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SHP-1 negatively regulates neuronal survival by functioning as a TrkA phosphatase

H. Nicholas Marsh,[1] Catherine I. Dubreuil,[1] Celia Quevedo,[2] Anna Lee,[3] Marta Majdan,[1] Gregory S. Walsh,[1,3] Sharon Hausdorff,[4] Farid Arab Said,[5] Olga Zoueva,[6] Maya Kozlowski,[6] Katherine Siminovitch,[7] Benjamin G. Neel,[4] Freda D. Miller,[1,3,8] and David R. Kaplan[1,2,8]

[1]Brain Tumor Research Centre, Montreal Neurological Institute, McGill University, Montreal, Quebec H3A 2B4, Canada
[2]Cancer Research Program and [3]Developmental Biology Program, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada
[4]Cancer Biology Program, Division of Medicine, Harvard Medical School, Beth Israel Hospital, Boston, MA 02215
[5]Aegera Therapeutics, Inc., Montreal, Quebec H3E 1A8, Canada
[6]Health Canada, Biologics and Genetics Therapies Directorate, Center for Biologics Research, Ottawa, Ontario K1A 0L2, Canada
[7]Department of Medicine, University of Toronto, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada
[8]Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada

Nerve growth factor (NGF) mediates the survival and differentiation of neurons by stimulating the tyrosine kinase activity of the TrkA/NGF receptor. Here, we identify SHP-1 as a phosphotyrosine phosphatase that negatively regulates TrkA. SHP-1 formed complexes with TrkA at Y490, and dephosphorylated it at Y674/675. Expression of SHP-1 in sympathetic neurons induced apoptosis and TrkA dephosphorylation. Conversely, inhibition of endogenous SHP-1 with a dominant-inhibitory mutant stimulated basal tyrosine phosphorylation of TrkA, thereby promoting NGF-independent survival and causing sustained and elevated TrkA activation in the presence of NGF. Mice lacking SHP-1 had increased numbers of sympathetic neurons during the period of naturally occurring neuronal cell death, and when cultured, these neurons survived better than wild-type neurons in the absence of NGF. These data indicate that SHP-1 can function as a TrkA phosphatase, controlling both the basal and NGF-regulated level of TrkA activity in neurons, and suggest that SHP-1 regulates neuron number during the developmental cell death period by directly regulating TrkA activity.

Introduction

The survival of cells of the nervous system is dependent on the action of neurotrophic factors that prevent both intrinsic and extrinsic cues from inducing cellular apoptosis. In developing peripheral neurons, neurotrophic target-derived factors, such as NGF, retrogradely determine neuronal survival by suppressing autocrine and $p75^{NTR}$-induced apoptotic signals. This interplay between life and death signals precisely regulates neuron number during development. The initial step in NGF survival signaling is the stimulation of the tyrosine kinase activity of the NGF receptor, TrkA, which in turn activates key survival and axonal growth regulatory proteins such as PI 3-kinase, Akt, and MEK/MAPK (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). The activated survival proteins suppress the activity of apoptotic proteins such as Bad, Forkhead, p53, and BAX (Datta et al., 1999; Mazzoni et al., 1999), and increase the levels and activities of pro-survival proteins such as CREB, Bcl-2, and ΔNp73 (Riccio et al., 1999; Pozniak et al., 2000). NGF-activated TrkA initiates survival signaling by binding to and phosphorylating on tyrosine proteins such as Shc, FRS-2, rAPS, and SH2-B that activate the Ras–PI 3-kinase and Ras–MAPK signaling pathways and PLC-γ1, which regulates protein kinase C activity and intracellular calcium levels (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). The major sites whereby Trk associates with and couples to intracellular signaling pathways in primary neurons are

C.I. Dubreuil and C. Quevedo contributed equally to this work.

Address correspondence to D. Kaplan, Cancer Research Program, Hospital for Sick Children, 555 University Ave., Toronto, Ontario, M5G 1X8. Tel.: (416) 813-7654, ext. 1433. Fax: (416) 813-2212.
email: dkaplan@sickkids.ca

H.N. Marsh's present address is Archemix Corporation, 1 Hampshire St., Cambridge, MA 02139.

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Abbreviations used in this paper: Ad, adenovirus; MOI, multiplicity of infection; P, postnatal day; SCG, superior cervical ganglia; wt, wild-type.

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the SHC/FRS-2 and the PLC-γ1 binding sites, Y490 and Y785, respectively (Atwal et al., 2000). Although TrkA functions by phosphorylating on tyrosine and thereby activating signaling proteins involved in neuronal survival and differentiation, paradoxically, it also activates the phosphotyrosine (pTyr) phosphatase SHP-1 (Vambutas et al., 1995). Because phosphatases such as SHP-1 can have both positive and negative effects on receptor tyrosine kinase signaling (Tonks and Neel, 2001), we asked about the function and mechanism of action of this protein in NGF signal transduction.

The SHP family of protein tyrosine phosphatases includes SHP-1, SHP-2, and the *Drosophila melanogaster* homologue Corkscrew (Tonks and Neel, 2001). SHP-1 is expressed in the hematopoietic system, the nervous system, epithelial cells, and the NGF-responsive PC12 cell line (Tonks and Neel, 2001). The *motheaten* (*me/me*) mouse, which lacks SHP-1 expression, displays an array of hematopoietic abnormalities resulting in severe immunodeficiency and systemic autoimmunity (Tsui and Tsui, 1994). The pathology of the *me/me* mouse, which is caused by the overproduction of multiple hematopoietic cell lineages, initially suggested that SHP-1 was primarily a negative regulator of cell proliferation. In this regard, SHP-1 has been shown to interact with and dephosphorylate a number of growth factor receptors, including those for insulin-like growth factor-1, platelet-derived growth factor, EGF (Tonks and Neel, 2001), and Ros (Keilhack et al., 2001). In contrast, SHP-1, like SHP-2, has been shown to positively regulate MAPK signaling (Krautwald et al., 1996; Wright et al., 1997), as well as EGF, interferon-γ, and Ras signaling (Su et al., 1996; You and Zhao, 1997). The positive effects of SHP-1 signaling may explain why the absence of SHP-1 in *me/me* mice leads to decreased numbers of central nervous system glia (Wishcamper et al., 2001).

Whereas the survival and growth-promoting aspects of neurotrophin signaling are dependent on the levels of TrkA receptor autophosphorylation initiated by NGF binding; the existence of phosphatases that dephosphorylate TrkA would suggest an additional and important mechanism of neurotrophin receptor regulation. In this regard, our previous work in PC12 cells showed that SHP-1 was activated after NGF treatment of PC12 cells (Vambutas et al., 1995). Here, we have asked about the biological importance of this activation in two cell types that require TrkA signaling for their survival, developing sympathetic neurons, and PC12 cells (Greene and Tischler, 1976; Chun and Patterson, 1977). Our results indicate that SHP-1 functions as a TrkA phosphatase, controlling the level of TrkA activity in cultured neurons and PC12 cells and regulating the number of NGF-dependent sympathetic neurons during development.

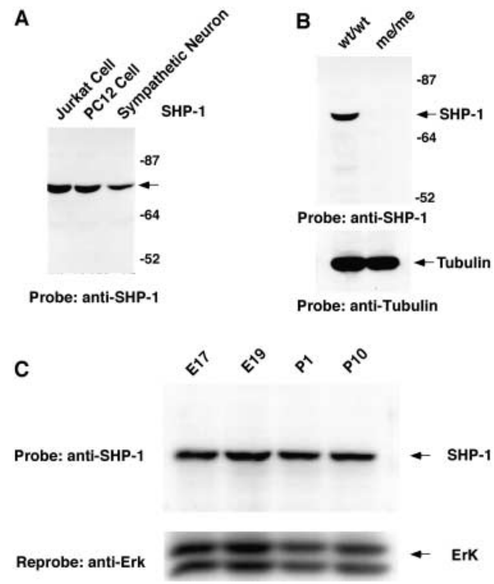

Figure 1. **SHP-1 is expressed in sympathetic neurons.** (A) SHP-1 expression in cultured sympathetic neurons. Cell lysates were prepared from Jurkat, PC12 cells, and sympathetic neurons. Sympathetic neurons were grown for 5 d in the presence of 20 ng/ml NGF before harvesting. 25 μg Jurkat, 100 μg PC12, and 100 μg of sympathetic neuron lysates were electrophoresed and probed in Western blots with monoclonal anti–SHP-1. (B and C) SHP-1 expression in the SCG. Lysates containing equivalent amounts of protein were prepared from SCG dissected from (B) mice that lack SHP-1 expression (*me/me*) or wild-type (*wt/wt*) mice at P15, or (C) from wt mice at E17, E19, P1, and P10. Western blot analysis was performed using anti–SHP-1. Equal loading of protein was confirmed by reprobing the blot in B with antitubulin and in C with anti-Erk1/2 (B and C, bottom blots).

