We termed this protein SNT, for *suc*-associated neurotrophic factor-induced tyrosine-phosphorylated target.

Neurotrophic factors and not mitogens induce SNT tyrosine phosphorylation. To determine whether SNT was phosphorylated on tyrosine in response to mitogenic or differentiative agents, we treated PC12 cells with the mitogens EGF and insulin (13, 31) or with the neurotrophic factors NGF and FGF. Cells were treated for 5 min with a factor (100 ng/ml) and lysed, and proteins were precipitated with $p13^{suc1}$-agarose. While NGF and FGF induced tyrosine phosphorylation of SNT, tyrosine phosphorylation of this protein was not evident in precipitates from EGF- or insulin-treated cells (Fig. 2A). Addition of 20% serum to serum-starved PC12 cells also did not stimulate SNT tyrosine phosphorylation (data not shown). Furthermore, SNT tyrosine phosphorylation was not observed in a 50-h time course (assayed at 5-h intervals) of serum treatment of serum-starved cells (data not shown). The NGF-related neurotrophins BDNF and NT-3 did not induce SNT tyrosine phosphorylation, since PC12 cells lack the receptors (TrkB and TrkC, respectively) for these factors (37). However, when Trk is overexpressed in PC12 cells, NT-3 is capable of interacting with Trk and stimulating neurite outgrowth responses (3, 35). In PC12 cells overexpressing Trk, NT-3 induced tyrosine phosphorylation of SNT (Fig. 2A).

NT-3, BDNF, and FGF stimulate neuronal differentiation of primary cultures of rat embryonic cortical neurons (74). EGF, NGF, and ciliary neurotrophic factor, however, do not induce differentiative responses in these neurons (74). The lack of response to NGF is presumably due to the absence of Trk expression in these cells. Primary cultures of rat embryonic cortical neurons do, however, express TrkB and EGF receptors (42, 73; data not shown). In rat cortical neurons, the neurotrophic factors NT-3, BDNF, and FGF induced tyrosine phosphorylation of SNT (Fig. 2B). EGF, NGF, and CNTF failed to elicit this response.

Tyrosine phosphorylation of SNT was also observed in two other cell lines undergoing neuronal differentiation. These included the human neuroblastoma cell line SH-SY5Y treated with NGF, and PDGF receptor-transfected PC12 cells treated with PDGF (data not shown). NGF and PDGF induce neuronal differentiation of SH-SY5Y cells and PDGF receptor-transfected PC12 cells, respectively (26, 63).

Each of the factors assayed in the experiments described above is known to stimulate protein tyrosine phosphorylation in responsive cells. To determine whether the mitogens and neurotrophic factors induced similar tyrosine phosphorylation responses with the exception of SNT, we assessed these responses in PC12 cells treated for 5 min with NGF, FGF, or EGF. Examination of cell lysates probed with anti-Ptyr antibody indicated that all three factors stimulated tyrosine phosphorylation of both common and unique proteins (Fig. 3A). Two of these proteins, PLC-γ1 and Erk-1, were phosphorylated on tyrosine in PC12 cells treated with NGF, FGF, or EGF (Fig. 3B and C). PLC-γ1 is also phosphorylated on tyrosine in primary rat embryonic cortical neurons treated with NT-3, BDNF, FGF, and EGF (42). Thus, while several proteins are phosphorylated on tyrosine in response to neurotrophic factor and mitogen treatment of neurons and PC12 cells, only neurotrophic factors induce SNT tyrosine phosphorylation.

