

Identification and Characterization of Novel Substrates of Trk Receptors in Developing Neurons

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Summary

Neurotrophins influence growth and survival of specific populations of neurons through activation of Trks, members of the receptor tyrosine kinase (RTK) family. In this report, we describe the identification and characterization of two substrates of Trk kinases, rAPS and SH2-B, which are closely related Src homolog 2 (SH2) domain-containing signaling molecules. rAPS and SH2-B are substrates of TrkB and TrkC in cortical neurons and SH2-B is a substrate of TrkA in sympathetic neurons. Moreover, rAPS and SH2-B bind to Grb2, and both are sufficient to mediate NGF induction of Ras, MAP kinase (MAPK), and morphological differentiation of PC12 cells. Lastly, antibody perturbation and transient transfection experiments indicate that SH2-B, or a closely related molecule, is necessary for NGF-dependent signaling in neonatal sympathetic neurons. Together, these observations indicate that rAPS and SH2-B mediate Trk signaling in developing neurons.

Introduction

A variety of extrinsic cues, including soluble neurotrophic factors, influence differentiation, survival, and plasticity of developing neurons. One family of neurotrophic factors that contributes to neuronal development is the neurotrophins, which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, and NT-6 (reviewed by Reichardt and Farinas, 1997). Cell surface receptors for neurotrophins are members of the Trk family of receptor tyrosine kinases (RTKs) (Kaplan et al., 1991a, 1991b; Klein et al., 1991). TrkA, TrkB, and TrkC are receptors for NGF, BDNF, and NT-4/5 and NT-3, respectively (reviewed by Barbacid, 1995; Bothwell, 1995; Reichardt and Farinas, 1997). In addition, a distinct neurotrophin receptor, p75^{LNGFR}, binds to all neurotrophins (reviewed by Barker, 1998). While the role of p75^{LNGFR} during neurodevelopment is not yet fully appreciated, Trk receptors appear to be the major mediators of neurotrophin signaling in developing and adult neurons.

Upon binding their ligands, Trk receptors dimerize and autophosphorylate on multiple tyrosine residues *trans*. Autophosphorylation sites within rat TrkA include tyrosine residues Y679, Y683, and Y684, which are located within the catalytic loop of the tyrosine kinase domain, and several tyrosine residues that lie outside

of the kinase domain, including Y499 and Y794 (Stephens et al., 1994; Peng et al., 1995). TrkA phosphotyrosines and their surrounding amino acid residues serve as specific recognition sites for effector molecules that contain either an Src homolog 2 (SH2) domain or a phosphotyrosine binding (PTB) domain (domains reviewed by Pawson and Scott, 1997). Recruitment of Trk effectors to the plasma membrane initiates signaling events that promote growth, differentiation, and survival.

Propagation of the TrkA signal within PC12 cells and neurons is mediated by multiple effectors that influence distinct intracellular signaling pathways (Kaplan and Stephens, 1994; Greene and Kaplan, 1995), including Ras-dependent signaling pathways and Ras-independent signaling pathways. Ras-dependent signaling pathways mediate NGF induction of MAP kinase (MAPK) (Qiu and Green, 1992; Thomas et al., 1992; Wood et al., 1992), morphological differentiation (Hagag et al., 1986; Szaberenyi et al., 1990) and expression of a variety of genes in PC12 cells (D'Arcangelo and Halegoua, 1993), and NGF-dependent survival of small-diameter dorsal root ganglion (DRG) sensory neurons (Borasio et al., 1989, 1993). Ras-independent signaling pathways mediate NGF induction of Na⁺ channel activity and expression of the type II and PN-1 Na⁺ channel and Thy-1 genes in PC12 cells (D'Arcangelo and Halegoua, 1993) and survival of sympathetic neurons (Borasio et al., 1993; Creedon et al., 1996). Although the identity of TrkA effectors that influence Ras-dependent and Ras-independent TrkA signaling in neurons remains unclear, two general tyrosine kinase effector molecules, Shc and PLC- γ , mediate NGF induction of Ras-dependent signaling in PC12 cells (Stephens et al., 1994). Shc is an adaptor protein that couples Trk receptors to Grb2, SOS, and the Ras-MAPK signaling cascade, and it associates with TrkA phosphotyrosine residue 499 (pY499) (Obermeier et al., 1993; Loeb et al., 1994) through its PTB domain (Kavanaugh and Williams, 1994). PLC- γ , an enzyme that catalyzes phosphoinositide turnover and promotes activation of protein kinase C and the Ras-MAPK signaling cascade, associates with TrkA phosphotyrosine residue 794 (pY794) through its SH2 domain (Anderson et al., 1990; Obermeier et al., 1993; Loeb et al., 1994). Both Shc and PLC- γ are widely expressed in neuronal and nonneuronal cells, and it is likely that they contribute to Trk signaling in neurotrophin-dependent neurons during neurodevelopment.

In support of the idea that Shc and PLC- γ are essential TrkA effector molecules, the integrity of both TrkA residues Y499 and Y794, the binding sites for Shc and PLC- γ , respectively, is essential for the NGF induction of Ras-MAPK signaling and morphological differentiation of PC12 cells (Stephens et al., 1994; Inagaki et al., 1995). However, considerable evidence supports the idea that Shc and PLC- γ do not mediate all aspects of NGF signaling, even in PC12 cells. In addition to rat TrkA residues Y499 and Y794, the integrity of several other TrkA tyrosine residues and of a small juxtamembrane domain of TrkA are essential for TrkA-mediated differentiation of PC12 cells (Inagaki et al., 1995; Peng et al.,

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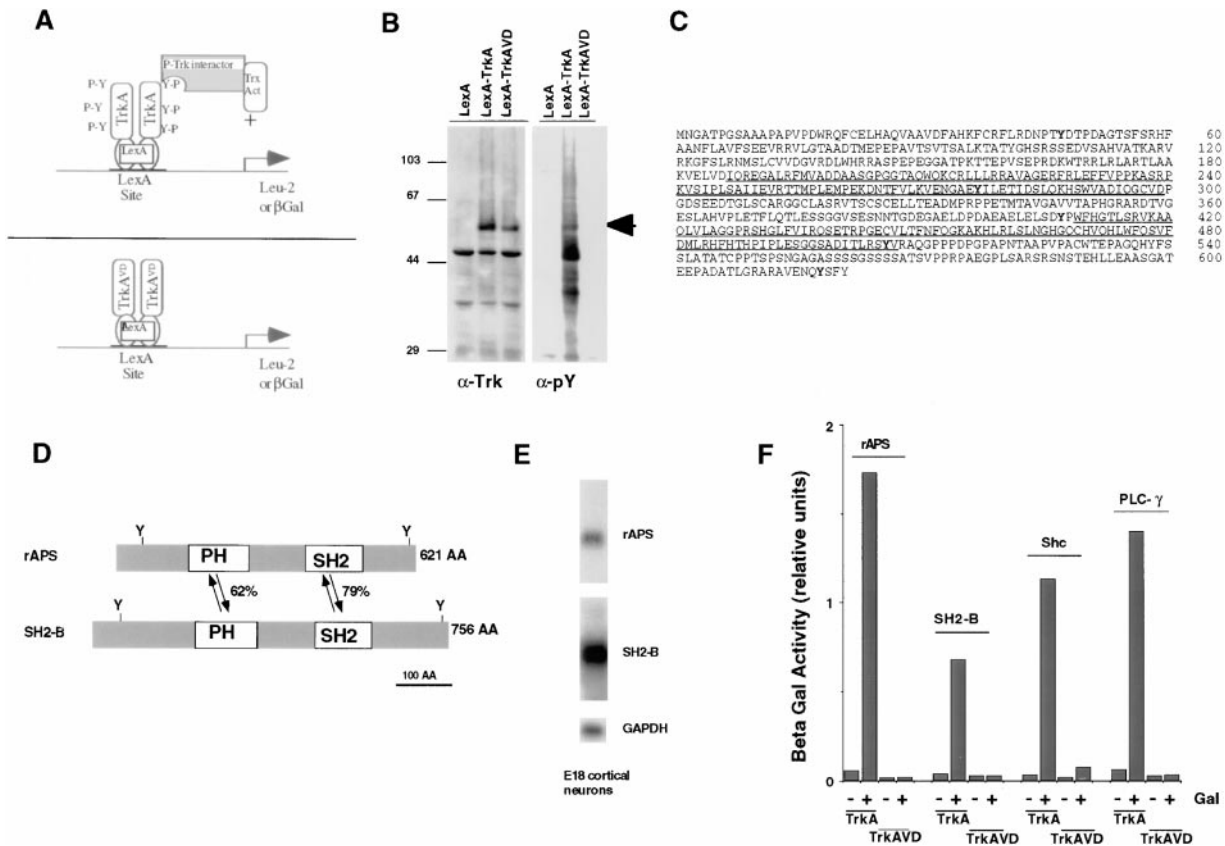


Figure 1. Identification of Phospho-Trk-Interacting Proteins by a Yeast Two-Hybrid Screen

(A) An overview of two-hybrid screen to identify novel Trk substrates. The baits consisted of the entire intracellular domains of either wild-type rat TrkA or kinase-deficient TrkA^{VD} mutant that were fused to the DNA binding and dimerization domain of LexA. First, clones that encode proteins that interacted with phospho-TrkA were identified and isolated. Then, those cDNAs were reintroduced into yeast expressing either LexA-TrkA or LexA-TrkA^{VD}. Clones that encode proteins that interacted with phospho-TrkA, but not TrkA^{VD}, were characterized further.