Results

SHP-1 is expressed in developing sympathetic neurons in culture and in vivo

SHP-1 expression has not been reported in the peripheral nervous system. To determine this, we cultured sympathetic neurons from postnatal day (P) 1 rat superior cervical ganglia (SCG), and assessed expression of SHP-1 by Western blot analysis with an antibody that does not recognize the related SHP-2 (Tomic et al., 1995). SHP-1 protein was detected in sympathetic neurons, and in Jurkat and PC12 cells known to express SHP-1 (Vambutas et al., 1995; Fig. 1 A). SHP-1 was also detected in freshly dissociated P15 mouse SCGs from wild-type (wt) mice but not from *me/me* mice, which are genetically deficient in SHP-1 (Fig. 1 B). A developmental time course revealed that SHP-1 levels remained constant in the SCG over the period of naturally occurring cell death from E17 to P10 (Fig. 1 C). Both endogenous SHP-1 and SHP-1, overexpressed using a recombinant adenovirus (Ad), were located predominantly in the cell bodies of sympathetic neurons (Fig. 2). The neuronal localization of SHP-1 was confirmed by costaining with the neuronal marker neurofilament. Therefore, we used sympathetic neurons and PC12 cells to assess SHP-1's function in NGF signal transduction.

Identification of sites on TrkA that regulate complex formation with and are dephosphorylated by SHP-1

Previously, we showed that in response to NGF-treatment of PC12 cells, SHP-1 was activated and formed complexes with TrkA in vitro (Vambutas et al., 1995). Therefore, we asked whether SHP-1 and TrkA would form complexes in NGF-treated PC12 cells and sympathetic neurons. PC12 cells

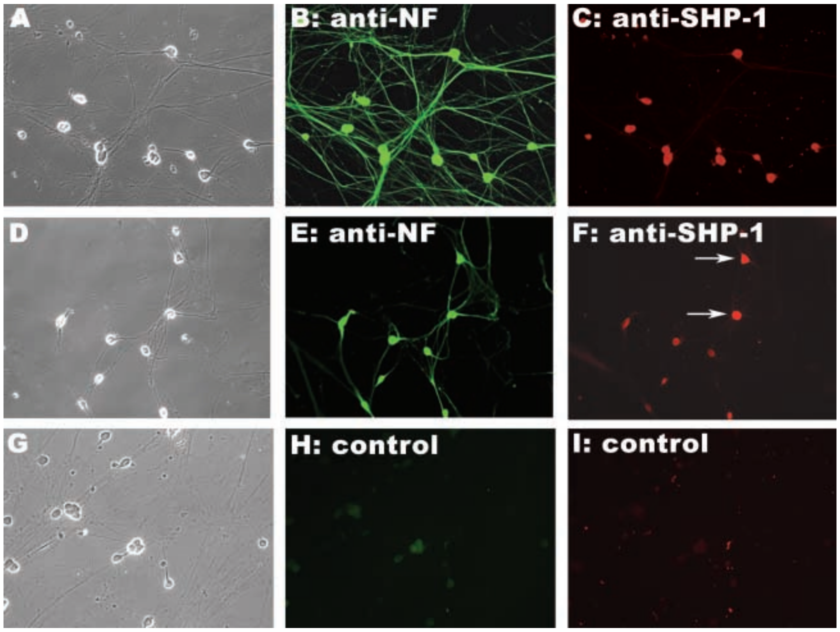

Figure 2. **Immunocytochemistry of SHP-1 expressed in sympathetic neurons.** Neurons from newborn rat SCGs were cultured in NGF and processed for double-label immunocytochemistry using antineurofilament (anti-NF; B and E) or anti–SHP-1 (C and F). A–C are photomicrographs of the same field of control neurons, whereas D–F are of the same field of neurons infected with SHP-1–expressing recombinant adenovirus for 24 h. arrows, Cells overexpressing SHP-1. A, D, and G are phase-contrast images. In G–I, the primary antibody was omitted.

overexpressing TrkA (clone 6-24; Hempstead et al., 1992) or sympathetic neurons were treated with NGF, and TrkA protein was immunoprecipitated with anti-Trk and subjected to Western blotting with anti–SHP-1. SHP-1 coimmunoprecipitated with TrkA from NGF-treated PC12 cells (Fig. 3 A), although this was much more evident in PC12 cells and sympathetic neurons overexpressing SHP-1 via recombinant Ad (Fig. 3, B–D). In both PC12 cells and sympathetic neurons, NGF treatment for 5–10 min enhanced complex formation between SHP-1 and TrkA (Fig. 3, B and D).

Next, we identified a site on TrkA that regulates SHP-1 complex formation. To determine this, we used a panel of TrkA proteins encoding mutations in the two primary binding sites for TrkA substrates, Y490 (Shc/FRS2 site) and Y785 (PLC-γ1 site) expressed via baculovirus in Sf9 cells (Stephens et al., 1994). The baculovirus-expressed TrkA was washed and phosphorylated on Y490 and/or Y785 in vitro, and was incubated with PC12 cell lysates expressing SHP-1. TrkA was immunoprecipitated from these lysates with anti-TrkA; SHP-1 present in the complexes was detected by Western blotting (Fig. 4 A). SHP-1 formed complexes with wt TrkA, with TrkA lacking Y785 (Y785F), and to a much lesser extent, with TrkA lacking Y490 (Y490F and Y490/785F) or kinase-inactive TrkA (K538N; Fig. 4 A). Thus, phosphorylated Y490 of TrkA is required for optimal complex formation between SHP-1 with TrkA.

Having defined a site regulating complex formation of TrkA and SHP-1, we asked whether SHP-1 dephosphorylated TrkA in vitro. ^{32}P-labeled TrkA was incubated in vitro with purified SHP-1. We observed that SHP-1 efficiently dephosphorylated TrkA (Fig. 4 B). Next, we asked whether SHP-1 could dephosphorylate TrkA in vivo and identify the sites of dephosphorylation. TrkA was coexpressed with SHP-1 in Sf9 cells, and the sites of TrkA dephosphorylation were determined by Western blotting with antibodies that recognize phosphorylated Y490 or Y674 and Y675 (Segal et al., 1996), the latter being sites critical for regulating the overall catalytic activity of the receptor (Cunningham et al., 1997). As a control, we coexpressed TrkA with SHP-2. TrkA expressed in Sf9 cells was constitutively tyrosine phosphorylated (Fig. 4 C), as we have previously reported (Stephens et al., 1994). SHP-1 efficiently dephosphorylated

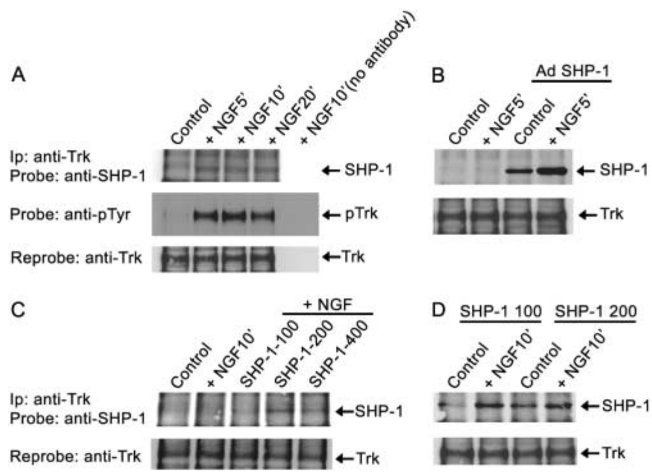

Figure 3. **SHP-1 coimmunoprecipitates with TrkA in PC12 cells and sympathetic neurons.** (A) PC12 cells treated with 50 ng/ml NGF for the indicated times were lysed and immunoprecipitated with anti-Trk, and precipitated proteins were probed in Western blots with anti–SHP-1 (top), anti-pTyr (middle), or anti-Trk (bottom) to detect Trk protein levels in the immunoprecipitates. (B) PC12 cells were infected with Ad SHP-1 for 24 h, and treated for 5 min with NGF before immunoprecipitation with anti-Trk and Western blotting with anti–SHP-1 (top) or anti-Trk (bottom). (C) Sympathetic neurons, either mock infected or infected with wild-type Ad SHP-1 at the indicated MOI, were treated with 50 ng/ml NGF for 10 min. Cells were lysed, the lysates were immunoprecipitated with anti-TrkA, and immunoprecipitated proteins were subjected to Western blotting with anti–SHP-1 (top) or anti-Trk (bottom). (D) Sympathetic neurons infected with Ad SHP-1 at the indicated MOIs were treated with NGF for 10 min, and cell lysates were immunoprecipitated with anti-Trk and probed in Western blots with anti–SHP-1 (top) or anti-TrkA (bottom).