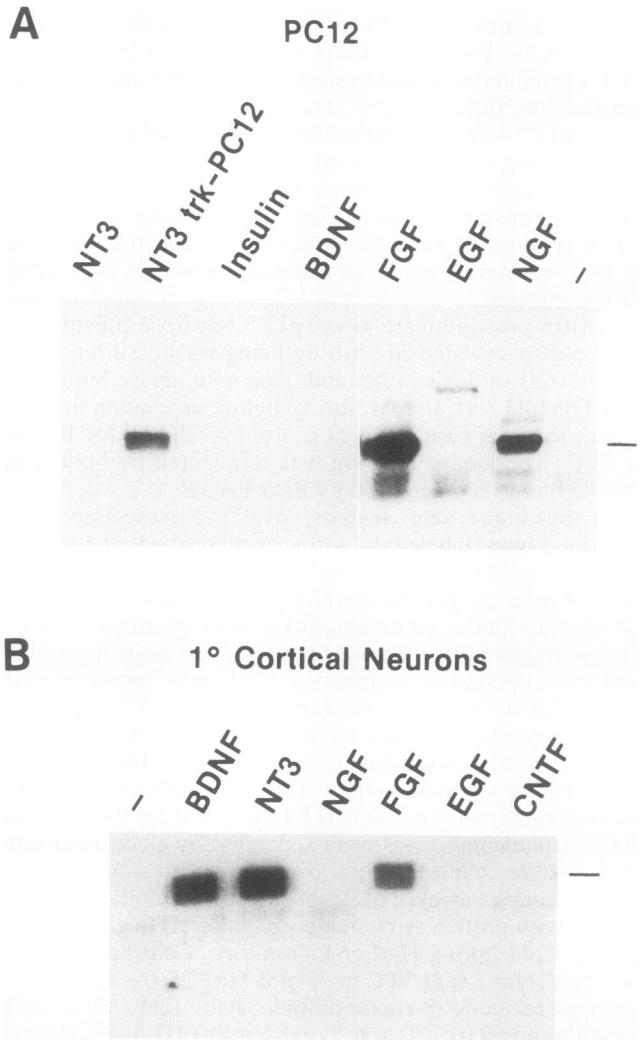

FIG. 2. Tyrosine phosphorylation of SNT in PC12 cells and primary (1°) rat cortical neurons treated with neurotrophic factors and mitogens. Cells were treated with factors for 5 min at 37°C and lysed, and cell lysates were precipitated with $p13^{suc1}$-agarose. Proteins were transferred to nitrocellulose and probed with anti-Ptyr antibody. (A) Growth factor specificity of SNT tyrosine phosphorylation in PC12 cells. Cells were treated with factors exhibiting neurotrophic (NGF, basic FGF, and NT-3) or mitogenic (EGF and insulin) activity or with BDNF. trk-PC12 cells (28) were stimulated with NT-3. Factors were used at 100 ng/ml. The position of SNT is indicated by the dash. (B) Growth factor specificity of SNT tyrosine phosphorylation in primary rat cortical neurons. Cultures were grown for 7 days and then treated with factors exhibiting neurotrophic activity (BDNF, 200 ng/ml; NT-3, 200 ng/ml; and FGF, 500 ng/ml) or mitogenic activity (EGF, 200 ng/ml) or with NGF (50 ng/ml) and ciliary neurotrophic factor (200 ng/ml).

NGF induces SNT tyrosine phosphorylation while acting as a differentiation factor and not as a mitogen. The PC12 variant cell line U7 responds to NGF by differentiating or proliferating on the basis of the concentration of serum in the medium (7). In serum-free medium, NGF induces differentiation of U7 cells, while in 1.5% serum, NGF stimulates cell proliferation. No effects of NGF are observed in 15%

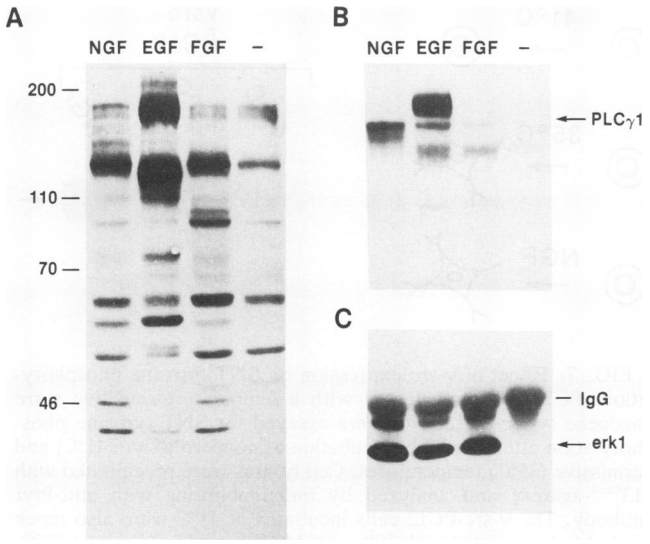

FIG. 3. Growth factor-induced tyrosine phosphorylation of cellular proteins in PC12 cells. Cells were treated with factors (100 ng/ml) for 5 min at 37°C and lysed as described in Materials and Methods. (A) Protein tyrosine phosphorylation in PC12 cells treated with NGF, EGF, or FGF. Cell lysates were fractionated on SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with anti-Ptyr antibody. Molecular masses are indicated on the left in kilodaltons. (B) Tyrosine phosphorylation of PLC-γ1. Growth factor-treated cells were lysed, and the lysates were immunoprecipitated with anti-PLC-γ1 antibody. PLC-γ1 immunoprecipitates were probed in Western blots (immunoblots) with anti-Ptyr antibody. The broad band above PLC-γ1 in the EGF lane is most likely the EGF receptor. The diffuse band below PLC-γ1 in the NGF lane is Trk (71). (C) Tyrosine phosphorylation of Erk-1. Growth factor-treated cells were lysed, and the lysates were immunoprecipitated with anti-Erk-1 antibody. Erk-1 immunoprecipitates were probed in Western blots with anti-Ptyr antibody. The positions of Erk-1 and immunoglobulin G (IgG) are indicated.