(B) LexA-TrkA, but not LexA-TrkA^{VD}, is catalytically active in yeast. Extracts of yeast expressing LexA, LexA-TrkA, or LexA-TrkA^{VD} were resolved by SDS-PAGE and immunoblotted with either anti-Trk (left) or anti-pY (right).

(C) Deduced amino acid sequence of rAPS. The PH domain and SH2 domain are underlined.

(D) Schematic representation of rAPS and SH2-B proteins. The degree of amino acid identity between several domains of rAPS and SH2-B are indicated.

(E) rAPS and SH2-B are expressed in E18 cortical neurons grown in vitro as determined by Northern blot.

(F) Shc, PLC-γ, rAPS, and SH2-B interact with phospho-TrkA, but not with the TrkA^{VD} mutant. Interactions between PTRIPs and LexA-Trks were assessed with a liquid β-gal assay using extracts of yeast that were grown in the presence or absence of D-galactose, which controls expression of interactors.

1995). Moreover, NGF and BDNF induce tyrosine phosphorylation of many proteins in sympathetic neurons and cortical neurons, respectively (for example, see Senger and Campenot, 1997), and several unidentified proteins coprecipitate with TrkA or TrkB from these neurons (unpublished data). Further, TrkA, TrkB, and TrkC mediate distinct responses, even within the same cell type (Carter et al., 1995; Iwasaki et al., 1997; McAllister et al., 1997). Since Shc and PLC-γ interact with all three Trk receptors, it is likely that each Trk receptor has one or more distinct unidentified substrate(s) that endows it with a unique signaling capacity. Together, these and other observations provide a compelling argument that unidentified Trk substrates, which are essential mediators of neurotrophin signaling, must exist. Here, we report the identification and characterization of two structurally related phospho-Trk-interacting proteins that are

substrates of Trk receptors in developing neurotrophin-dependent neurons.

Results

Identification of Proteins that Interact with Phospho-TrkA, -TrkB, and -TrkC

We employed a yeast two-hybrid screen to identify novel substrates of Trk receptor tyrosine kinases (Figure 1A). The entire intracellular domains of rat TrkA, TrkB, or TrkC were fused to the DNA binding and dimerization domain of the bacterial transcription factor LexA. Dimerization of the LexA-TrkA fusion protein through the LexA dimerization domain resulted in tyrosine phosphorylation of LexA-TrkA when expressed in yeast (Figure 1B and data not shown). In addition, a kinase-inactive LexA-

Table 1. Identification of Phospho-Trk-Interacting Proteins (PTRIPs)

Name	Interaction with		Number of Clones (different fragments)	ID
	LexA-TrkA	LexA-TrkA ^{VD}		
PTRIP-1	yes	no	7	novel
PTRIP-2	yes	no	5	SH2-B
PTRIP-3	yes	no	2	Shc
PTRIP-4	yes	no	2	PLC- γ
PTRIP-5 to -8	yes	no	1	novel

ID, identity of clone.

Number of Clones, number of distinct cDNA fragments isolated.

TrkA^{VD} variant was generated by altering the coding determinants of a valine at position 527 (Val-527) of TrkA to an aspartic acid. TrkA Val-527 resides within the invariant glycine loop of all protein kinases and is highly conserved among protein tyrosine kinases (Hanks et al., 1988). Mutation of the analogous valine residue of erbB to an aspartic acid completely abolished its catalytic activity (Shu et al., 1994). The LexA-TrkA^{VD} mutant was expressed in yeast, but it was not catalytically active (Figure 1B). Expression of LexA-TrkA, LexA-TrkB, LexA-TrkC, or LexA-TrkA^{VD} did not substantially affect the growth of yeast (data not shown), even though several endogenous yeast proteins were phosphorylated by the catalytically active LexA-Trk proteins (Figure 1B).

To identify proteins that associate with autophosphorylated TrkA, we employed LexA-TrkA as a bait and a yeast cDNA expression library prepared from mRNA isolated from rat embryonic day 14 (E14) spinal cord and DRG. mRNA from E14 spinal cord and DRG was used as a template for cDNA synthesis because all three Trks are expressed in one or more subsets of neurons within this tissue, and mice lacking either TrkA, TrkB, or TrkC exhibit severe neuropathies of specific classes of DRG neurons (reviewed by Reichardt and Farinas, 1997). Thus, natural substrates of all three Trk receptors are expressed in embryonic spinal cord and DRG. A total of 19 cDNAs were identified that encode proteins that conferred Leu-independent growth of LexA-TrkA but not LexA-TrkA^{VD} yeast. Sequence analysis revealed that these cDNAs encode eight distinct proteins. We have named the TrkA-interacting clones PTRIPs (phospho-Trk interacting proteins). Distinct fragments of cDNAs encoding PTRIP-1 and PTRIP-2 were identified most often, two distinct cDNAs encoding either PTRIP-3 or -4 and one cDNA fragment encoding PTRIP-5-PTRIP-8 were obtained (Table 1). Sequence analysis revealed that PTRIP-3 encodes Shc and PTRIP-4 encodes PLC- γ , both of which are previously identified substrates of TrkA (see Introduction). Like Shc and PLC- γ , PTRIP-1 and PTRIP-2 interacted strongly with LexA-TrkA, LexA-TrkB, and LexA-TrkC in yeast (Figure 1F and data not shown). Since multiple distinct cDNAs encoding PTRIP-1 and PTRIP-2 were obtained most often in our screen (Table 1), and since these PTRIPs are expressed in developing brain, spinal cord, and primary embryonic cortical neurons (Figure 1E and data not shown), we have focused our attention on PTRIP-1 and PTRIP-2 as mediators of Trk signaling in developing neurons.

PTRIP-1 and PTRIP-2 Encode rAPS and SH2-B, Two Highly Related Adaptor Proteins

One of the cDNA clones encoding PTRIP-1 rescued from yeast was used as a probe to screen a phage cDNA library prepared from adult rat hippocampus mRNA. The largest PTRIP-1 cDNA isolated was 2.9 kb. The coding sequence of PTRIP-1 starts at nucleotide 272 of this clone; the first methionine codon is located within a Kozak consensus sequence followed by an open reading frame (ORF) of 1866 bp. Conceptual translation of the nucleotide sequence of the ORF revealed that it encodes a novel 621 amino acid protein with a Pleckstrin homolog (PH) domain and an SH2 domain. The deduced amino acid sequence of PTRIP-1 is presented in Figure 1C. PTRIP-1 is likely to be the rat ortholog of human APS (Yokouchi et al., 1997) because the two proteins are overall 82% identical, and therefore we have used the name rAPS to describe this protein. We also isolated a full-length cDNA clone encoding PTRIP-2. Sequence analysis of this clone revealed that PTRIP-2 is identical to SH2-B, a previously identified rat protein that can bind to the γ subunit of the high-affinity IgE receptor, Fc ϵ R1 (Osborne et al., 1995). Interestingly, rAPS and SH2-B are overall 37% identical and 60% similar to each other. The N-terminal proline-rich domains, the PH domains, the SH2 domains, and the C-terminal tyrosine-containing motifs are all highly conserved between rAPS and SH2-B, as is the general organization of these domains (Figure 1D). Besides three conserved tyrosine residues within the PH domains and SH2 domains, two other tyrosine residues are conserved in both proteins; one in the context of an N/HPXY motif (Y55 in SH2-B) and the other in an YXXL motif (Y753 in SH2-B). These tyrosine-containing motifs may be recognition sites for other signaling proteins that contain PTB or SH2 domains, respectively.

NGF Promotes an Association between rAPS and SH2-B and TrkA

Effectors of RTKs bind directly to specific phosphotyrosine residues, and insight into their function can be obtained from structure-function studies that identify specific domains that mediate the association between RTKs and their effectors. Thus, we sought to identify TrkA tyrosine residues critical for the interaction between TrkA and rAPS and SH2-B. Since rAPS and SH2-B contain SH2 domains, and since they interact with phospho-Trks, but not unphosphorylated Trks, it is likely that the SH2 domains mediate their association with TrkA. In support of this idea, one clone isolated in our screen encodes only the SH2 domain and the short C terminus of SH2-B (data not shown). To identify tyrosine residues of TrkA that mediate its association with the rAPS and SH2-B SH2 domains, we performed yeast two-hybrid experiments employing a series of TrkA mutants in which individual or multiple tyrosine residues were changed to phenylalanine (Figure 2A and data not shown; Inagaki et al., 1995). Interestingly, rAPS and SH2-B interacted with the LexA-TrkA mutant F8 in which all conserved tyrosine residues within the intracellular TrkA domain are mutated to phenylalanine, except Y679, Y683, and Y684 within the catalytic loop (Figure 2B). In contrast,