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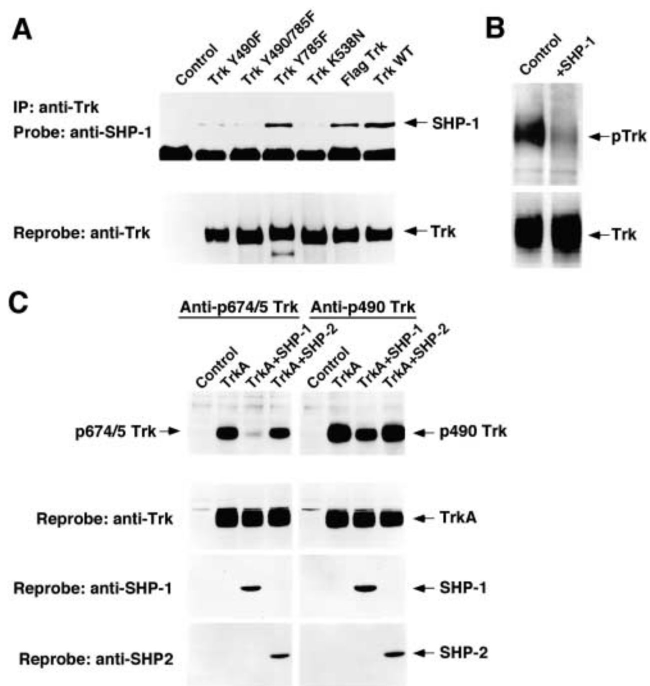

Figure 4. **SHP-1 forms complexes with TrkA at Y490 and dephosphorylates TrkA in Sf9 cells at Y674/Y675 and in vitro.** (A) WT TrkA, but not TrkA mutated at Y490, forms complexes with SHP-1 in vitro. Human TrkA expressed in Sf9 cells was immunoprecipitated, subjected to autophosphorylation in vitro, and incubated with PC12 cell lysates as a source of SHP-1. TrkA-containing immune complexes were washed extensively and probed on Western blots with anti–SHP-1. The TrkA receptors were WT TrkA, Flag epitope-tagged WT TrkA (Flag TrkA), or receptors encoding mutations at Y490 (Y490F), Y785 (Y785F), Y490 and Y785 (Y490/785F), or K538 (K538N), a kinase inactive form of TrkA. The control lane has no input TrkA protein. The blot was reprobed with anti-Trk to show that similar amounts of Trk protein were present in each lane. (B) SHP-1 dephosphorylates TrkA in vitro. SHP-1 or TrkA were expressed in Sf9 cells, and immunoprecipitated with anti–SHP-1 or anti-Trk. The purified TrkA was autophosphorylated with ^{32}P-labeled ATP in vitro and incubated with or without immunopurified SHP-1, and the reaction products were electrophoresed and exposed to film. The blot was reprobed with anti-Trk to show equivalent levels of TrkA in each lane. (C) SHP-1 dephosphorylates TrkA in vivo at Y674 and Y675. WT TrkA was expressed with SHP-1 or SHP-2 in Sf9 cells, and TrkA phosphorylation levels were assessed by Western blots using anti-phosphorylated Y490 Trk (p490, top right) and antiphosphorylated Y674/675 Trk (p674/675, top left). The blots were reprobed with anti-TrkA (second panels), anti–SHP-1 (third panels), and anti–SHP-2 (bottom panels) to indicate the levels of each of these proteins.

TrkA at sites Y674/Y675, but only partially dephosphorylated TrkA at the Y490 site (Fig. 4 C). SHP-2 was unable to substantially dephosphorylate either site under these conditions, even though SHP-2 was able to dephosphorylate exogenous substrates other than TrkA (unpublished data). Therefore, SHP-1 can dephosphorylate TrkA on specific tyrosine residues required for optimal activity.

SHP-1 overexpression suppresses the survival of sympathetic neurons and PC12 cells

The biochemical data indicate that SHP-1 negatively regulates TrkA tyrosine phosphorylation and activation. To determine whether this has functional consequences, we examined sympathetic neurons that require TrkA signaling for survival. Neurons were infected with Ad SHP-1 or a dominant-inhibitory SHP-1 mutant (SHP-1ΔP; Neel and Tonks, 1997), and after 2 d, they were switched into media with or without 20 ng/ml NGF. After 72 h, survival was quantified using MTT, which measures mitochondrial function (Manthorpe et al., 1986; Fig. 5 A). SHP-1WT expression decreased sympathetic neuron survival by 70% (100 multiplicity of infection [MOI]), whereas SHP-1ΔP expression had little effect on survival in 20 ng/ml NGF relative to a LacZ-expressing control virus (Fig. 5 A). The specificity of the SHP-1 effect was demonstrated by coinfecting neurons with the Ad SHP-1ΔP; this completely rescued neurons from SHP-1WT–induced death (Fig. 5 A). Similarly, coexpression of Bcl-xL, a protein that prevents NGF withdrawal-induced apoptosis of sympathetic neurons (Gonzalez-Garcia et al., 1995), prevented SHP-1WT–mediated suppression of neuronal survival. The expression of Ad SHP-1 proteins in sympathetic neurons was confirmed by Western blot analysis with anti–SHP-1 (Fig. 5 B).

To determine whether SHP-1 induced the apoptosis of sympathetic neurons, we examined the cells by TUNEL (Fig. 5 C). Approximately 85% of SHP-1WT (200 MOI)–infected sympathetic neurons were TUNEL positive, confirming the results obtained by MTT assay (Fig. 5 D). Uninfected sympathetic neurons maintained in 20 ng/ml NGF had few TUNEL-positive cells (∼5%), whereas neurons deprived of NGF for 48 h were 95% TUNEL positive. Approximately 12% of the neurons infected with the Ad SHP-1ΔP (200 MOI) were TUNEL positive, similar to neurons infected with Ad LacZ (7% positive; Fig. 5 D). Cell death was also accompanied by neurite fragmentation (unpublished data). Thus, overexpression of SHP-1 causes apoptosis of NGF-dependent sympathetic neurons in culture.

We turned to PC12 cells, whose survival can be promoted either by NGF or by serum factors such as IGF-1 (Greene and Tischler, 1976), to find out whether this suppressive effect of SHP-1 was specific for TrkA-mediated survival. PC12 cells were infected with Ad SHP-1WT, and cultured in serum or in NGF without serum for 72 h. MTT analysis demonstrated that overexpression of SHP-1WT decreased survival of PC12 cells maintained in NGF by 50% (100 MOI; Fig. 6 A), but had no effect on PC12 cells maintained in serum (Fig. 6 B). Thus, SHP-1 is specific for TrkA-mediated survival, and does not apparently inhibit survival promoted by other exogenous factors present in serum. Based on these results, together with the biochemical analysis, we hypothesized that SHP-1 directly inhibited TrkA and TrkA-mediated downstream survival signals.

SHP-1 dephosphorylates TrkA in PC12 cells and sympathetic neurons

To determine if SHP-1 could dephosphorylate TrkA in PC12 cells and sympathetic neurons as we had hypothesized, the cells were infected with Ad SHP-1WT or, as controls, Ad SHP-2 or Ad LacZ (Fig. 6 C). Cells were exposed to NGF for 5 min, and the levels of TrkA tyrosine phosphorylation were assessed by anti-TrkA immunoprecipitations followed by Western blotting with anti-pTyr (Fig. 6, D and E). This analysis revealed that, as seen in Sf9 cells,