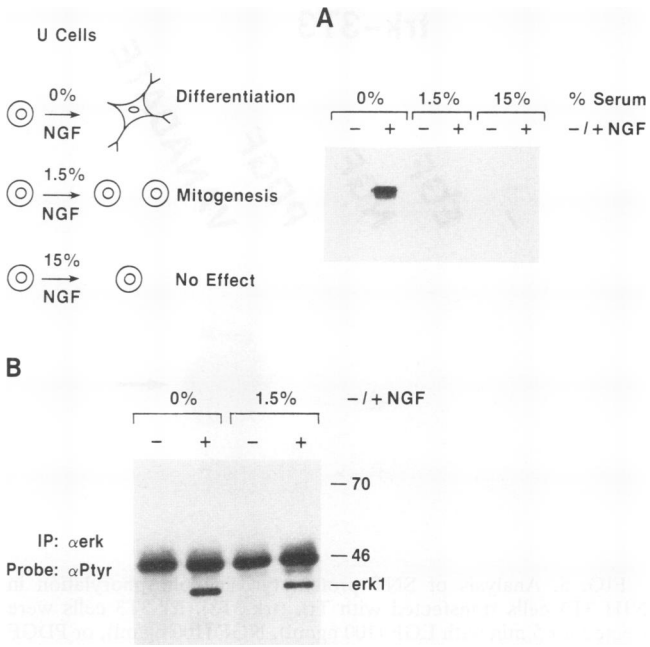

FIG. 4. Analysis of SNT and Erk-1 tyrosine phosphorylation in U7 (U) cells. The PC12 variant cell line U7 responds to NGF by differentiating or proliferating on the basis of the concentration of serum in the medium (7). NGF induces differentiation in serum-free medium and mitogenesis in 1.5% serum-containing medium. U7 cells were grown for 3 days in 0, 1.5, or 15% serum-containing medium, treated with NGF for 5 min at 37°C, and lysed. Cell lysates were precipitated with $p13^{suc1}$-agarose or anti-Erk-1, and proteins were transferred to nitrocellulose and probed with anti-Ptyr antibody. (A) Tyrosine phosphorylation of SNT. (B) Tyrosine phosphorylation of Erk-1. IP, immunoprecipitation. The numbers on the right are molecular sizes in kilodaltons.

serum-containing medium. We assayed SNT tyrosine phosphorylation in NGF-treated U7 cells in different serum concentrations. NGF induced significant SNT tyrosine phosphorylation only in serum-free medium (0% serum), where this factor promotes differentiation (Fig. 4A). Small amounts of tyrosine-phosphorylated SNT were observed in NGF-treated U7 cells grown in 1.5 or 15% serum-containing medium. NGF stimulated the tyrosine phosphorylation of Erk-1 and Trk in both serum-free medium and 1.5% serum-containing medium, indicating that the NGF signal transduction machinery was functioning in cells grown in both serum conditions (Fig. 4B and data not shown).

NGF and PDGF act as mitogens for NIH 3T3 cells transfected with Trk (Trk-3T3) (12, 35). Following treatment of these cells with NGF or PDGF, only small amounts of SNT tyrosine phosphorylation were observed (Fig. 5). SNT was present in Trk-3T3 cells, since treatment of the cells with the phosphatase inhibitor sodium orthovanadate (40 μM for 16 h) stimulated the tyrosine phosphorylation of this protein (Fig. 5). The amount of vanadate-induced SNT tyrosine phosphorylation in Trk-3T3 cells was similar to that observed in PC12 cells treated with NGF or sodium orthovanadate (data not shown).

SNT tyrosine phosphorylation is modulated by the level of Trk tyrosine kinase activity in PC12 cells. NGF-induced differentiation of PC12 cells is dependent upon the presence of functional Trk receptors. PC12 variant cell lines that lack Trk fail to differentiate in response to NGF (20). Transfection of Trk into these cell lines restores NGF responsiveness (49). The level of Trk tyrosine kinase activity also controls the timing of NGF-induced neuronal differentiation. Overexpression of Trk in PC12 cells results in rapid acceleration of neurite outgrowth in response to NGF (28). PC12 cells overexpressing Trk (trk-PC12) also show sustained NGF-mediated tyrosine phosphorylation of Trk and Trk substrates (28). These responses may be due to 10-fold higher levels of Trk tyrosine kinase activity in NGF-treated trk-PC12 cells compared with normal PC12 cells. We assayed SNT tyrosine phosphorylation in PC12 cells expressing only kinase-inactive Trk receptors or overexpressing wild-type Trk. In cells expressing kinase-inactive Trk (PC12-trk^{KIN-}), NGF failed to induce SNT tyrosine phosphorylation. FGF, however, stimulated both SNT tyrosine phosphorylation and neurite outgrowth of PC12-trk^{KIN-} cells (Fig. 6 and not shown). In PC12 cells overexpressing Trk (trk-PC12), SNT tyrosine phosphorylation was elevated for at least 16 h (O/N) in the presence of NGF (Fig. 6). Thus, the level of Trk tyrosine kinase activity in PC12 cells affects the appearance and kinetics of induction of SNT tyrosine phosphorylation in a manner similar to that of other components of the NGF signal transduction pathway.