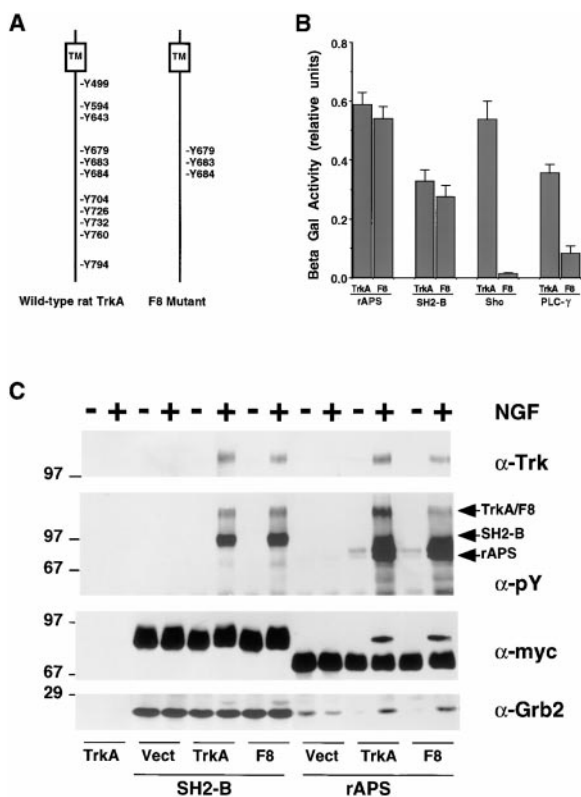


Figure 2. rAPS and SH2-B Interact with TrkA and F8 in Yeast and 293 Cells

(A) Schematic diagram of the intracellular domains of TrkA and TrkA mutant F8 (rat sequence).

(B) Interactions between Shc, PLC-γ, rAPS, or SH2-B and wild-type TrkA or the F8 mutant in yeast. The degree of interaction was assessed by a yeast two-hybrid liquid β-gal assay. The β-gal activity is shown in relative units.

(C) rAPS and SH2-B associate with TrkA and the TrkA mutant F8 in an NGF-dependent manner in HEK 293 cells. Either myc-tagged rAPS or SH2-B was expressed in 293 cells in the presence or absence of either TrkA or F8. Cell extracts were subjected to immunoprecipitation using anti-myc monoclonal antibodies. The immune complexes were resolved by SDS-PAGE and immunoblotted with anti-Trk, anti-pY, anti-myc, and anti-Grb2 antibodies.

Shc and PLC-γ interacted with TrkA mutants only when Y499 or Y794 are intact. For example, Shc and PLC-γ did not interact with F8 (Figure 2B). Therefore, rAPS and SH2-B, Shc, and PLC-γ can associate with TrkA through distinct domains of TrkA. While the interactions between Shc and PLC-γ and TrkA require TrkA phosphotyrosine residues Y499 or Y794, rAPS and SH2-B can interact with catalytically active TrkA through association of other domains, possibly through direct binding to TrkA phosphotyrosine residues Y679, Y683, and/or Y684 TrkA within the catalytic loop domain. Alternatively, rAPS and SH2-B may bind to a distinct TrkA domain that is made available for binding following autophosphorylation of the catalytic loop tyrosines.

If rAPS and SH2-B are substrates of TrkA, then they should associate with TrkA upon exposure of cells to NGF. To address this possibility, we coexpressed either myc-tagged rAPS (myc-rAPS) or myc-SH2-B with either full-length TrkA or the TrkA mutant F8 in HEK 293 cells. TrkA coprecipitated with both myc-rAPS and myc-SH2-B

from extracts of NGF-treated 293 cells but not untreated cells. In addition, NGF induced tyrosine phosphorylation of rAPS and SH2-B (Figure 2C). Similar results were obtained from 293 cells that coexpressed either rAPS or SH2-B with TrkB or TrkC and were treated with BDNF or NT-3, respectively (data not shown). Lastly, NGF triggered an association between the TrkA mutant F8 and myc-rAPS and myc-SH2-B as well as F8-mediated tyrosine phosphorylation of both of these PTRIPs (Figure 2C). Taken together, these results indicate that neurotrophins can induce an association between rAPS and SH2-B and membrane-bound Trk receptors.

NGF, BDNF, and NT-3 Induce Tyrosine Phosphorylation of rAPS and SH2-B in Neurons

To determine whether rAPS and SH2-B are natural Trk substrates in neurons, immunoprecipitation and immunoblot experiments were performed using extracts of neurotrophin-treated cortical neurons or sympathetic neurons. For these experiments, rabbit polyclonal antibodies directed against rAPS and SH2-B fusion proteins were generated. Anti-rAPS and anti-SH2-B, but not pre-immune sera, recognized their respective recombinant proteins (Figures 3A and 3B), and they did not cross-react (data not shown).

SH2-B and rAPS are substrates of Trk receptors in embryonic cortical neurons. Anti-SH2-B antibodies, but not preimmune sera, detected multiple proteins in the range of 90–110 kDa from extracts of cortical neurons by immunoblot analysis. Upon exposure of cortical neurons to BDNF for 5 min, there was a marked mobility shift of these proteins, most likely due to their tyrosine phosphorylation (Figure 3A). An identical pattern was detected in immunoblot experiments using a different SH2-B antibody (data not shown). One of the major proteins detected by anti-SH2-B by immunoblot analysis of extracts of cortical neurons comigrated with SH2-B detected in extracts of 293 cells transfected with an SH2-B expression vector (Figure 3A). These results demonstrate that the SH2-B antibodies are specific and are consistent with the idea that SH2-B is rapidly post-translationally modified following Trk receptor activation. To directly establish whether rAPS and SH2-B are phosphorylated on tyrosine residues following Trk receptor activation, rAPS and SH2-B antibodies were used to immunoprecipitate rAPS and SH2-B from extracts of cortical neurons, immune complexes were resolved by SDS-PAGE, and immunoblot analyses were done with anti-phosphotyrosine. BDNF effectively induced tyrosine phosphorylation of a protein recognized by the rAPS antisera but not preimmune antisera (Figure 3B). Identical results were obtained in experiments employing a goat antibody raised against a distinct domain of human APS (data not shown). Moreover, rAPS is tyrosine phosphorylated upon exposure of cortical neurons to either BDNF or NT-3 (Figure 3B), and BDNF induction of phosphorylation of rAPS occurs within 1 min and persists for more than 60 min (Figure 3C). Likewise, BDNF induction of tyrosine phosphorylation of SH2-B peaked within 2 min of exposure of cortical neurons to BDNF and persisted for at least 1 hr (Figure 3C). In contrast to these results with cortical neurons, we failed

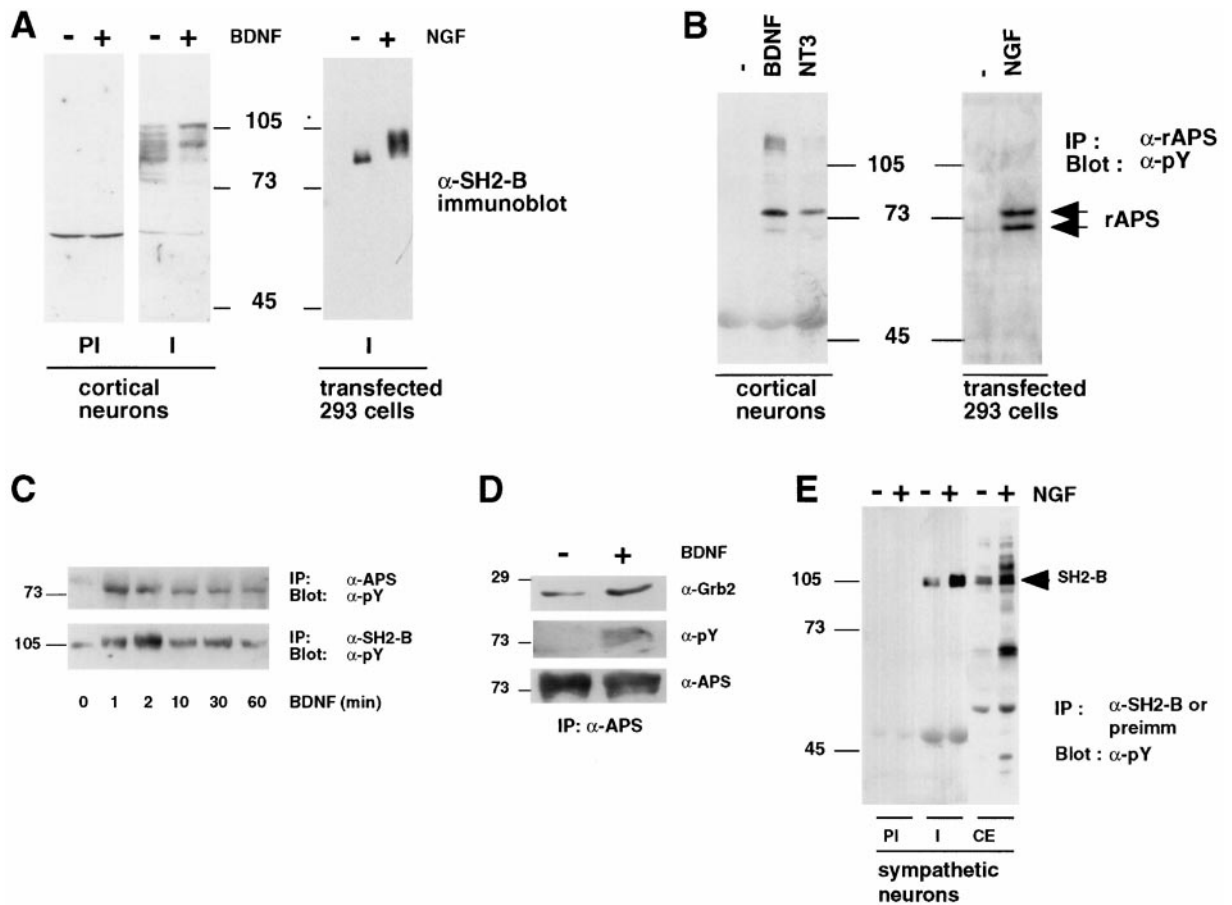


Figure 3. Neurotrophins Induce Tyrosine Phosphorylation of rAPS and SH2-B in Cortical and Sympathetic Neurons