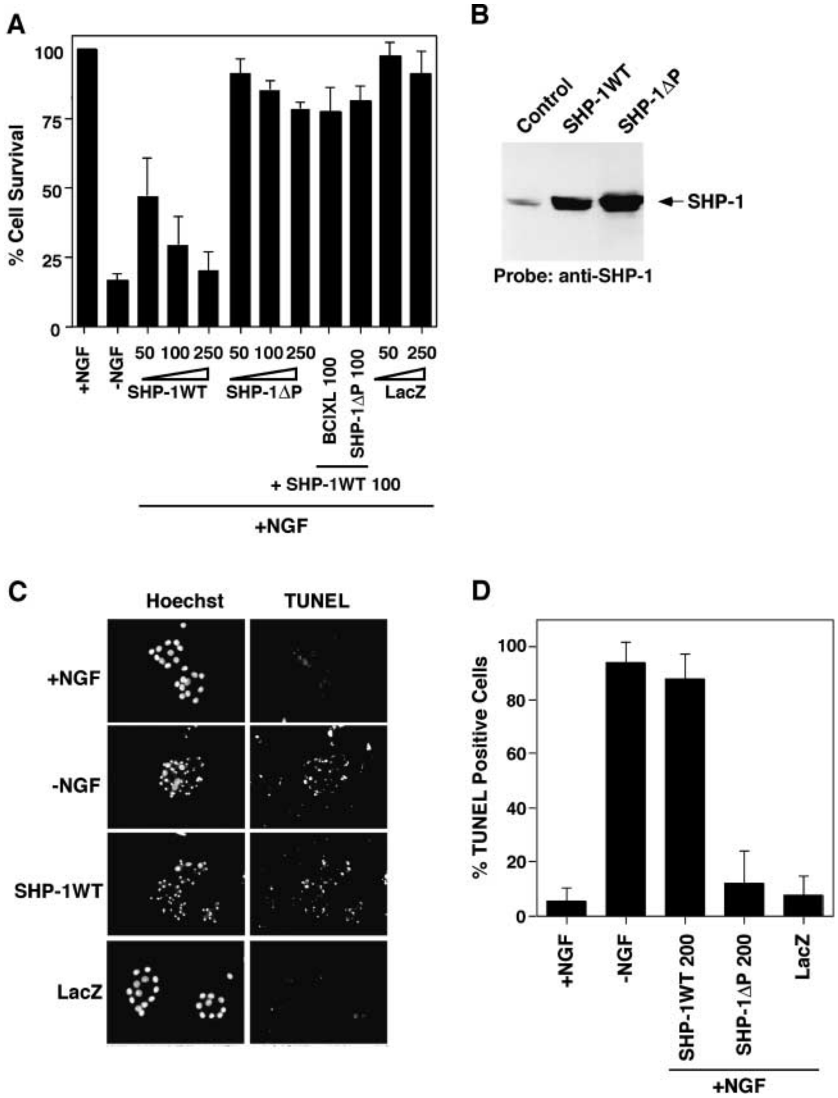

Figure 5. **SHP-1 overexpression suppresses the survival of cultured sympathetic neurons.** (A) MTT survival assays of sympathetic neurons infected with various MOIs of adenovirus encoding SHP-1WT, SHP-1ΔP (dominant-negative), BCl-XL (pro-survival protein), or LacZ (control virus) in the presence of NGF. In brief, neurons were grown in 20 ng/ml NGF for 5 d, infected with the indicated Ad, and 48 h later, assayed for survival by MTT assay. Values were normalized to that of 20 ng/ml NGF (100% survival). (B) Western blot analysis of equivalent amounts of protein from sympathetic neurons infected with Ad SHP-1WT or Ad SHP-1ΔP. NGF-selected sympathetic neurons were infected with Ad for 48 h at 50 MOI. Neurons were lysed, and equal amounts of protein were electrophoresed and probed in Western blots with anti–SHP-1. (C) TUNEL of sympathetic neurons transduced with Ad SHP-1WT. Cultured sympathetic neurons were either mock infected (top four panels) or infected with 200 MOI of Ad SHP-1WT or Ad LacZ, and were maintained in 20 ng/ml NGF for 48 h before TUNEL and Hoechst labeling to detect all of the nuclei in the field. Each set of two panels represents the same field photographed under one set of fluorescence filters for Hoechst (left) and a second set for TUNEL (right). Neurons deprived of NGF for 48 h were included as a control (−NGF). (D) Quantification of TUNEL data is similar to that shown in C. Error bars represent the SEM.

SHP-1 overexpression completely blocked the ability of NGF to stimulate TrkA tyrosine phosphorylation in both PC12 cells (Fig. 6 D) and sympathetic neurons (Fig. 6 E). In contrast, neither SHP-2 nor LacZ expression had any detectable effect. These results were confirmed by performing similar experiments and probing the Western blots with antiphospho-Y674/Y675 Trk (Fig. 6 F); this analysis indicated that dephosphorylation of TrkA occurred at these two sites in sympathetic neurons as it had in Sf9 cells. These results suggest that a potential mechanism used by SHP-1 to cause cell death is dephosphorylating TrkA.

SHP-1 decreases the activity of NGF-stimulated signaling proteins

We first examined NGF-induced changes in tyrosine phosphorylation, to determine how overexpression of SHP-1 affects NGF-mediated TrkA survival signals. Sympathetic neurons were infected with Ad SHP-1WT, washed free of NGF, and then treated for 5 min with 100 ng/ml NGF. Western blot analysis with anti-pTyr revealed that SHP-1WT had little effect on overall basal tyrosine phosphorylation, but that it suppressed the tyrosine phosphorylation of NGF-stimulated signaling proteins (Fig. 7 A). Particularly evident was the decreased phosphorylation of two bands that migrated at the molecular weight of the MAPKs, Erk1, and Erk2.

To find whether SHP-1 overexpression specifically inhibited downstream TrkA signaling pathways, we examined tyrosine phosphorylation of PLC-γ1, a direct TrkA substrate (Kaplan and Miller, 2000; Fig. 7 B), and the phosphorylation of Akt (Fig. 7 C), which occurs after NGF treatment of sympathetic neurons and which regulates NGF-dependent cell survival (Kaplan and Miller, 2000). Neurons were infected and treated with NGF, and lysates were immunoprecipitated with anti–PLC-γ1 and probed with anti-pTyr (Fig. 7 B), or were analyzed by Western blots with anti–phospho-S473 Akt (Fig. 7 C). This analysis revealed that SHP-1 overexpression inhibited NGF-mediated phosphorylation of both of these downstream TrkA targets. Thus, SHP-1 is a potent inhibitor of multiple TrkA-activated signaling proteins, suggesting that it exerts its effects at the level of the receptor itself.

Finally, we examined whether overexpression of SHP-1 resulted in the activation of cell death–inducing proteins, as would be predicted if it induces apoptosis by suppressing Trk signaling. Two such proteins that are induced or activated by

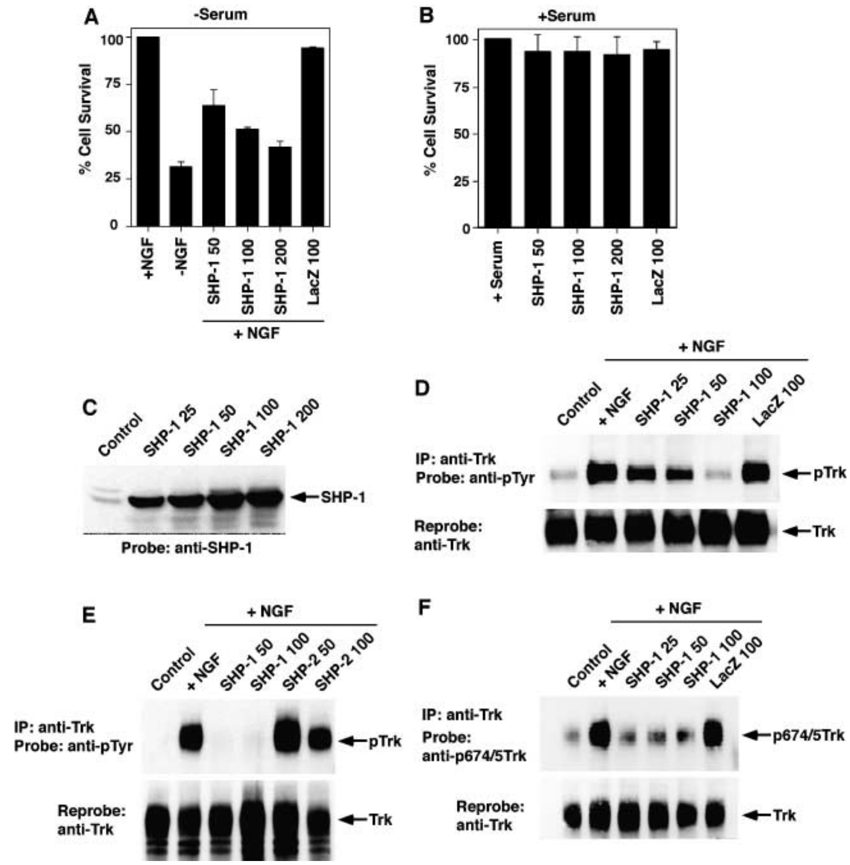

Figure 6. **SHP-1 inhibits NGF-induced, but not serum-induced, survival of PC12 cells and dephosphorylates TrKA in PC12 and neurons.** (A and B) MTT survival assays of PC12 cells infected with Ad SHP-1WT or Ad LacZ at the indicated MOIs. Cells were washed free of serum and incubated in the absence (A) or presence (B) of serum, and in the presence or absence of NGF as indicated. 48 h later, MTT assays were performed and the data were normalized to survival induced by 20 ng/ml NGF or serum. Ad SHP-1 suppressed NGF-induced, but not serum-induced, survival. (C–F) SHP-1 dephosphorylates NGF-activated TrkA in PC12 cells and sympathetic neurons. (C) Western blot analysis with anti–SHP-1 to determine the levels of SHP-1 expression in PC12 cells infected with Ad SHP-1WT. (D–F) PC12 cells (D) or sympathetic neurons (E and F) were established in 20 ng/ml NGF and were infected with Ad SHP-1WT, SHP-2, or LacZ at the indicated MOIs. Cells were washed free of NGF and exposed to 100 ng/ml NGF for 5 min. TrkA was immunoprecipitated and subjected to Western blot analysis (D and E) with anti-pTyr to assess tyrosine phosphorylation, or (F) with antibodies specific to phosphorylated residues Y674/Y675 of Trk (p674/5Trk). In all experiments, the blots were reprobed with anti-Trk to detect TrkA protein levels in the immunoprecipitates.