Analysis of SNT tyrosine phosphorylation in PC12 cells expressing $pp60^{v\text{-}src}$, $p21^{ras}$, or dominant inhibitory *ras*. PC12 cells may be induced to differentiate by expression of the oncogenes v-*src* or activated H-*ras*. We asked whether

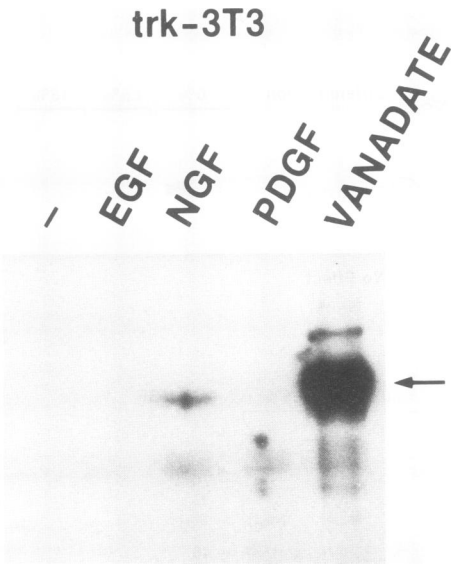

FIG. 5. Analysis of SNT protein tyrosine phosphorylation in NIH 3T3 cells transfected with Trk (trk-3T3). trk-3T3 cells were treated for 5 min with EGF (100 ng/ml), NGF (100 ng/ml), or PDGF (2 nM) or for 16 h with 40 μM sodium vanadate (vanadate). Cell lysates were precipitated with $p13^{suc1}$-agarose, and proteins were transferred to nitrocellulose and probed with anti-Ptyr antibody. The position of SNT is indicated by the arrow.

expression of these oncogenes also induces tyrosine phosphorylation of SNT. To determine whether v-*src* stimulates SNT tyrosine phosphorylation, we used PC12 cells expressing a temperature-sensitive v-*src*-encoded protein (ts:v-src3) (70). This cell line elaborates neurites at 35°C, probably owing to the presence of elevated $pp60^{v\text{-}src}$ kinase activity. At 41°C, no neurites are evident in the cells. ts:v-src3 cells were grown at 35 or 41°C, lysed, and precipitated with $p13^{suc1}$-agarose. Constitutive tyrosine phosphorylation of SNT was observed in differentiated cells grown at 35°C but not in undifferentiated cells cultured at 41°C (Fig. 7). NGF

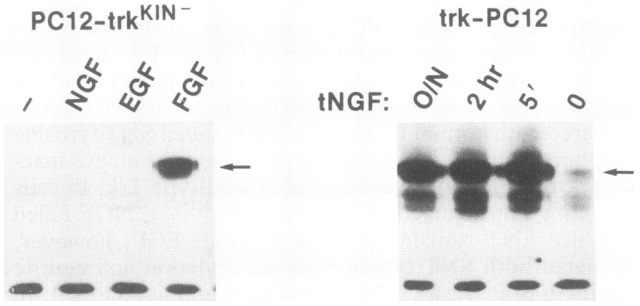

FIG. 6. Analysis of SNT tyrosine phosphorylation in cells expressing kinase-inactive Trk receptors (PC12trk^{KIN-}) or overexpressing Trk protein (trk-PC12). Cells were treated with NGF, EGF, or FGF (100 ng/ml) for 5 min at 37°C and lysed, and cell lysates were precipitated with $p13^{suc1}$-agarose. Proteins were transferred to nitrocellulose and probed with anti-Ptyr antibody. (A) SNT tyrosine phosphorylation in PC12trk^{KIN-} cells. This cell line expresses only kinase-inactive Trk receptors (PC12trk^{KIN-}). (B) SNT tyrosine phosphorylation in trk-PC12 cells. PC12 cells overexpressing Trk receptors were treated with 100 ng of NGF per ml at 37°C for the indicated times. The position of SNT is indicated by the arrows. O/N, overnight. tNGF, time of NGF treatment.