(A) BDNF or NGF induce a mobility shift of SH2-B in cortical neurons or HEK 293 cells, respectively. 293 cells expressing recombinant SH2-B and TrkA were stimulated with vehicle or NGF (100 ng/ml, 10 min). Cell extracts were analyzed by immunoblotting with anti-SH2-B antisera (right). Alternatively, extracts of cultured embryonic cortical neurons stimulated with vehicle control or BDNF (50 ng/ml, 10 min) were prepared and analyzed by immunoblotting with either preimmune sera (PI) or anti-SH2-B antisera (I) (left). Anti-SH2-B did not recognize any protein in extracts of mock-transfected 293 cells (data not shown).

(B) rAPS is a substrate of both TrkB and TrkC in cortical neurons. Rat E18 cortical cultures were stimulated with BDNF or NT3 (100 ng/ml, 10 min) (left), and 293 cells expressing rAPS and TrkA were stimulated with NGF (right). Cell extracts were immunoprecipitated with anti-rAPS antibodies and immune complexes analyzed by immunoblot with anti-pY.

(C) Time course of BDNF induction of tyrosine phosphorylation of rAPS and SH2-B in cortical neurons. Cortical neurons were treated with BDNF for indicated times, cell extracts prepared and subjected to immunoprecipitation with either anti-rAPS or anti-SH2-B, and immune complexes analyzed by immunoblot with anti-pY.

(D) Grb2 coprecipitates with rAPS from extracts of cortical neurons. Cortical neurons were untreated or treated with BDNF, and extracts were prepared and subjected to immunoprecipitation with anti-rAPS. Immune complexes were subjected to immunoblot using either anti-Grb2, anti-pY, or anti-APS.

(E) SH2-B is a substrate of TrkA in sympathetic neurons. Rat neonatal sympathetic neurons were grown in low NGF (2 ng/ml) medium for 48 hr and then exposed to a high concentration of NGF (200 ng/ml, 10 min). Immunoprecipitation with anti-SH2-B or preimmune antisera and immunoblot with anti-pY were performed as above. Abbreviations: PI, preimmune sera; I, immune sera; CE, cell extracts; and IP, immunoprecipitation.

to detect tyrosine-phosphorylated rAPS or SH2-B in extracts prepared from NGF-treated PC12 cells (data not shown).

Since neonatal sympathetic neurons require NGF for growth and survival, we next sought to determine whether either rAPS or SH2-B is a substrate of TrkA in sympathetic neurons. RT-PCR experiments demonstrated that SH2-B is expressed in these neurons (data not shown). Anti-SH2-B precipitated a protein from NGF-treated sympathetic neurons that comigrated with recombinant SH2-B and that was phosphorylated on tyrosine residues following exposure of these neurons to

NGF (Figure 3E). Importantly, this protein comigrates with one of the major tyrosine phosphorylated proteins in extracts of NGF-treated sympathetic neurons (Figure 3E). Taken together, these results indicate that rAPS and SH2-B are substrates of TrkB and TrkC in embryonic cortical neurons, and SH2-B is a substrate of TrkA in neonatal sympathetic neurons.

rAPS and SH2-B Are Mediators of NGF Signaling to the Ras-MAPK Pathway

Since rAPS and SH2-B have conserved structural domains and tyrosine residues that are likely to mediate

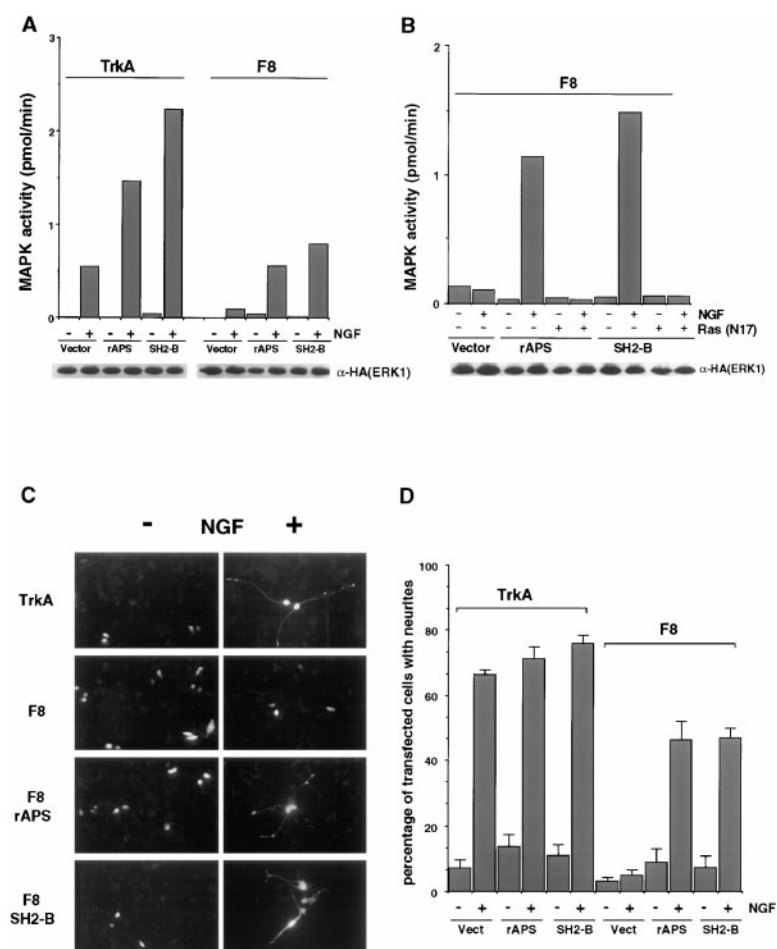


Figure 4. rAPS and SH2-B Mediate NGF Induction of MAPK and Neuritogenesis

(A) rAPS and SH2-B mediate TrkA and F8 activation of MAPK. 293 cells transfected with expression vectors encoding rAPS or SH2-B and either TrkA or F8 and HA-tagged ERK1 were stimulated with NGF (100 ng/ml, 10 min). HA-ERK1 was immunoprecipitated from cell extracts with an anti-HA antibody, and its activity was measured by an immune complex kinase assay. The immune complexes were then immunoblotted with anti-HA antibody (bottom). Similar results were obtained in three experiments performed in duplicate.

(B) RasN17 inhibits the rAPS and SH2-B-mediated MAPK activation. rAPS or SH2-B were transiently expressed with F8 and ERK1 in the absence or presence of an expression vector encoding RasN17 in 293 cells. Cells were treated and MAPK activity was measured as above. Similar results were obtained in three experiments performed in duplicate.

(C) rAPS and SH2-B can mediate NGF induction of morphological differentiation of PC12 cells. Wild-type TrkA or the TrkA mutant F8 in the presence or absence of either rAPS or SH2-B was transiently expressed in PC12nr5 cells along with GFP. Cells were incubated with NGF (100 ng/ml) for 3 days after transfection. Then, cells were fixed and transfected cells were identified by fluorescence microscopy.

(D) Quantitation of neurite outgrowth was performed as described in the Experimental Procedures. The results are means \pm SEM of three experiments performed in duplicate.

protein-protein interactions, we investigated whether rAPS and/or SH2-B interact with other known signaling molecules. We found that Grb2, an adaptor protein that contains one SH2 domain flanked by two SH3 domains (Lowenstein et al., 1993), associates with both myc-rAPS and myc-SH2-B expressed in 293 cells (Figure 2C) as well as endogenous rAPS expressed in cortical neurons (Figure 3D). The possibility that rAPS and SH2-B can couple Trk receptors to the Ras-dependent signaling pathway was tested next. rAPS or SH2-B were coexpressed with HA-tagged ERK1 and either TrkA or F8 in 293 cells, and then the ability of NGF to induce MAPK activity in transfected cells was determined by an immune complex kinase assay. As expected, NGF did not induce the activity of MAPK in cells that were not transfected with the TrkA expression vector. However, NGF did evoke robust activation of MAPK in 293 cells that coexpressed TrkA and HA-ERK1, and coexpression of TrkA with either rAPS or SH2-B enhanced NGF induction of MAPK activity (Figure 4A). In contrast, the TrkA mutant, F8, did not mediate NGF induction of MAPK activity in the absence of rAPS and SH2-B. However, coexpression of either rAPS or SH2-B with F8 conferred NGF induction of MAPK (Figure 4A). In addition, NGF induction of MAPK was dependent on Ras; the dominant-inhibitory Ras mutant, RasN17 (Feig and Cooper, 1988), completely blocked NGF induction of MAPK activity in

cells that expressed F8 and either rAPS or SH2-B (Figure 4B). Together, these experiments demonstrate that rAPS and SH2-B can mediate NGF/TrkA activation of the Ras-dependent MAPK signaling cascade.