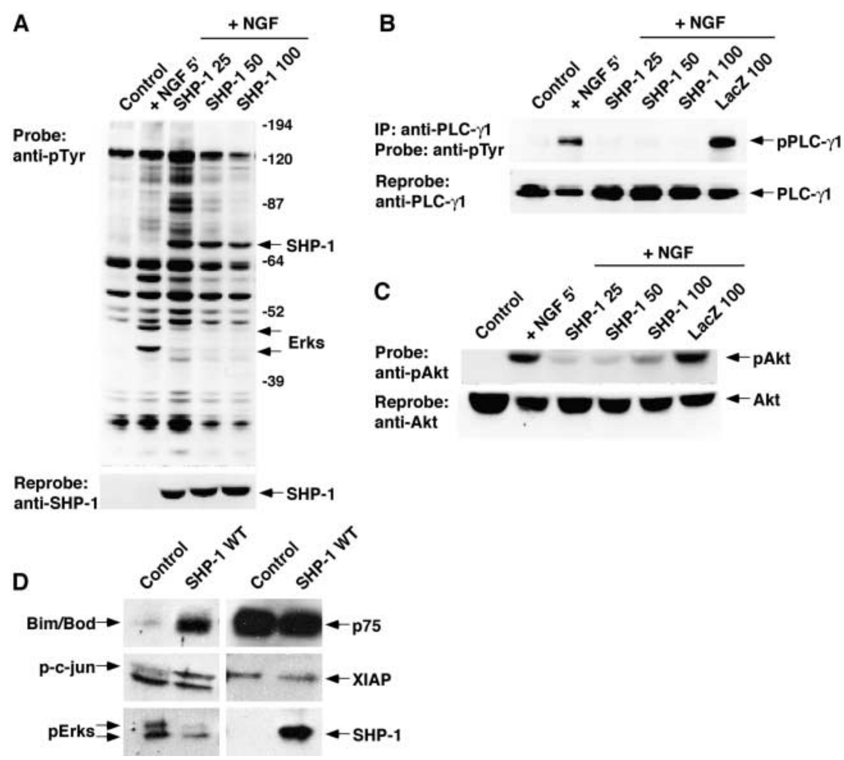

Figure 7. **SHP-1 inhibits NGF-induced phosphorylation of MAPK/Erk, PLC-γ1, and Akt, and increases Bim/Bod expression and c-jun phosphorylation.** Sympathetic neurons grown in 20 ng/ml NGF were infected with Ad SHP-1WT or LacZ, washed free of NGF, and treated for 5 min with 100 ng/ml NGF. (A) Western blot analysis for total tyrosine-phosphorylated proteins in lysates of neurons infected with Ad SHP-1WT at an MOI of 25 (which induced 50% cell death in this experiment) or of 50 (which induced 75% cell death; top panel). The positions of proteins migrating at the molecular weights of SHP-1 and MAPK (Erk1/2) are indicated. The blots were reprobed with anti–SHP-1 (bottom panel) to show the levels of recombinant protein expressed in each sample. (B) Ad SHP-1WT suppresses PLC-γ1 tyrosine phosphorylation. Neurons infected with Ad SHP-1WT or LacZ were treated with 100 ng/ml NGF, and PLC-γ1 was immunoprecipitated from the lysates. NGF-induced tyrosine phosphorylation of PLC-γ1 was determined by probing Western blots of the precipitates with anti-pTyr (top). The blot was reprobed with anti–PLC-γ1 to indicate PLC-γ1 levels in each lane (bottom). (C) Ad SHP-1WT suppresses Akt phosphorylation. Neurons were infected with Ad SHP-1WT at various MOIs, induced with 100 ng/ml NGF for 5 min, and the phosphorylation of Akt was determined by probing Western blots with antiphosphorylation-specific Akt directed at S473 (top). The level of Akt in each lane was determined by reprobing with anti-Akt (bottom). (D) Ad SHP-1 increases the levels of Bim/Bod and the phosphorylation of c-jun, and suppresses the phosphorylation of Erk1/2. Ad SHP-1WT did not alter the levels of $p75^{NTR}$ or XIAP. Neurons were infected with Ad SHP-1WT or Ad LacZ at 200 MOI in the presence of 20 ng/ml NGF, and equivalent amounts of protein from cell lysates were examined using antibodies to the indicated proteins in Western blots.

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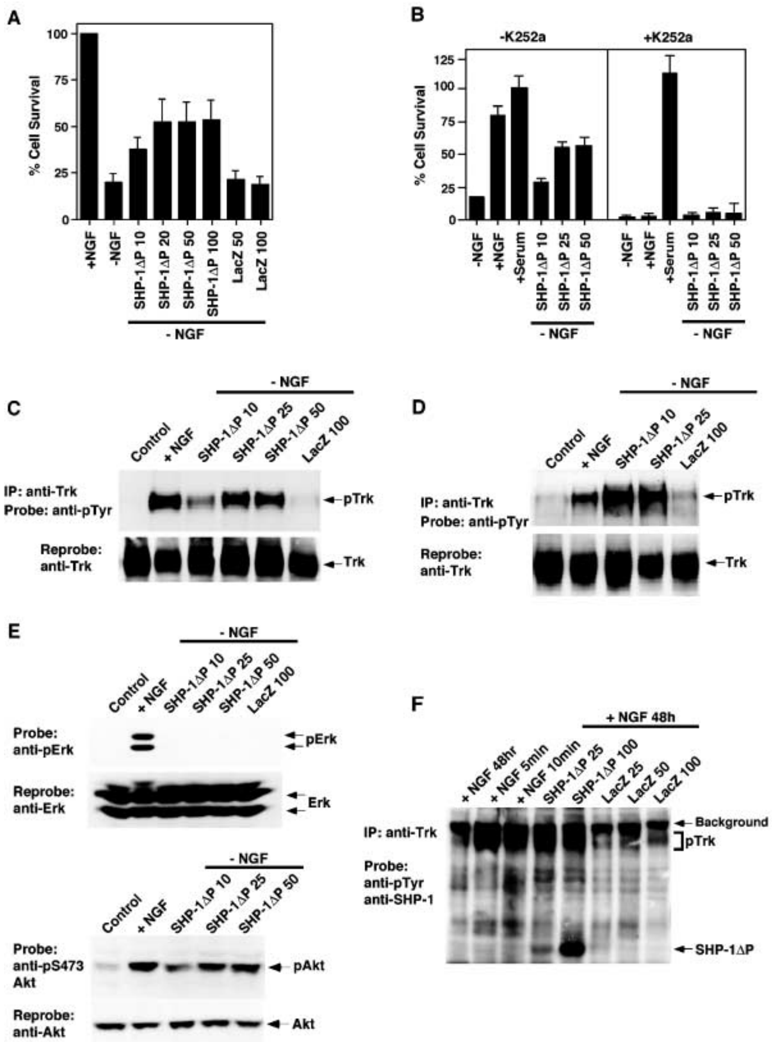