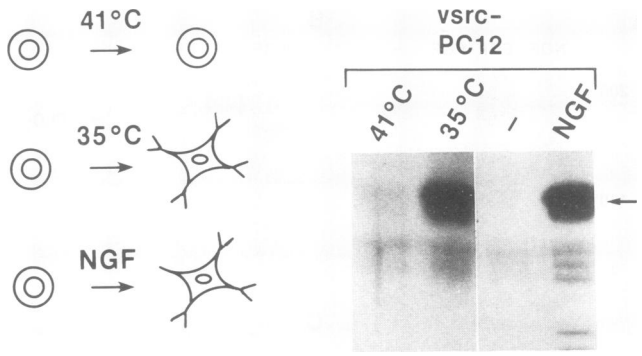

FIG. 7. Effect of v-*src* expression on SNT tyrosine phosphorylation. PC12 cells transfected with a temperature-sensitive v-*src* oncogene (v-src-PC12) (70) were assayed for SNT tyrosine phosphorylation after overnight incubation at nonpermissive (41°C) and permissive (35°C) temperatures. Cell lysates were precipitated with $p13^{suc1}$-agarose and analyzed by immunoblotting with anti-Ptyr antibody. The v-src-PC12 cells incubated at 41°C were also mock treated (−) or treated with 100 ng of NGF per ml for 5 min at 37°C. The position of SNT is indicated by the arrow.

induced both neurite outgrowth and SNT tyrosine phosphorylation in cells grown at 41°C (70; Fig. 7). Expression of activated H-*ras*, however, did not induce constitutive tyrosine phosphorylation of SNT. In this experiment, PC12 cells transfected with an activated H-*ras* gene under control of a dexamethasone-inducible mouse mammary tumor virus promoter (44) were assayed for SNT tyrosine phosphorylation in cells grown in the presence or absence of dexamethasone (300 nM for 16 h). In PC12 cells expressing activated H-*ras* owing to dexamethasone treatment, no tyrosine-phosphorylated SNT was observed (Fig. 8A, right panel). In contrast, NGF induced tyrosine phosphorylation of SNT in these cells. Furthermore, expression of a dominant inhibitory *ras* mutant in PC12 cells (44) did not block tyrosine phosphorylation of SNT after NGF treatment (Fig. 8A, left panel). Expression of this dominant inhibitory *ras* mutant has been shown to block NGF-induced neurite outgrowth responses and tyrosine phosphorylation of Erk-1 (17, 69, 75).

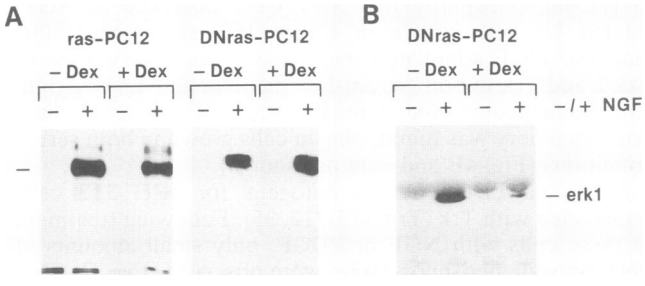

FIG. 8. Effect of oncogenically activated $p21^{ras}$ and dominant inhibitory *ras* on SNT tyrosine phosphorylation in PC12 cells. (A) PC12 cells stably transfected with activated H-*ras* (ras-PC12) or dominant inhibitory *ras* (DNras-PC12) genes under the control of a mouse mammary tumor virus dexamethasone-responsive promoter (44) were mock treated (−Dex) or treated with 300 nM dexamethasone (+Dex) for 16 h. Cells were then treated for 5 min at 37°C with 100 ng of NGF per ml and lysed, and cell lysates were precipitated with $p13^{suc1}$-agarose or anti-Erk-1. Precipitates were transferred to nitrocellulose and probed with anti-Ptyr antibody. (A) Tyrosine phosphorylation of SNT. The position of SNT is indicated with a dash. (B) Tyrosine phosphorylation of Erk-1.