rAPS and SH2-B Mediate NGF Induction of Neurite Outgrowth in PC12nr5 Cells

To determine whether rAPS and SH2-B are sufficient to mediate NGF induction of morphological differentiation, we performed experiments employing PC12nr5 cells, which express little or no TrkA and are unresponsive to NGF (Green et al., 1986). As shown previously (Loeb et al., 1991), expression of exogenous TrkA in PC12nr5 cells restored NGF sensitivity (Figures 4C and 4D). Because rAPS and SH2-B can associate with F8 (Figure 2), and because the TrkA mutant F8 cannot mediate NGF induction of morphological differentiation of PC12nr5 cells (Figures 4C and 4D and Inagaki et al., 1995), we determined whether rAPS and SH2-B can mediate NGF induction of morphological differentiation of these cells when coexpressed with F8. NGF did not promote morphological differentiation of PC12nr5 cells that expressed rAPS, SH2-B, or F8 alone (Figures 4C and 4D and data not shown). In contrast, NGF elicited robust outgrowth of neurites in cells that coexpressed F8 with either rAPS or SH2-B (Figures 4C and 4D). Consistent with the finding that rAPS and SH2-B can mediate NGF

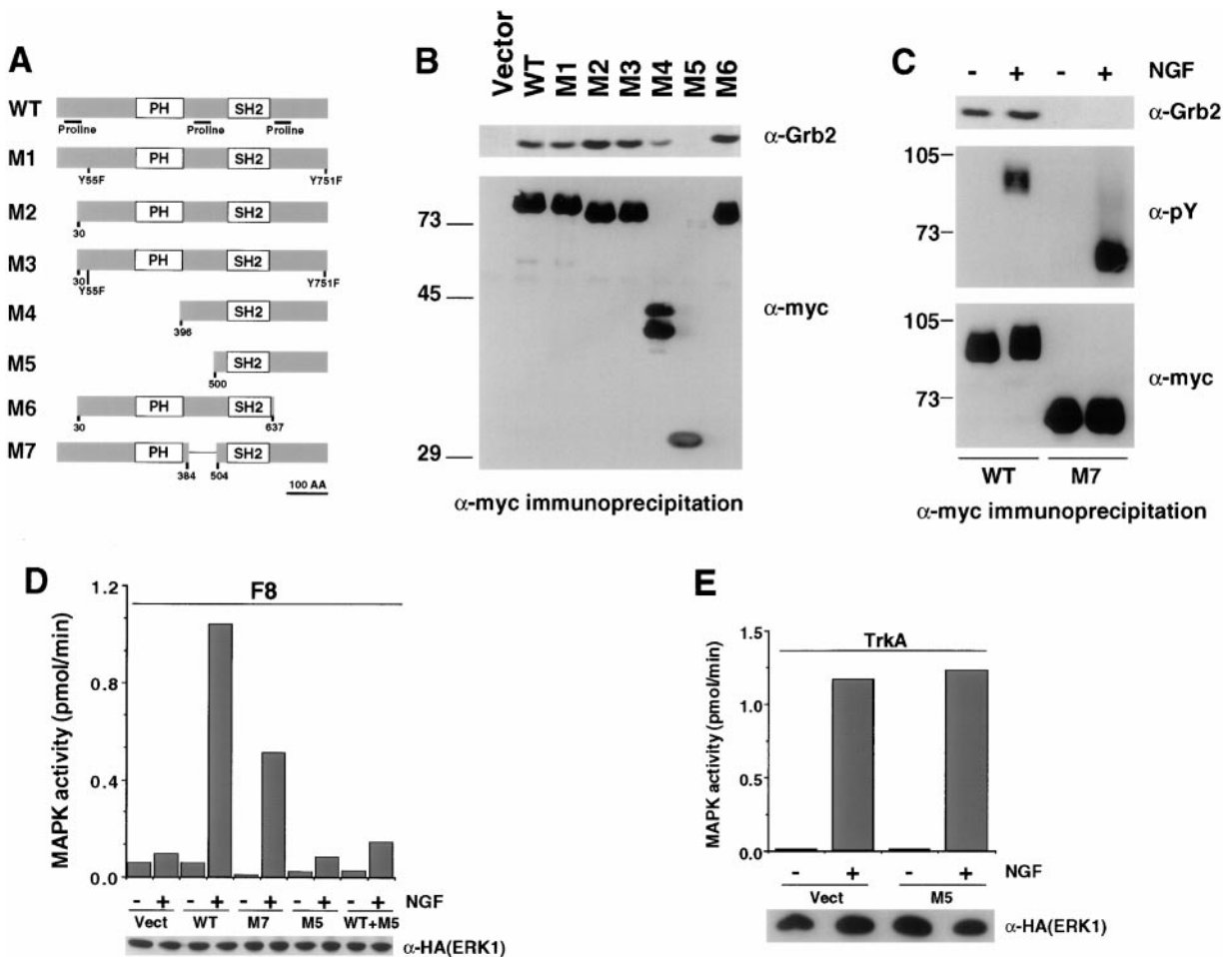


Figure 5. SH2-B Constitutively Binds to Grb2 through a Proline-Rich Domain that Contributes to SH2-B Signal Transduction

(A) Schematic diagrams of SH2-B deletion and tyrosine mutants. The proline-rich motifs are underlined in wild-type SH2-B. (B) Grb2 associates with SH2-B via the proline-rich motif located between the PH and SH2 domains. Myc-tagged SH2-B mutation and deletion constructs were expressed in 293 cells. Cell extracts were then subjected to immunoprecipitation using anti-myc antibody. Immune complexes were immunoblotted with anti-Grb2 (upper) and anti-myc antibodies (bottom). (C) The internal deletion mutant (M7) was coexpressed with TrkA in 293 cells, and cells were stimulated with NGF (100 ng/ml, 10 min). Cell extracts were subjected to immunoprecipitation using anti-myc antibody. Immune complexes were immunoblotted with anti-Grb2 (upper), anti-pY (middle), and anti-myc (bottom) antibodies. (D) The N terminus and a proline-rich domain of SH2-B contribute to NGF induction of SH2-B-mediated MAPK activation. NGF induction of MAPK mediated by vector, SH2-B, M7, M5, or SH2-B + M5 was assessed in 293 cells. DNA amounts used for transfections were: HA-ERK1, 5 μ g; F8, 2 μ g; WT SH2-B, 2 μ g; M7, 2 μ g; M5, 2 μ g; and WT SH2-B + M5, 2 μ g + 10 μ g, respectively (total amount of DNAs including empty vector was 17 μ g). Each experiment was performed three times in duplicate or triplicate. (E) M5 does not block NGF induction of MAPK mediated by wild-type TrkA in 293 cells. Cells were transfected with expression vectors encoding TrkA (5 μ g) and either empty vector or M5 (5 μ g) and HA-ERK1 (5 μ g), and MAPK immune complex kinase assays and immunoblotting were done as above.

activation of Ras-dependent signaling (Figures 4A and 4B), these results indicate that rAPS and SH2-B have the capacity to mediate TrkA induction of morphological differentiation in PC12 cells.

SH2-B Mediates Grb2-Dependent and Grb2-Independent TrkA Signaling

While the interaction between rAPS and Grb2 is partially dependent on tyrosine phosphorylation of rAPS, unphosphorylated SH2-B can bind constitutively to Grb2 (Figure 2C). This observation suggests that Grb2 binds SH2-B through its SH3 domain. To identify domains

of SH2-B that mediate its association with Grb2, we expressed a series of altered myc-SH2-B proteins (Figure 5A) in 293 cells and performed immunoprecipitation and immunoblotting experiments. An SH2-B mutant lacking the conserved tyrosine residues Y55 and Y751 (mutant M1) bound normally to Grb2 (Figure 5B), demonstrating that the SH2-B-Grb2 association is not dependent upon phosphorylation of these SH2-B residues. In addition, deletion of the conserved N-terminal proline-rich domain (amino acids 1-28) (M3), both the N-terminal and the C-terminal proline-rich domains (M6), and the PH domain of SH2-B did not affect its association with Grb2. However, an SH2-B deletion mutant lacking the

N-terminal half of SH2-B (M5), which contains the N-terminal proline-rich domain, the PH domain, and a proline-rich motif located between the PH and SH2 domains, did not interact with Grb2 (Figure 5B). Moreover, a mutant lacking only the proline-rich region located between the PH and SH2 domains did not bind to Grb2 (Figure 5C). Together, these results indicate that SH2-B associates directly and constitutively with Grb2, and that the proline-rich motif located between the PH and SH2 domains of SH2-B is responsible for this association. To test whether the association between SH2-B and Grb2 is essential for Trk-activated SH2-B-mediated signal transduction events, we employed the SH2-B mutants M5 and M7 in MAPK activation experiments. Upon exposure of cells to NGF, mutant M7 associates with TrkA and becomes phosphorylated on tyrosine residues, but it can no longer bind to Grb2 (Figure 5C). M7 and, to a greater extent, M5 were compromised in their ability to mediate NGF induction of MAPK (Figure 5D). Moreover, M5 effectively blocked NGF induction of F8/SH2-B-mediated MAPK activity (Figure 5D). In contrast, M5 did not block the ability of wild-type TrkA to mediate NGF induction of MAPK (Figure 5E). These observations demonstrate that SH2-B mediates NGF induction of MAPK, in part, through its constitutive association with the previously characterized signaling molecule Grb2. Moreover, these results demonstrate that the SH2-B mutant M5 specifically blocks SH2-B-mediated signaling to MAPK; it does not block SH2-B-independent TrkA signaling to the Ras/MAPK pathway.