Figure 8. **Inhibition of endogenous SHP-1 enhances TrkA survival signaling and rescues sympathetic neurons and PC12 cells from death induced by NGF withdrawal.** (A) Dominant-negative Ad SHP-1ΔP rescues sympathetic neurons from NGF withdrawal-induced cell death. Sympathetic neurons cultured for 5 d in 20 ng/ml NGF were infected with Ad SHP-1ΔP or LacZ. 48 h later, NGF was washed out, and the cells were fed with 10 ng/ml NGF or medium alone. Cell viability was assessed by MTT assay. (B, left) SHP-1ΔP rescues PC12 cells from NGF withdrawal-induced cell death in a manner dependent on TrkA. PC12 cells were infected with SHP-1ΔP, and incubated in the absence of serum and NGF. Survival was compared with uninfected cells incubated in 50 ng/ml NGF or serum (B, right). SHP-1ΔP will not rescue cells from apoptosis when TrkA activity is suppressed. Cells treated as in the right panel were maintained in the presence of the selective Trk kinase inhibitor K252a (200 nM). Cell survival was measured using MTT assay. Neither NGF nor SHP-1ΔP could maintain survival in the absence of Trk activity, suggesting that SHP-1ΔP enhances survival by increasing TrkA activity. (C) SHP-1ΔP increases the tyrosine phosphorylation of TrkA in the absence of NGF in PC12 cells. Cells cultured in media lacking NGF were infected with SHP-1ΔP at the indicated MOIs or treated with 50 ng/ml NGF for 5 min (+NGF). TrkA was immunoprecipitated with anti-Trk and probed in Western blots with anti-pTyr (top). The levels of TrkA in each lane were visualized by reprobing the blots with anti-TrkA. (D) SHP-1ΔP increases the tyrosine phosphorylation of TrkA in the absence of NGF in sympathetic neurons. Neurons were infected with various MOIs of Ad SHP-1WT, maintained in NGF for 2 d, and withdrawn from NGF. As a control, neurons were treated with 50 ng/ml NGF for 5 min. Lysates were immunoprecipitated with anti-Trk and probed with anti-pTyr. (E and F) SHP-1ΔP expression increases Akt and not MAPK phosphorylation in PC12 cells in the absence of NGF. (E) Western blot analysis with an antibody specific to the phosphorylated, activated forms of MAPK1/2 (Erk, top panel) and Akt (bottom panel) in PC12 cells infected and treated as in panel D. The blot was reprobed for total Erk1/2 or Akt protein to demonstrate that equal amounts of protein were present in each lane. (F) Suppression of endogenous SHP-1 activity by SHP-1ΔP expression results in sustained and elevated NGF-activated TrkA tyrosine phosphorylation. Sympathetic neurons cultured for 5 d in 20 ng/ml NGF were infected with SHP-1ΔP or Ad LacZ at the indicated MOI. 48 h later, NGF was washed out, and the cells were treated with 50 ng/ml NGF for 5 min, 10 min, or 48 h. TrkA tyrosine phosphorylation in equivalent amount of protein from cell lysates was assessed by immunoprecipitating with anti-Trk and probing in Western blots with anti-pTyr. Phosphorylated TrkA migrates underneath the background band seen in all lanes. Error bars represent the SEM.

the lack of TrkA signaling are c-jun and Bim/Bod (Putcha et al., 2001; Whitfield et al., 2001). Western blot analysis of sympathetic neurons expressing SHP-1WT, and maintained in 20 ng/ml NGF, showed that SHP-1 overexpression caused increased levels of Bim/Bod and the phosphorylation (activation) of c-jun (Fig. 7 D), coincident with a decrease in Erk1/2 phosphorylation. In contrast, the levels of the $p75^{NTR}$ and the pro-survival protein XIAP (Wiese et al., 1999) were not affected by SHP-1 overexpression (Fig. 7 D).

Inhibition of endogenous SHP-1 rescues sympathetic neurons and PC12 cells from NGF withdrawal by increasing basal TrkA activation

These data indicate that overexpression of SHP-1 acts to decrease TrkA tyrosine phosphorylation, resulting in decreased downstream survival signals and subsequent neuronal apoptosis. To ask whether endogenous SHP-1 plays a similar role in regulating TrkA activity, we used the dominant-inhibitory mutant SHP-1ΔP. Sympathetic neurons were cultured for 4 d, infected with Ad SHP-1ΔP or LacZ, and either withdrawn from NGF or treated with 10 ng/ml NGF. In the absence of NGF, SHP-1ΔP expression maintained the survival of 50% of the neurons, whereas survival of LacZ-infected neurons was similar to controls (Fig. 8 A). Similar results were obtained using PC12 cells (Fig. 8 B, left). PC12 cells maintained in serum were infected for 24 h with the SHP-1ΔP virus at various MOIs. After infection, the cells were washed free of serum and cell survival was assessed after 72 h using MTT (Manthorpe et al., 1986). Expression of SHP-1ΔP increased PC12 cell survival in the absence of serum or NGF in a dose-dependent manner (Fig. 8 B, left). Survival with 50 MOI of SHP-1ΔP was 50% of that seen with 50 ng/ml NGF.

The enhanced survival seen with SHP-1ΔP in the absence of NGF could be due to increased ligand-independent basal activation of TrkA. To address this possibility, we infected sympathetic neurons and PC12 cells with Ad SHP-1ΔP, and examined TrkA tyrosine phosphorylation. This experiment demonstrated that for both PC12 cells (Fig. 8 C) and sympathetic neurons (Fig. 8 D), inhibition of SHP-1 led to TrkA tyrosine phosphorylation and therefore activation in the absence of exogenous NGF. To determine whether this increased basal TrkA activation was the mechanism underlying the SHP-1ΔP–mediated survival effect, we asked whether inhibition of SHP-1 still caused PC12 cell survival in the presence of K252a, a selective pharmacological Trk inhibitor (Tapley et al., 1992; Fig. 8 B, right). The rationale for this experiment is that SHP-1ΔP should no longer induce survival in the presence of K252a if SHP-1 functions solely to inhibit TrkA. However, if SHP-1 suppresses the activity of other survival-promoting receptors than TrkA, then SHP-1ΔP should continue to induce survival in the absence of TrkA activity. As predicted, if SHP-1ΔP functioned only via TrkA, MTT assays revealed that K252a treatment completely blocked SHP-1ΔP's ability to increase the survival of NGF-deprived PC12 cells. In contrast, K252a had no effect on serum-regulated survival of PC12 cells.

Inhibition of endogenous SHP-1 differentially activates downstream TrkA signaling pathways in the absence of NGF

These experiments indicated that endogenous SHP-1 normally functions to keep basal TrkA activation low, and thereby to maintain the NGF dependence of PC12 cells and sympathetic neurons. However, SHP-1ΔP did not cause PC12 cells to extend neurites in the presence or absence of serum (unpublished data), suggesting that in the absence of NGF, SHP-1 inhibition might only activate a subset of TrkA signaling pathways. In PC12 cells, survival is an Akt-dependent process, whereas neurite outgrowth is a MEK/MAPK-dependent process (Klesse et al., 1999; Kaplan and Miller, 2000). Therefore, we examined the effect of SHP-1ΔP on MAPK1/2 and Akt activity. PC12 cells were infected with Ad SHP-1ΔP, and the phosphorylation state of Akt and MAPK1/2 in the absence of NGF was examined in Western blots with phosphospecific antibodies. SHP-1ΔP caused an increase in Akt (Fig. 8 E, bottom panel) but not of MAPK (Erk1/2) phosphorylation (Fig. 8 E, top panel), which is consistent with the cell biology data.

Because TrkA forms complexes with and activates SHP-1 after NGF treatment of PC12 cells, we asked whether endogenous SHP-1 plays a role in attenuating TrkA activity in the continued presence of its NGF ligand. In sympathetic neurons, TrkA tyrosine phosphorylation is maximal after 5 min of exposure to NGF, with this phosphorylation being largely attenuated by 48 h, even in the continued presence of NGF (Belliveau et al., 1997; Fig. 8 F). Suppression of SHP-1 activity after SHP-1ΔP expression led to sustained and elevated TrkA tyrosine phosphorylation in the continuous presence of 100 ng/ml NGF, with the levels at 48 h being similar to those seen at 5 min in neurons infected with Ad LacZ (Fig. 8 F). These results suggest that, in the presence of NGF, TrkA activates SHP-1, which in turn functions to attenuate TrkA activity and downstream signaling, thereby participating in a negative feedback loop.

Sympathetic neuron number is increased in the *me/me* mouse

These experiments using cultured cells indicate that endogenous SHP-1 functions both to keep basal levels of TrkA activity low in the absence of NGF and to attenuate TrkA activity in the presence of NGF. If SHP-1 plays a similar role in vivo, then sympathetic neurons in the *me/me* mouse, which is genetically deficient in SHP-1, should have up-regulated TrkA activity, and should not die appropriately during naturally occurring cell death. To test this hypothesis, we analyzed the number of neurons in SCGs taken from P15 *me/me* mice; the major period of sympathetic neuron death occurs in the first few weeks postnatally, and SCG neuron number decreases from ~25,000 at birth to ~15,000 at P15. SCGs from *me/me* and wt mice were removed and sectioned at 7-μm thickness, and neuronal numbers were determined by counting all neuronal profiles with nucleoli on every fourth section, as described by Coggeshall et al. (1984).