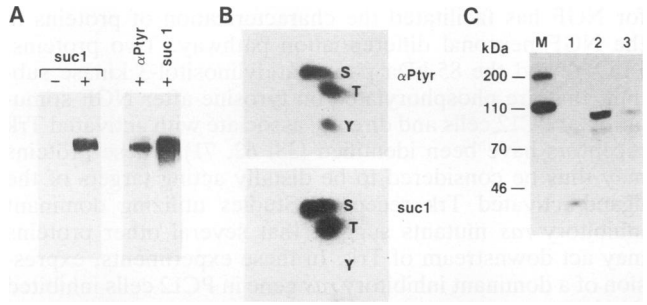

FIG. 9. Tyrosine, serine, and threonine phosphorylation of SNT in vitro. p13^{suc1}-agarose precipitates from mock (−)- or NGF (+)-treated PC12 cell lysates were incubated with [γ-^{32}P]ATP in kinase assays in vitro. Phosphoproteins were resolved on SDS–7.5% polyacrylamide gels, and SNT was excised and eluted. (A) Left panel: autoradiograph of gel-purified SNT phosphoprotein reprecipitated with p13^{suc1}-agarose. Right panel: gel-purified SNT phosphoprotein from NGF-treated PC12 cells immunoprecipitated with anti-Ptyr conjugated to Sepharose. Following immunoprecipitation with anti-Ptyr antibody, the cleared cell lysate was reprecipitated with p13^{suc1}-agarose. (B) Phosphoamino acid analysis of reprecipitated SNT from the experiment in the right half of panel A. S, phosphoserine. T, phosphothreonine. Y, phosphotyrosine. (C) Phosphatase assay. p13^{suc1}-agarose precipitates from NGF-treated PC12 cells were incubated with tyrosine-specific phosphatase 1B for 1 h at 30°C. Untreated (lane 1), NGF-treated (lane 2), and phosphatase-treated (lane 3) samples were analyzed by immunoblotting with anti-Ptyr antibody.

In our experiments, Erk-1 tyrosine phosphorylation was also greatly reduced in PC12 cells expressing the dominant inhibitory *ras* mutant (Fig. 8B). Thus, the activity of pp60$^{v\text{-}src}$, but not that of p21^{ras}, results in constitutive SNT tyrosine phosphorylation in PC12 cells.

SNT from NGF-treated PC12 cells is phosphorylated on serine, threonine, and tyrosine in vitro. p13^{suc1}-agarose binds at least several protein kinases, including p34^{cdc2}. We determined whether SNT bound to p13^{suc1}-agarose coprecipitates with kinase activities capable of phosphorylating this protein in vitro. p13^{suc1}-agarose precipitates from NGF or mock-treated PC12 cells were incubated in vitro with $MnCl_2$ and [γ-^{32}P]ATP. The precipitates were boiled and subjected to SDS-PAGE, and proteins with the molecular weight of SNT were eluted from the gel. The eluted protein was reprecipitated with p13^{suc1}-agarose, and phosphoproteins were visualized following electrophoresis. A phosphorylated 78- to 90-kDa species was observed in p13^{suc1}-agarose precipitates from NGF-treated PC12 cells (Fig. 9A). Phosphorylated SNT was not evident in precipitates from untreated PC12 cells. To determine whether SNT was phosphorylated on tyrosine in the in vitro kinase assay, gel-eluted SNT was immunoprecipitated with anti-Ptyr antibody (Fig. 9A). Phosphoamino acid analysis of this protein indicated that it was phosphorylated on serine, threonine, and tyrosine (Fig. 9B, top panel). Following immunoprecipitation with anti-Ptyr antibody, the cleared eluate was reprecipitated with p13^{suc1}-agarose (Fig. 9A). Phosphoamino acid analysis of the reprecipitated protein showed that this subpopulation of SNT proteins was phosphorylated only on serine and threonine (Fig. 9B, bottom panel). These results indicate that from NGF-treated cells, p13^{suc1} associates with kinase activities capable of phosphorylating SNT on multiple residues in vitro. Furthermore, the gel-purified and denatured SNT could be reprecipitated with p13^{suc1}-agarose, indicating that this reagent directly binds to SNT.

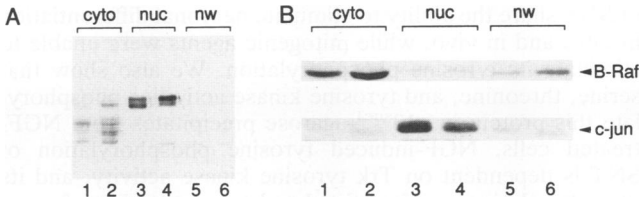

FIG. 10. Association of tyrosine-phosphorylated SNT with nuclear fractions. PC12 cells were treated with NGF (100 ng/ml) for 1 (lanes 1, 3, and 5) or 5 (lanes 2, 4, and 6) min at 37°C and fractionated into cytoplasmic (cyto), nuclear (nuc), and nuclear wash (nw) fractions as described in Materials and Methods. (A) Samples were precipitated with p13^{suc1}-agarose, and the presence of SNT was assessed by immunoblotting with anti-Ptyr antibody. (B) Aliquots of cell fractions were probed with an antibody to cytoplasmic protein B-Raf (top panel) or with an antibody to nuclear protein c-Jun (bottom panel).

To confirm that SNT was phosphorylated on tyrosine in vivo, p13^{suc1}-agarose precipitates from NGF-treated PC12 cells were incubated with protein tyrosine phosphatase 1B prior to probing with anti-Ptyr antibody. Treatment of the precipitates with tyrosine phosphatase greatly diminished the ability of the anti-Ptyr antibody to bind to SNT (Fig. 9C).