SH2-B Is Necessary for NGF Signaling in Primary Sympathetic Neurons

Since SH2-B is a TrkA substrate in sympathetic neurons (Figure 3E), and since neonatal sympathetic neurons require NGF and TrkA for growth and survival, we sought to determine whether the TrkA–SH2-B interaction was necessary for TrkA signaling in these neurons. Two complementary approaches were taken. First, anti-SH2-B antibodies, described above, were introduced into dissociated sympathetic neurons by the trituration method (see Experimental Procedures), and then survival of neurons was determined over the course of several days. Two control experiments were performed in parallel to establish specificity of this approach. First, the effects of preimmune IgG trituration on survival of sympathetic neurons were assessed. Second, the effects of anti-SH2-B and preimmune antibodies on growth and survival of sympathetic neurons grown in the presence of forskolin were determined. Because NGF/TrkA signaling is not required for survival of sympathetic neurons grown in the presence of forskolin and other agents that increase intracellular levels of cyclic AMP (cAMP) (Rydell and Greene, 1988), inhibition of TrkA effectors should not affect cAMP-dependent growth and survival of these neurons.

Neonatal sympathetic neurons trituated with anti-SH2-B antibodies, but not preimmune IgG, exhibited a reduced rate of survival when grown in NGF-containing media (Figure 6). Thirty-six hours after plating, 32% fewer MAP-2-positive neurons were identified in cultures derived from cells trituated with anti-SH2-B compared to cells trituated with preimmune IgG. Likewise,

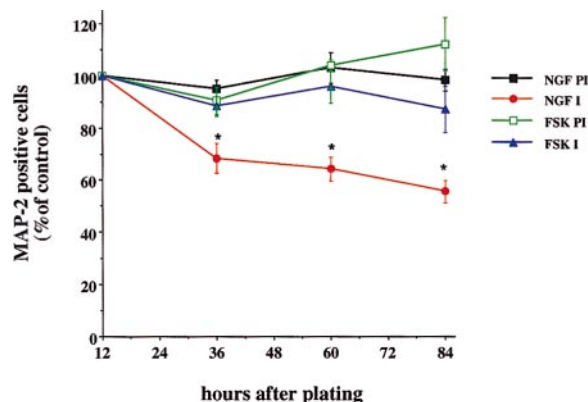


Figure 6. SH2-B Is Necessary for NGF-Dependent, but Not cAMP-Dependent, Survival of Sympathetic Neurons

Anti-SH2-B (I) but not preimmune (PI) antibodies kill dissociated sympathetic neurons grown in the presence of NGF but not in the presence of forskolin. Dissociated sympathetic neurons were injected with either anti-SH2-B antibodies or preimmune IgG by the trituration method. Subsequently, cells were plated, grown in the presence of either NGF (200 ng/ml) or forskolin (FSK) (10 μ M), and fixed at the indicated times. Fixed cells were stained for MAP-2 immunoreactivity and MAP-2-immunoreactive cells counted. Each data point represents the average \pm SEM of three experiments, each performed with three cultures. Asterisk indicates statistical difference from preimmune-treated neurons ($p < 0.05$).

35% and 43% fewer neurons were found in anti-SH2-B-trituated cultures 60 and 84 hr after plating, respectively. In contrast, neither SH2-B antibodies nor preimmune IgG affected survival of sympathetic neurons grown in the presence of forskolin (Figure 6). Similar results were obtained when cells were stained with acridine orange and MAP-2 to identify viable neurons (data not shown). Together, these data indicate that SH2-B is required for NGF/TrkA-dependent, but not cAMP-dependent, survival of neonatal sympathetic neurons.

As a second approach to establish a role for TrkA–SH2-B signaling in NGF/TrkA-dependent growth of sympathetic neurons, we performed experiments in which an SH2-B mutant that blocks SH2-B signaling was introduced into neurons within explants of sympathetic ganglia. As shown in Figures 5D and 5E, the SH2-B mutant M5 is incapable of mediating TrkA signaling to MAPK, and it blocks SH2-B-dependent, but not SH2-B-independent, signaling to MAPK when expressed in 293 cells. Therefore, M5 is a dominant-interfering SH2-B mutant useful for blocking SH2-B-mediated signaling events. Expression vectors encoding either wild-type SH2-B or mutant M5 were introduced into sympathetic neurons within explants of neonatal superior cervical ganglia (SCG) together with an expression vector encoding green fluorescent protein (GFP) by particle-mediated gene transfer. Up to 200 transfected neurons within the explants were readily detected by fluorescence microscopy (Figure 7A), which represents fewer than 5% of the total neurons within the explants. Neurons transfected with an expression vector encoding wild-type SH2-B thrived and had elaborate, long-branching axonal processes that extended up to 2 mm in length when grown in the presence of either NGF or forskolin for the duration of the experiment (Figures 7B and 7C), which

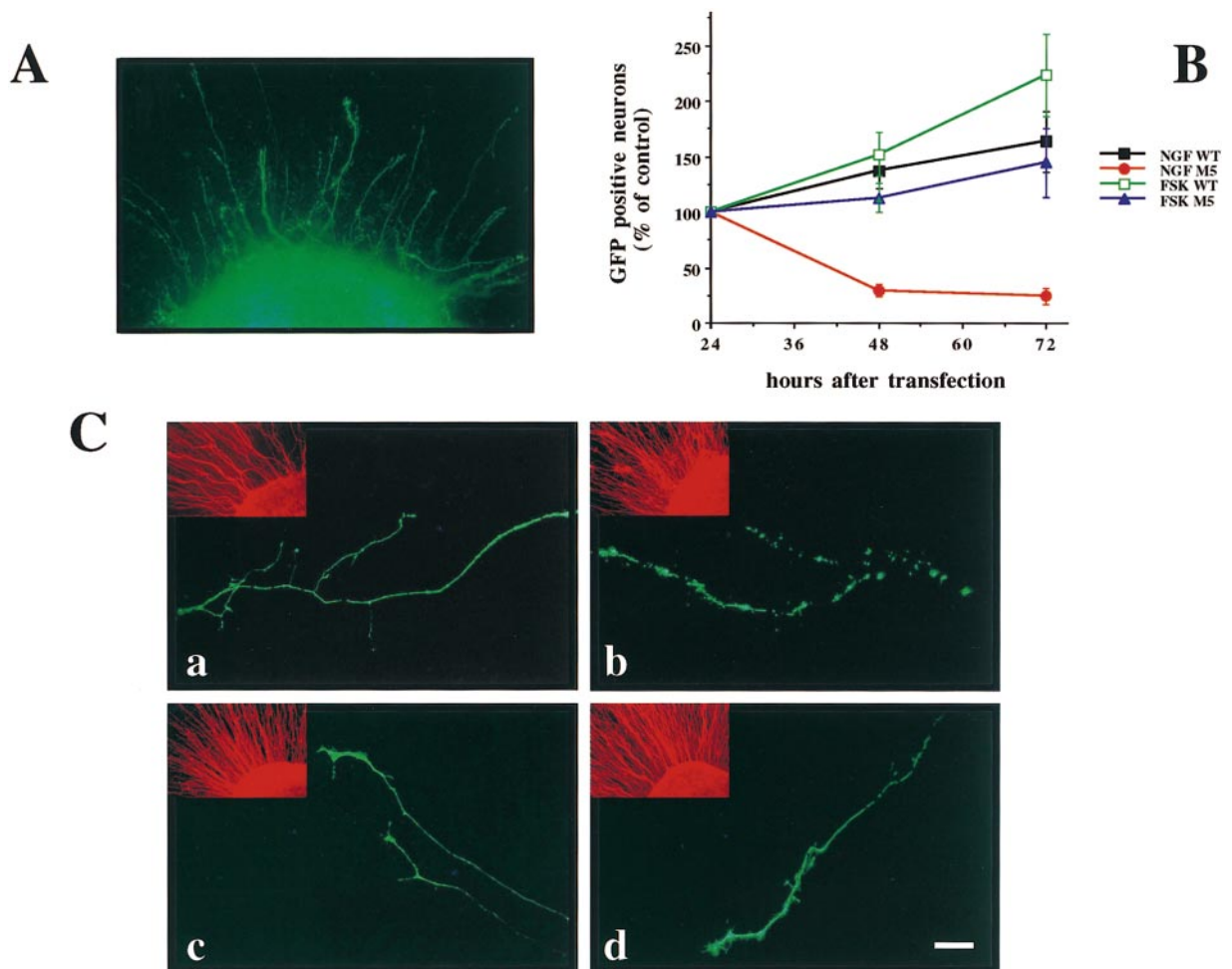


Figure 7. A Dominant-Interfering Mutant of SH2-B Promotes Degeneration of Axons of Sympathetic Neurons in Explants of SCG Grown in the Presence of NGF but Not Forskolin

SCG explants were grown for 16 hr in growth medium containing NGF (200 ng/ml). Then, expression vectors encoding GFP and either wild-type SH2-B or SH2-B mutant M5 were introduced into neurons by particle-mediated gene transfer. Subsequently, medium was replaced with growth medium containing either NGF (200 ng/ml) or forskolin (10 μ M).