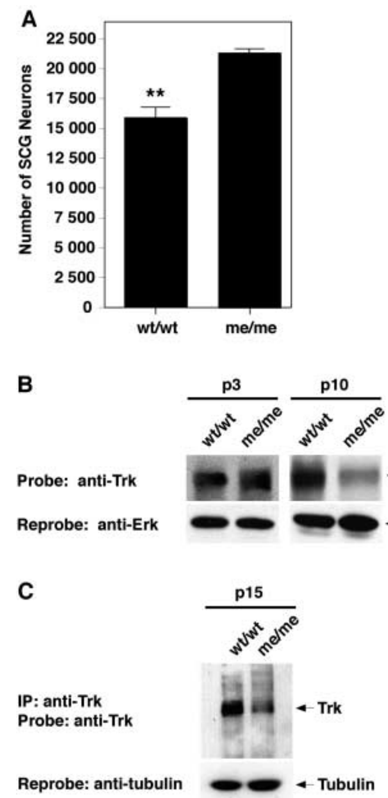

Figure 9. **The number of sympathetic neurons in the superior cervical ganglia (SCG) is increased in the absence of SHP-1 in vivo.** (A) The number of neurons in the SCG of P15 mice from wild type (wt/wt) or that lack SHP-1 (me/me) is shown. Results are expressed as the mean ± SEM for *wt/wt*, 15,813 ± 813, $n = 9$; and for *me/me*, 21,289 ± 452, $n = 4$. Asterisks indicate values significantly different from wt mice of the same age. $P < 0.05$. (B and C) TrkA levels are decreased in the SCG of *me/me* mice during the period of naturally occurring cell death at P10 and P15, but not at P3. Naturally occurring cell death in the SCG from mice has just commenced at P3, is maximal at P10, and is completed at P15. Lysates were prepared from SCGs dissected from wt/wt and *me/me* mice. Western blot analysis was performed using anti-TrkA (RTA; B and C, top panels). Equal loading of protein was confirmed by reprobing the blot with anti-Erk1/2 or antitubulin (B and C, bottom panels).

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This analysis demonstrated a statistically significant increase of 35% in the relative number of sympathetic neurons in *me/me* (21,289 ± 452; *n* = 4) relative to wt mice (15,813 ± 1033; *n* = 9; Fig. 9 A). Therefore, this analysis suggests that in vivo, SHP-1 regulates sympathetic neuron apoptosis. To ascertain whether increased TrkA expression could account for the increases in neuron number, we determined the levels of TrkA protein in sympathetic ganglia at P3, soon after the commencement of naturally occurring cell death, and at P10 and P15, when cell death is maximal or complete. TrkA protein levels were equivalent in wt and *me/me* SCG at P3, but were reduced in the *me/me* SCG by ~50% at P10 and P15 (Fig. 9, B and C). Thus, the increase in neuron number could not be accounted for by an increase in TrkA expression level. Due to the low levels of TrkA, we could not assess TrkA autophosphorylation in *me/me* SCGs.

Sympathetic neurons from mice lacking SHP-1 require less NGF to survive than neurons from wt mice

Because the *me/me* mice showed increased numbers of sympathetic neurons during development, we predicted that neurons cultured from these mice should survive better than wt neurons in the absence of NGF. Neurons from newborn *me/me* mice, wt littermates, or wt C3H mice were cultured in 50 ng/ml NGF for 3 d, the NGF was washed away, and the cells were incubated in 0, 5, or 15 ng/ml NGF. Sympathetic neuron survival is optimal in 15 ng/ml NGF and half-maximal in 5 ng/ml NGF (Belliveau et al., 1997). Sympathetic neurons from the *me/me* mice survived much better than wt neurons in both 5 ng/ml NGF (Fig. 10 A, dashed lines; and Fig. 10 C) and NGF-deprived conditions (Fig. 10 B). Both *me/me* and wt neurons survived to similar extents in optimal (15 ng/ml) NGF (Fig. 10 A, solid lines). Thus, neurons lacking SHP-1 survive in suboptimal amounts of NGF, consistent with increased TrkA activity in these cells.

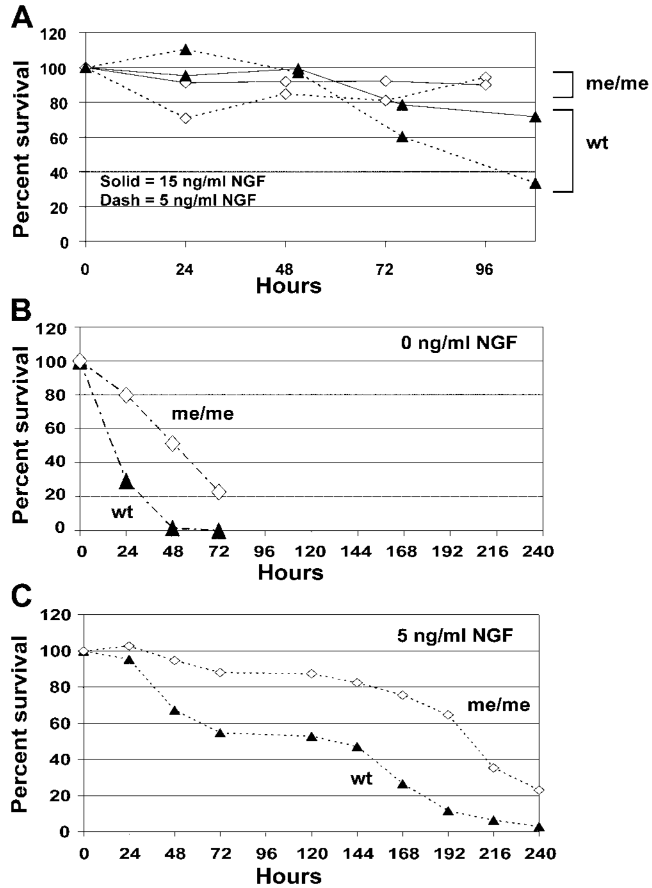

Figure 10. **Sympathetic neurons from mice lacking SHP-1 require less NGF to survive than neurons from wt mice.** (A) *me/me* neurons survive better than wt neurons in suboptimal amounts of NGF. Neurons from newborn *me/me* mice (open diamonds) or wt mice (closed triangles) were cultured in 50 ng/ml NGF for 3 d. The NGF was washed away, and the neurons were placed in suboptimal (5 ng/ml) NGF (dashed lines) or optimal (15 ng/ml) NGF (solid lines) for survival. The percentage of surviving neurons was determined by counting phase-bright (live) cells in preselected randomly chosen fields. Results were normalized such that the number of neurons at the time of NGF withdrawal was 100% (0 h). The *me/me* and wt data were from two separate experiments. (B) *me/me* neurons survive better than wt neurons in the absence of NGF. Percentage of survival of neurons from *me/me* (open diamonds) or wt (closed triangles) mice established in NGF for 5 d, and then withdrawn completely from NGF (0 h). (C) *me/me* neurons survive better than wt neurons in suboptimal NGF. Percentage of survival of *me/me* (open diamonds) and wt (closed triangles) neurons switched into 5 ng/ml NGF. The experiments in A–C represent three separate experiments using neurons from litters obtained at different times.

Discussion

In this work, we examined the role of the SHP-1 pTyr phosphatase in neurotrophin-mediated cell survival and signal transduction. Our results indicate that SHP-1 is a TrkA phosphatase in PC12 cells and in sympathetic neurons in culture and in vivo, and that it functions to ensure low levels of basal TrkA activation and to attenuate long-term TrkA signaling in the presence of NGF. These conclusions are supported by four findings. First, we show that SHP-1 dephosphorylated TrkA in vivo and in vitro, and that dephosphorylation was predominantly at sites that controlled TrkA activity (Y674 and Y675). Second, overexpression of SHP-1 in sympathetic neurons and PC12 cells resulted in apoptosis as a consequence of TrkA dephosphorylation. Third, inhibition of endogenous SHP-1 activity was sufficient to support NGF-independent neuronal survival as a consequence of enhanced basal TrkA phosphorylation and downstream Akt activation. Inhibition of endogenous SHP-1 also led to sustained and elevated TrkA activation in the presence of NGF. Fourth, sympathetic neuron number was higher in mice genetically deficient in SHP-1, presumably as a consequence of enhanced TrkA activation during the period of naturally occurring neuronal death; neurons from these mice survived in limiting amounts of NGF. Together, these results argue that SHP-1 is a key negative regulator of TrkA-initiated signal transduction, and that it mediates this negative regulation largely at the level of the TrkA receptor. Such regulation is critical for maintaining the trophic factor dependence of at least one population of neurons during the naturally occurring cell death period, a dependence that is essential for establishing appropriate neuronal connectivity.

How does SHP-1 regulate TrkA activity? We propose that SHP-1 regulates both basal and NGF-stimulated TrkA activity. Because inhibition of endogenous SHP-1 stimulates the NGF-independent phosphorylation of TrkA, SHP-1 can regulate the basal, nonliganded activity of TrkA. TrkA activity, in the absence of NGF, is thus normally controlled and suppressed by SHP-1 activity. In the presence of NGF, TrkA is efficiently dimerized and hyperactivated, and TrkA tyrosine kinase activity predominates over basal SHP-1 tyrosine phosphatase activity. The enhanced TrkA activity results in receptor transphosphorylation, followed by recruitment of cytoplasmic signaling proteins to TrkA transphosphorylation sites, and TrkA-induced tyrosine phosphorylation of these substrates that in turn stimulates survival and growth pathways. However, SHP-1 is also recruited to and stimulated by NGF-bound TrkA, resulting in an increase in SHP-1 tyrosine phosphatase activity. The increase in SHP-1 tyrosine phosphatase activity would result in an attenuation of TrkA activity. Thus, we propose a model whereby SHP-1 either directly or indirectly associates with TrkA, resulting in an increase in SHP-1 activity followed by dephosphorylation of TrkA at the Y674 and Y675 sites; a similar mechanism is used by the tyrosine phosphatase PTP1B to regulate the insulin receptor (Salmeen et al., 2000). The dephosphorylation of these sites results in decreased TrkA biochemical and biological activity (Cunningham et al., 1997) and subsequent decreased activation of NGF-signaling proteins. Therefore, we suggest that SHP-1 has two functions: (1) to keep TrkA in an "off" state in the absence of ligand, and (2) to modulate TrkA activity after dimerization and activation of TrkA by NGF.