SNT is localized to nuclear fractions from NGF-treated PC12 cells. Determination of the intracellular location of SNT may suggest a possible role for this polypeptide. We therefore fractionated PC12 cells treated for 1 or 5 min with NGF into cytosolic and nuclear fractions and precipitated the tyrosine-phosphorylated SNT with p13^{suc1}-agarose. Most of the tyrosine-phosphorylated SNT was observed in the nuclear fraction (Fig. 10A). The effectiveness of Dounce homogenization and then centrifugation for separation of the cytosolic and nuclear fractions was assessed by monitoring the intracellular locations of several previously characterized proteins. The B-Raf protein was found almost exclusively in the cytosolic fraction, as previously reported (66a), whereas the nuclear transcription factor c-Jun was localized primarily in the nuclear fraction (Fig. 10B). To determine the subcellular fraction that contained the plasma membrane, the various fractions were assayed for the presence of the membrane-associated 75-kDa NGF receptor and EGF receptor. The majority of both receptors was found in the cytosolic fractions (data not shown). Tyrosine-phosphorylated SNT is thus localized primarily to nuclear fractions in NGF-treated PC12 cells. This experiment, however, only measured the distribution of SNT phosphoprotein capable of binding to p13^{suc1}-agarose. Therefore, it is not known whether the unmodified form of the protein is associated with nuclear fractions or whether the protein translocates to the cell structures contained in these fractions after addition of NGF.

DISCUSSION

To understand fully the signal transduction pathways utilized by growth factors, differences between pathways that initiate divergent cellular responses must first be identified. Previous studies of neuronal proliferation and differentiation pathways in PC12 cells did not identify any significant differences in early signal transduction responses (10). Here, we report the identification of a species, SNT, in PC12 cells and neurons that is rapidly phosphorylated on tyrosine in response to differentiation factors and directly associates with p13^{suc1}. Factors that promote tyrosine phosphorylation

of SNT share the ability to stimulate neuronal differentiation in vitro and in vivo, while mitogenic agents were unable to stimulate its tyrosine phosphorylation. We also show that serine, threonine, and tyrosine kinase activities phosphorylate this protein in $p13^{suc1}$-agarose precipitates from NGF-treated cells. NGF-induced tyrosine phosphorylation of SNT is dependent on Trk tyrosine kinase activity, and its phosphorylation can be stimulated by expression of v-*src* and is unaffected by expression of a dominant inhibitory *ras* mutant.

SNT was phosphorylated on tyrosine in neuronal cells expressing several receptor and nonreceptor tyrosine kinases that mediate neurotrophic factor activity. These include the FGF receptor and the receptors of the Trk family. However, two receptor tyrosine kinases (EGF and insulin receptors) that promote only mitogenic responses in PC12 cells or in primary cortical neurons were incapable of stimulating SNT tyrosine phosphorylation, indicating specificity among tyrosine kinases for the phosphorylation of this protein. NGF did not induce significant SNT tyrosine phosphorylation in NIH 3T3 fibroblasts transfected with Trk, where it elicits a proliferative response. PDGF also did not induce SNT tyrosine phosphorylation in NIH 3T3 cells, where it is a potent mitogen. However, PDGF did stimulate tyrosine phosphorylation of this protein when acting as a neurotrophic factor for PC12 cells transfected with the PDGF receptor. Thus, significant tyrosine phosphorylation of SNT by neurotrophins and PDGF is limited to cells in which these factors induce differentiative responses. It is possible that SNT is phosphorylated on tyrosine in response to mitogen treatment, but if so, SNT does not in this case associate with $p13^{suc1}$-agarose.

Expression of $pp60^{v\text{-}src}$ or overexpression of Trk in PC12 cells led to constitutive tyrosine phosphorylation of SNT (Fig. 6 and 7). PC12 cells overexpressing Trk (trk-PC12) show greatly accelerated cell differentiation in response to NGF treatment (28). In addition, tyrosine phosphorylation of PLC-γ1 and Erk-1, phosphatidylinositol-3 kinase activity, and transcription of the *transin* gene are sustained for many hours in NGF-treated trk-PC12 cells (28). In wild-type PC12 cells, these responses are extremely transient. For example, PLC-γ1 tyrosine phosphorylation appears within 1 min of NGF treatment, is maximal by 5 min, and declines by 30 min to levels only slightly above that in untreated cells (28, 38, 71). SNT tyrosine phosphorylation was also transient in NGF-treated wild-type PC12 cells (Fig. 1). The elevated levels of Trk tyrosine kinase activity in trk-PC12 cells may result in acceleration of NGF-induced differentiation due to the sustained tyrosine phosphorylation of proteins such as SNT.