(A) Photograph of an SCG explant transfected with wild-type SH2-B and grown in medium containing NGF for 2 days. The photograph was taken under fluorescence microscopy to identify GFP-positive axons.

(B) The number of fluorescent axons identified from ganglia transfected with either wild-type SH2-B (WT) or SH2-B mutant M5 and grown in the presence of either NGF or forskolin (FSK). The data represent means \pm SEM of fluorescent axons identified in 15 transfected ganglia. Statistical analysis indicated that there were fewer GFP-positive neurons in explants transfected with M5 and grown in the presence of NGF as compared to the other three groups at 48 and 72 hr ($p < 0.001$).

(C) Representative axons from neurons that were transfected with either wild-type SH2-B (a and c) or SH2-B mutant M5 (b and d) and grown in medium containing either NGF (200 ng/ml [a and b]) or forskolin (10 μ M [c and d]) for 2 days following particle-mediated gene transfer. Scale bar, 100 μ m (A); 25 μ m (C).

was for 3 days following the transfection. These axonal processes were not obviously different from processes of neurons transfected with GFP alone. In dramatic contrast, expression of M5 resulted in a near complete elimination of axonal processes of neurons grown in the presence of NGF. After 2 days, many of the axons of M5-expressing neurons had a broken, granulated appearance (Figure 7C). This degeneration-promoting effect of M5 was only seen in transfected neurons; axons of untransfected neurons were normal in appearance, as determined by phase contrast microscopy or following immunostaining with anti-neurofilament antibodies (Figure 7C, insets). Importantly, while dramatic axonal degeneration resulted following expression of M5 in sympathetic neurons maintained in the presence of NGF,

neurons expressing M5 survived and maintained long, healthy axonal processes, often with large growth cones, when grown in the presence of forskolin (Figure 7C). Therefore, M5 was not generally toxic to sympathetic neurons; it was only toxic for neurons grown in the presence of NGF. Quantitation of the total number of GFP-positive axons emanating from explants transfected with expression vectors encoding either wild-type SH2-B or mutant M5, and grown in the presence of either NGF or forskolin, is presented in Figure 7B. The total number of GFP-positive axons was greatly reduced in M5-transfected neurons grown in the presence of NGF, but not forskolin ($p < 0.001$), measured 2 or 3 days following transfection. Together with the results of antibody perturbation experiments described above,

these results indicate that SH2-B, rAPS, or a closely related molecule is a critical mediator of NGF/TrkA signals, but not cAMP signals, that support axon growth of sympathetic neurons.

Discussion

Neurotrophins influence growth, differentiation, and survival of neurons through activation of their cell surface receptors, members of the Trk family of RTKs. In this study, we describe the identification and characterization of two substrates of Trk receptors, rAPS and SH2-B. rAPS and SH2-B interact with TrkA, TrkB, and TrkC, suggesting that they are general substrates of Trk receptors. Moreover, these proteins are endogenous substrates of Trk receptors in developing cortical neurons, and, importantly, SH2-B is a substrate of TrkA in neonatal sympathetic neurons. rAPS and SH2-B are found complexed with Grb2, and these adaptor proteins are sufficient to mediate NGF/TrkA induction of Ras-dependent signaling and morphological differentiation, in part, through their association with Grb2. Lastly, SH2-B is necessary for NGF-dependent signaling in neonatal sympathetic neurons *in vitro*. Together, these observations provide evidence for critical roles of rAPS and SH2-B in Trk receptor signaling in developing neurons.

Lnks, a Family of Tyrosine Kinase Signaling Molecules

rAPS and SH2-B are highly related proteins with very similar domain structures. Both contain a PH domain, an SH2 domain, a C-terminal tyrosine-containing motif, and several proline-rich motifs. rAPS is likely to be the rat ortholog of a recently identified human protein, APS, which interacts with *c-kit* RTK (Yokouchi et al., 1997); SH2-B was first identified as a protein that binds to the high-affinity IgE receptor, FcεRI (Osborne et al., 1995). In addition, two splice variants of SH2-B, SH2-Bβ (Rui et al., 1997) and PSM (Riedel et al., 1997), were recently identified. Lastly, parts of the PH domains, the SH2 domains, and the C-terminal regions of rAPS, APS, and SH2-B/SH2-B are similar to a previously described protein, Lnk, which associates with the T cell receptor complex (Huang et al., 1992). Thus, rAPS/APS, SH2-B/SH2-Bβ/PSM, and Lnk are members of a growing family of structurally related signaling molecules. Interestingly, members of this family have the capacity to directly interact with RTKs (this study and Riedel et al., 1997; Yokouchi et al., 1997), the nonreceptor tyrosine kinase JAK2 (Rui et al., 1997), and substrates of tyrosine kinases (Osborne et al., 1995) through their highly conserved SH2 domains. These observations, together with the widespread expression patterns of members of this gene family (data not shown and Osborne et al., 1995; Yokouchi et al., 1997), suggest that its members function as general mediators of phosphotyrosine signaling cascades in neuronal and nonneuronal cells.

Function of rAPS and SH2-B during Trk Signaling

The present study provides evidence that SH2-B and rAPS are sufficient, and SH2-B is necessary for at least

some aspects of neurotrophin signal transduction. Either of these Trk substrates can mediate Trk signaling to MAPK and morphological differentiation when expressed in 293 cells or PC12 cells. Evidence in support of a role for SH2-B in TrkA signaling in sympathetic neurons comes from two different sets of experiments. First, injection of anti-SH2-B antibodies, but not preimmune antibodies, by trituration led to death of sympathetic neurons that were grown in the presence of NGF. However, cAMP-mediated survival of sympathetic neurons was unaffected by the introduction of anti-SH2-B antibodies. Therefore, anti-SH2-B antibodies were not simply toxic to the cells; rather, the anti-SH2-B antibodies killed the sympathetic neurons in a TrkA-dependent manner. Similarly, expression of the dominant interfering SH2-B mutant M5 led to axonal degeneration of sympathetic neurons grown in the presence of NGF but not in the presence of forskolin. Thus, like anti-SH2-B, M5 selectively led to degeneration of axons under conditions that require TrkA signaling. Moreover, experiments presented in Figure 5 demonstrate that while M5 effectively blocked SH2-B-mediated signaling events, it did not block SH2-B-independent TrkA signaling. Therefore, M5 appears to function as a specific and effective inhibitor of TrkA signaling. How M5 blocks SH2-B signaling is unclear. In addition to its ability to associate with Grb2, our preliminary studies have indicated that SH2-B forms homodimers as well as heterodimers with rAPS; M5 is incapable of forming homo- and heterodimers (X. Q. and D. D. G., unpublished data), and it cannot associate with Grb2 (Figure 5). Therefore, it is possible that M5 blocks SH2-B function because it interacts with the SH2-B binding site on TrkA, but it cannot form dimers or bind to downstream signaling molecules, such as Grb2, that may be necessary for its signaling function. Regardless of the mechanism of action of M5, results of these inhibition experiments indicate that SH2-B or a closely related molecule is necessary for TrkA signal transduction events that support growth of sympathetic neurons.

Mechanism of rAPS and SH2-B Action

rAPS and SH2-B have no readily identifiable catalytic activity, and it is likely that they function solely as adaptor proteins. We have shown that the signaling molecule Grb2 associates with both rAPS and SH2-B, and structure-function experiments suggest that the association between SH2-B and Grb2 is at least partly responsible for its mediation of Ras-dependent MAPK (Figure 5) and morphological differentiation of PC12 cells (data not shown). Our preliminary studies suggest that Y55, Y753, and at least one additional tyrosine residue of SH2-B are phosphorylated by TrkA (data not shown). Y55 lies within the context of a putative PTB binding site, an N/HPXY motif, and this residue is not essential for the association between SH2-B and Grb2. Rather, this tyrosine residue and the C-terminal residue Y753 may contribute to the binding sites for other unidentified signaling molecules. Indeed, when the analogous tyrosine residue in Lnk, Y297, is phosphorylated, it mediates an interaction between Lnk and PI-3 kinase in lymphocytes (Huang et al., 1992). However, we have been unsuccessful in detecting an interaction between either

rAPS or SH2-B and the regulatory subunit of PI-3 kinase in extracts of either transiently transfected 293 cells or extracts of cortical neurons. The identity of proteins that may associate with either SH2-B and/or rAPS and mediate aspects of neurotrophin signaling in developing neurons remains unknown.