What is the role of SHP-1 during sympathetic neuron development? We propose that SHP-1 has two functions: (1) to control TrkA activity in the absence of NGF, and (2) to "fine-tune" TrkA-mediated survival signals in the presence of NGF. Correct neuron number during sympathetic development is dependent on the functional interplay of TrkA-induced survival signals and $p75^{NTR}$-induced apoptotic signals (Kaplan and Miller, 2000; Majdan et al., 2001). Mice deficient in TrkA lack most sympathetic neurons, whereas mice deficient in $p75^{NTR}$ have twice the number of sympathetic neurons per ganglia in the SCG at P20. *me/me* mice that lack SHP-1 have 35% more neurons than wt mice (Fig. 9 A), indicating that SHP-1 functions during development to either suppress TrkA activity or the activity of other apoptotic signals. On the basis of our work in cultured SCG neurons, we favor the former hypothesis. In particular, we propose that SHP-1 is essential to keep TrkA off in neurons that have not contacted the correct targets and/or are late arriving, and subsequently, have not sequestered sufficient levels of NGF. Any basal TrkA activation in these neurons would serve to undermine the biological purpose of the cell death period, which is to ensure that only those neurons that are appropriately connected are maintained. Moreover, even in neurons that have sequestered limited NGF, SHP-1 regulation of TrkA signaling may well serve to regulate the precise balance between "positive" TrkA and "negative" $p75^{NTR}$ signaling, a balance that is essential for establishment of appropriate neuron numbers.

Until recently, SHP-1 expression was largely thought to be restricted to the hematopoietic system. However, recent studies have demonstrated that SHP-1 is expressed throughout the central nervous system in both neurons (Jena et al., 1997; Horvat et al., 2001) and glia (Massa et al., 2000). SHP-1 plays a key role in oligodendrocyte and glial development, as *me/me* mice display decreased numbers of central nervous system glia and dysmyelination (Massa et al., 2000; Wishcamper et al., 2001). Together, these observations suggest an important role for SHP-1 in the development and maintenance of the nervous system, a role that we propose is mediated at least partially via regulation of the TrkA neurotrophin receptor.

Materials and methods

Cells, growth factors, and antibodies

PC12 cells (clone 6-24) and Sf9 insect cells were grown as described previously (Kaplan et al., 1990; Hempstead et al., 1992). The antibodies used were as follows: pTyr (4G10) and PLC-γ1 monoclonal, and $p75^{NTR}$, XIAP, Akt, and SHP-1 polyclonal antibodies (all from Upstate Biotechnology); SHP-1 monoclonal (Transduction Laboratories); MAPK monoclonal (Santa Cruz Biotechnology, Inc.); phosphospecific Akt and phosphospecific c-jun (Cell Signaling Technology); phosphospecific MAPK (Promega); Bim/Bod polyclonal (StressGen Biotechnologies); and anti-pan Trk 203 (Hempstead et al., 1992). The phosphospecific TrkA (Tyr490; 674/675) antibodies were a gift from R. Segal (Harvard University, Cambridge, MA) or were purchased from Cell Signaling Technology. Anti-TrkA RTA was a gift from L. Reichardt (University of California, San Francisco, San Francisco, CA). NGF was obtained from Cedarlane Labs, Ltd.

SCG neuronal cultures

Mass cultures of pure sympathetic neurons derived from the SCG of P1 rats (Sprague-Dawley) or *me/me* mice were prepared and cultured as described previously (Ma et al., 1992; Bamji et al., 1998).

Expression of recombinant TrkA in Sf9 cells

TrkA association assays were performed as described previously (Kaplan et al., 1990; Stephens et al., 1994) with the following modifications. Wild-type and phosphorylation site mutant human TrkA proteins (Stephens et al., 1994) immunoprecipitated from Sf9 cells and autophosphorylated in vitro were resuspended in 1 ml of lysate prepared from 10^7 PC12 cells lysed in NP-40 lysis buffer. The immune complexes were incubated with the lysate for 3 h at 4°C and washed three times with NP-40 lysis buffer and once with 10 mM Tris, pH 7.4.

TrkA dephosphorylation assays

Sf9 insect cells infected with SHP-1, SHP-2, or TrkA baculoviruses for 48 h were lysed in NP-40 lysis buffer, and SHP or TrkA proteins were immunoprecipitated. TrkA immunoprecipitates were washed once with RIPA, twice with NP-40 lysis buffer, and once with phosphatase buffer (25 mM Hepes, pH 7.3, 5 mM EDTA, and 10 mM DTT). Washed TrkA immunoprecipitates were incubated for 30 min at 30°C in phosphatase buffer containing 5 μCi γ-[^{32}P]ATP (5 μM ATP final) per reaction. SHP immunoprecipitates were washed once with RIPA, twice with NP-40 lysis buffer, and once with phosphatase buffer. To detect TrkA dephosphorylation, TrkA and SHP immunoprecipitates were incubated together for 30 min at 30°C. The reaction was stopped with Laemmli SDS sample buffer and boiled for 5 min.

Recombinant adenoviruses and viral infections

Replication-defective recombinant Ad SHP-1 WT and ΔP were prepared and purified as described previously (Slack et al., 1996; Mazzoni et al., 1999). Recombinant adenoviruses were amplified and purified on CsCl gradients and titered by plaque assay. GFP (Aegera Therapeutics Inc.) or *Escherichia coli* β-galactosidase (LacZ)–expressing recombinant adenoviruses (provided by F. Graham, McMaster University, Hamilton, ON) were prepared in the same backbone as the SHP-1 adenoviruses. Viral infections of sympathetic neurons were performed as described previously (Mazzoni et al., 1999; Wartiovaara et al., 2002). For PC12 infections, cells plated on poly-L-lysine were infected with adenoviruses for 48 h.

Immunoprecipitation, immunoblotting, and immunocytochemistry

Cells were treated with NGF, lysates were prepared, and immunoprecipitations and Western blotting were performed as described previously

(Kaplan et al., 1991; Vambutas et al., 1995; Mazzoni et al., 1999). Lysates were prepared from normal and *me/me* SCGs as follows. Freshly dissected ganglia were homogenized in 100 μl of lysis buffer (Kaplan et al., 1991), transferred to microfuge tubes, rocked gently at 4°C for 30 min, and microcentrifuged at 13,000 rpm for 10 min at 4°C. Immunoprecipitations and Western blots were performed as described by Majdan et al. (2001).

Immunocytochemistry

Fluorescence immunocytochemistry was performed essentially as described previously (Wartiovaara et al., 2002). Cells were double labeled with a polyclonal antibody to neurofilament (1:200; Chemicon) and an mAb to SHP-1 (1:50; Transduction Laboratories). All the secondary antibodies were obtained from Alexa (goat α Rb Alexa-488 and goat α ms Alexa-555, both 1:1,000; Molecular Probes, Inc.). The pictures were taken using a digital camera (model Retiga Exi; Q-Imaging) mounted directly on a microscope (model Axioplan 2; Carl Zeiss MicroImaging, Inc.) in a room with a temperature between 21 and 24°C and with an air objective (model Plan Neofluor, 20×/0,5; Carl Zeiss MicroImaging, Inc.). The acquisition software used was Northern Eclipse, and the pictures were assembled in Adobe Photoshop.

Cell survival assays and analysis of the *me/me* mice

Survival assays were performed 72 h after NGF withdrawal as described previously using MTT (Slack et al., 1996), TUNEL (Wartiovaara et al., 2002), or by counting phase-bright cells. Survival assays with *me/me* SCG neurons were performed by counting phase-bright (live) cells in premarked, randomly selected fields at 24-h intervals. The same fields were followed for the entire experiment with five to seven fields counted per well (30–300 cells/field). The percentage of survival was calculated by dividing the number of phase-bright cells remaining at each time point by the number of phase-bright cells at 0 h. Control neurons used for these experiments were obtained from either wt littermate or wt C3H mice. The number of sympathetic neurons per ganglia of the *me/me* was determined as described previously (Bamji et al., 1998).

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