The discovery of PC12 U-cell variants demonstrated that these cells contain the signal transduction machinery to respond to NGF as a mitogen or differentiation factor (7). The ability to change the specificity of NGF responses by altering the serum concentration of these cells provided an important system for analyzing SNT phosphorylation in different cellular environments. Significantly, SNT undergoes only very weak tyrosine phosphorylation when NGF acts as a mitogen and significantly greater tyrosine phosphorylation when NGF acts as a differentiation factor for these cells (Fig. 4). SNT may therefore be required for the neuronal differentiation of U cells by NGF. The variability of SNT tyrosine phosphorylation in U cells is evidence for the existence of multiple NGF signal transduction pathways in these cells.

The identification of the Trk tyrosine kinase as a receptor for NGF has facilitated the characterization of proteins in the NGF neuronal differentiation pathway. Two proteins, PLC-γ1 and the 85-kDa phosphatidylinositol-3 kinase subunit, that are phosphorylated on tyrosine after NGF stimulation of PC12 cells and directly associate with activated Trk receptors have been identified (38, 62, 71). These proteins may thus be considered to be distally acting targets of the ligand-activated Trk receptor. Studies utilizing dominant inhibitory *ras* mutants suggest that several other proteins may act downstream of Trk. In these experiments, expression of a dominant inhibitory *ras* gene in PC12 cells inhibited the NGF-induced activation or hyperphosphorylation of $p21^{ras}$ and the serine-threonine kinases Erk-1 and -2, $p90^{rsk}$, and Raf-1 but not the tyrosine phosphorylation of Trk and PLC-γ1 (57, 69, 75). Thus, $p21^{ras}$ may function downstream of Trk and PLC-γ1 and upstream of Raf-1, Erk-1 and -2, and $p90^{rsk}$ in the NGF signal transduction pathway. The NGF-mediated tyrosine phosphorylation of SNT is dependent upon the tyrosine kinase activity of Trk and is not affected by expression of a dominant inhibitory *ras* gene (Fig. 6 and 8). Therefore, tyrosine-phosphorylated SNT may act upstream or in a signal transduction pathway parallel to that of $p21^{ras}$. We do not know whether SNT is a direct substrate of Trk or $pp60^{c\text{-}src}$ tyrosine kinase activity. We did not detect Trk in $p13^{suc1}$-agarose precipitates from PC12 cells, indicating that SNT may not be a direct target of Trk or bind to Trk with sufficient affinity to be detected in precipitation assays. Furthermore, cell fractionation studies indicate that this protein localizes to nuclear fractions in NGF-treated PC12 cells (Fig. 10), where ligand-activated Trk receptors have not been reported. The coprecipitation of SNT with serine, threonine, and tyrosine kinase activities suggests that this protein is either a kinase itself or a substrate of one or more protein kinases in $p13^{suc1}$-agarose precipitates.

In addition to differentiation-specific tyrosine phosphorylation, one of the most interesting properties of SNT is its binding to $p13^{suc1}$. $p13^{suc1}$ is associated with the cell cycle regulator protein $p34^{cdc2}$ in both yeast and mammalian cells, although its role in controlling cell division is unclear (14, 16). Injection of $p13^{suc1}$ into rat fibroblasts or overexpression of this protein in fission yeast inhibits cell growth, indicating a negative regulatory role (16, 29, 56). However, overexpression of $p13^{suc1}$ can also rescue several *cdc2* mutants, suggesting that $p13^{suc1}$ exerts positive cell cycle effects (6, 25). Since one of the effects of NGF on PC12 cells is cessation of cell division, the association of SNT with $p13^{suc1}$ raises the possibility that SNT is involved in mediating the antimitogenic properties of NGF. If SNT acts as a negative regulator of $p34^{cdc2}$, tyrosine phosphorylation of this protein might stimulate its ability to bind $p34^{cdc2}$-$p13^{suc1}$ complexes and thereby inhibit $p34^{cdc2}$ function, initiating cell cycle arrest. Alternatively, SNT may compete with $p34^{cdc2}$ for $p13^{suc1}$ binding sites, preventing formation of functional $p34^{cdc2}$-$p13^{suc1}$ complexes. However, we have been unable to coimmunoprecipitate tyrosine phosphorylated SNT with either anti-$p34^{cdc2}$ or anti-$p13^{suc1}$ antibodies from NGF-treated PC12 cells. These studies have been hindered by the lack of a specific antibody to SNT and by the poor efficiency of labeling of the protein with [^{35}S]methionine and cysteine. Purification of SNT and determination of its sequence and gene of origin may be the most direct way of characterizing this protein and its role in neuronal signal transduction.

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