Surprisingly, rAPS and SH2-B were found to associate with TrkA variants lacking all tyrosines except the three phosphotyrosine residues within the catalytic loop of the TrkA kinase domain. The TrkA mutant F8, which has all tyrosine residues changed to phenylalanine except Y679, 683, and 684 within the catalytic loop, interacts with both rAPS and SH2-B in yeast and in mammalian cells (Figure 2). However, the possibility that rAPS and SH2-B can associate with tyrosine residues outside of the catalytic loop was not ruled out in the present study because TrkA mutants lacking tyrosine residues within the catalytic loop are deficient in kinase activity and, therefore, cannot effectively autophosphorylate on sites within or outside of the kinase domain (Cunningham et al., 1997). The fact that members of the Lnk family bind to distinct phosphotyrosine-containing motifs in various molecules, including RTKs, the nonreceptor tyrosine kinase JAK2, and the IgE receptor, suggests that their highly conserved SH2 domains are promiscuous in their ability to bind to structurally distinct domains. On the other hand, the observation that SH2-B mutant M5 blocked SH2-B signaling but not signaling emanating from wild-type TrkA in 293 cells (Figure 5) suggests that the SH2-B SH2 domain does not bind to the TrkA effector binding sites that mediate SH2-B-independent signaling in those cells. If the SH2-B SH2 domain could bind to the other TrkA effector binding sites, M5 would be expected to occlude binding of other TrkA effectors, such as Shc and PLC- γ , to their respective binding sites on TrkA and block all aspects of TrkA signaling. Phosphopeptide binding and competition experiments will be needed to identify domains of Trk that mediate the highest-affinity and most physiologically relevant interactions between phosphotyrosines in Trk receptors and rAPS and SH2-B.

In summary, we have identified rAPS and SH2-B as substrates of Trk receptors in developing cortical and sympathetic neurons. Both rAPS and SH2-B associate with Grb2, and each has the capacity to mediate MAPK activation and morphological differentiation of PC12 cells via a novel mechanism that is independent of Shc and PLC- γ . Importantly, SH2-B, or a closely related molecule, is necessary for TrkA signaling in neonatal sympathetic neurons. Members of this new family of adaptor molecules are likely to function as general mediators of phosphotyrosine signaling in both neuronal and nonneuronal cells and contribute to cell growth, differentiation, and survival.

Experimental Procedures

Cell Lines and Primary Neuron Cultures

HEK 293 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and penicillin and streptomycin (Pen/Strep). PC12nr5 cells were provided by Dr. Lloyd Greene and cultured in DMEM containing 10% FBS, 5% horse serum, and Pen/Strep. Primary cortical neurons were cultured as described (Rosen et al., 1994). Sympathetic neurons were isolated from superior cervical ganglia of neonatal rats (Mains and Patterson, 1973). Dissociated neurons were

plated on collagen-coated 60 mm dishes at a density of 1×10^6 cells per plate and grown in growth medium (DMEM containing 10% fetal bovine serum, 5 μ M arabinosylcytosine [Ara-C], and NGF [200 ng/ml]). Medium was replaced every 3 days. After 8–10 days, neurons were starved in medium containing a low concentration of NGF (2 ng/ml) for 48 hr prior to stimulation with NGF (200 ng/ml, 10 min).

Yeast Two-Hybrid Screening and cDNA Cloning of rAPS and SH2-B

To identify novel substrates of Trk family RTKs, a yeast two-hybrid screen was used (Gyuris et al., 1993). Briefly, the entire intracellular domains of TrkA, TrkB, or TrkC were inserted into the yeast expression vector PEG202 as a fusion to the LexA DNA binding and dimerization domain and introduced into the yeast strain PJK103. In addition, a mutant LexA-Trk^{V527D} was generated by PCR-mediated site-directed mutagenesis. A cDNA library generated from mRNA isolated from rat E14 spinal cord and DRG (Kolodkin et al., 1997) was cloned into the yeast expression vector pJG4/5, which confers expression of the cDNA fused with a strong transcription activator (Golemis et al., 1994). A screen of 30 million transformants was performed. Interaction with LexA-TrkA and/or LexA-TrkA^{VD} was assessed by a liquid β -galactosidase assay (Sambrook et al., 1989). The full-length clones of both rAPS and SH2-B were obtained by screening a rat hippocampus cDNA phage library provided by Dr. Paul Worley.

Generation of DNA Constructs

A series of TrkA mutants were obtained from Naoyuki Inagaki (Inagaki et al., 1995), and cDNAs encoding the intracellular domains of the TrkA mutants were PCR amplified and inserted into the EcoRI and BamHI sites of the yeast expression vector PEG202. Expression of the LexA-TrkA mutants was confirmed by immunoblot analysis. For coimmunoprecipitation experiments using HEK 293 cells, full-length wild-type TrkA or the TrkA mutant F8 cloned into the mammalian expression vector p139 were used. The full coding regions of rAPS and SH2-B fused to a myc epitope-tagged sequence at the N terminus were subcloned into the mammalian expression vector pRK5. Various deletions and mutations of SH2-B were constructed in pRK5 using PCR amplification and site-directed mutagenesis.

Generation of rAPS and SH2-B Antibodies

Anti-rAPS and SH2-B sera were generated in rabbits using 6-histidine-tagged fusion proteins containing either amino acids 66–254 of rAPS or amino acids 176–341 of SH2-B as immunogens. The corresponding cDNA fragments of rAPS and SH2-B were PCR amplified and inserted into the SacI and KpnI sites of bacterial expression vector pTrcHisA (Invitrogen). Fusion proteins expressed in *E. coli* were purified using Ni-NTA resin (Qiagen), and rabbits were immunized as described previously (Ginty et al., 1993). The IgG fraction of the antisera was purified by protein A-agarose chromatography.

HEK 293 Cell Transfection, Immunoprecipitation, and Immunoblot

HEK 293 cells were cotransfected with expression vectors encoding TrkA and myc-rAPS or myc-SH2-B using lipofectamine (GibcoBRL). Two days after transfection, cells were stimulated with NGF (100 ng/ml) for 10 min and then lysed in 1 ml of lysis buffer (1% NP-40 in TBS containing 10% glycerol, 1 mM PMSF, 1 mM sodium orthovanadate, 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin [pH 7.4]). All subsequent steps were at 4°C. Lysates were clarified by centrifugation (16,000 \times g for 20 min) and supernatants used for immunoprecipitations using anti-myc monoclonal antibodies (9E10). For immunoprecipitations from primary neurons, cortical neurons or sympathetic neurons were stimulated with BDNF (50 ng/ml), NT-3 (100 ng/ml), or NGF (200 ng/ml) for 10 min. Extracts were prepared as described above. Lysate supernatants were subjected to immunoprecipitation with indicated antibodies, and the immune complexes were resolved by SDS-PAGE gel and blotted with anti-phosphotyrosine antibodies.

MAP Kinase Assays

rAPS or SH2-B were transiently expressed with TrkA or F8 and an HA-tagged ERK1 expression vector in 293 cells. Two days after the

transfection, cells were stimulated and lysed with the lysis buffer (1% NP-40, 20 mM HEPES, 10 mM EGTA, 40 mM glycerophosphate, 2.5 mM MgCl₂, 1 mM DTT, 2 mM sodium orthovanadate, 1 mM PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin [pH 7.4]). HA-ERK1 was immunoprecipitated from the lysates with anti-HA antibodies (2 μg). The catalytic activity of HA-ERK1 under various stimulation conditions was measured in an immune complex kinase assay as described (Rosen et al., 1994).

PC12nr5 Cell Transfection and NGF-Induced Neurite Outgrowth

PC12nr5 cells were grown on 35 mm plates coated with polylysine. Cells were transfected with the indicated plasmids together with a cDNA encoding GFP. NGF (100 ng/ml) was applied immediately following transfection to induce morphological differentiation of PC12nr5 cells. Three days after NGF application, cells were fixed, and GFP-positive cells were scored for the presence of neurites. Cells with processes longer than two times the diameter of the cell body were considered positive.

SCG Explants and Particle-Mediated Gene Transfer into Sympathetic Neurons

SCG were dissected from E18 rats and grown on poly lysine/laminin-coated plates in sympathetic neuron growth medium in the presence of NGF (200 ng/ml) for 16–20 hr. Gold particles (1.6 μM particles, 25 mg) were coated with DNA (75 μg of expression vector encoding GFP and either wild-type SH2-B or SH2-B mutant M5). For transfection, medium was temporarily removed from the SCG cultures, and gene transfer was achieved following evacuation of the transfection chamber with a single pulse of helium (0.5 s, 100 psi) using the Helios gene gun device (BioRad). Subsequently, fresh medium containing either NGF (200 ng/ml) or forskolin (10 mM) was added to the plates. Typically, more than 50 neurons per ganglion were transfected as determined by GFP fluorescence 2 days following transfection.

Primary Sympathetic Neuron Antibody Trituration

Primary dissociated sympathetic neurons were injected with anti-SH2-B antibodies or preimmune IgG by the trituration method, essentially as described (Borasio et al., 1989). Briefly, ganglia were harvested from E18 rat pups, incubated in dissociation buffer, washed extensively in DMEM containing 10% fetal bovine serum, and resuspended in a small volume of medium (100 μl) containing a high concentration of either anti-SH2-B or preimmune IgG (50 mg/ml) prior to dissociation. Cells were mechanically triturated by drawing the entire solution through a pasteur pipette positioned at the bottom of an Eppendorf tube 50 times. This procedure resulted in incorporation of Texas red-labeled dextran (70 kDa) in more than 80% of sympathetic neurons as assessed by fluorescence microscopy (data not shown). Trituration did not irreversibly damage the neurons since they thrived and extended long axonal processes in the presence of either NGF or forskolin.

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