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# SLAM-associated Protein as a Potential Negative Regulator in Trk Signaling*

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Neurotrophin signaling plays important roles in regulating the survival, differentiation, and maintenance of neurons in the nervous system. Binding of neurotrophins to their cognate receptors Trks induces transactivation and phosphorylation of the receptor at several tyrosine residues. These phosphorylated tyrosine residues then serve as crucial docking sites for adaptor proteins containing a Src homology 2 or phosphotyrosine binding domain, which upon association with the receptor initiates multiple signaling events to mediate the action of neurotrophins. Here we report the identification of a Src homology 2 domain-containing molecule, SLAM-associated protein (SAP), as an interacting protein of TrkB in a yeast two-hybrid screen. SAP was initially identified as an adaptor molecule in SLAM family receptor signaling for regulating interferon-γ secretion. In the current study, we found that SAP interacted with TrkA, TrkB, and TrkC receptors *in vitro* and *in vivo*. Binding of SAP required Trk receptor activation and phosphorylation at the tyrosine 674 residue, which is located in the activation loop of the kinase domain. Overexpression of SAP with Trk attenuated tyrosine phosphorylation of the receptors and reduced the binding of SH2B and Shc to TrkB. Moreover, overexpression of SAP in PC12 cells suppressed the nerve growth factor-dependent activation of extracellular signal-regulated kinases 1/2 and phospholipase Cγ, in addition to inhibiting neurite outgrowth. In summary, our findings demonstrated that SAP may serve as a negative regulator of Trk receptor activation and downstream signaling.

Neurotrophins are a family of trophic factors that have been demonstrated to serve important roles in multiple aspects of neuronal functions. Not only are neurotrophins required for neuronal survival and development; they are also indispensable for the regulation of synaptic transmission via modulation of neuronal architecture and synapse formation (1, 2). To date, five members of the neurotrophin family have been identified. Members of the family include the prototypic member nerve growth factor (NGF),[3] brain-derived neurotrophic factor, neurotrophin (NT)-3, NT-4/5, and NT-6/7 (3). Actions of neurotrophins are mediated by a family of receptor tyrosine kinase known as Trks. Members of Trks, TrkA, TrkB, and TrkC, share high sequence homology and functional similarity but are activated by distinct neurotrophins. TrkA binds to NGF, NT-3, and NT-6/7, whereas TrkB activation requires association with brain-derived neurotrophic factor, NT-3, or NT-4/5. TrkC, on the other hand, binds only to NT-3 (1, 3).

Similar to other receptor tyrosine kinases, activation of Trk receptors involves ligand-induced receptor dimerization (4). This leads to the subsequent trans-phosphorylation of five tyrosine residues (Tyr$^{484}$, Tyr$^{670}$, Tyr$^{674}$, Tyr$^{675}$, and Tyr$^{785}$ on TrkB) on the adjacent receptor (5). Among the five phosphotyrosines, Tyr$^{484}$ and Tyr$^{785}$ are located outside the kinase domain, whereas Tyr$^{670}$, Tyr$^{674}$, and Tyr$^{675}$ are situated in the activation loop of the kinase domain. These phosphorylated tyrosine residues then serve as important docking sites for a myriad of adaptor molecules. For example, Shc and FRS-2 interact with TrkB via phosphorylated Tyr$^{490}$, whereas SH2B and rAPS associate with the phosphorylated tyrosine residues in the activation loop. Tyr$^{785}$, on the other hand, serves as docking site for PLCγ. Association of these adaptor molecules with activated Trk receptors results in the initiation of signaling pathways, including the mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and PLCγ pathways, thereby mediating the actions of neurotrophins (5–12).

Among the Trk receptors, TrkB has received increasing attention as a modulator of synaptic plasticity. TrkB was recently demonstrated to exhibit a regulatory role on dendritic spine formation (13) and dendritic growth (14), in addition to regulating synaptic transmission and plasticity (15–20). To further elucidate the diverse functions of TrkB at synapses, we performed a yeast two-hybrid screen with the intracellular region of TrkB to search for potential TrkB interacting partners at the neuromuscular junction using a postnatal day 12 mouse muscle cDNA library. Interestingly, we identified SLAM-associated protein (SAP) as a novel TrkB-interacting protein. SAP is expressed abundantly in immune T cells and thymus and was originally identified as a regulatory molecule of the SLAM family receptor. SAP acts as an endogenous inhibitor of SLAM signaling by blocking the recruitment of SHP-2 to the receptor, thereby modulating interferon-γ production (21). Indeed, mice deficient in SAP exhibit aberrant development of certain immune cells such as NK cells and elevated production of interferon-γ (22). Nonetheless, whether neuronal development or survival is altered in SAP$^{-/-}$ mice remains to be examined. Importantly, mutation of the SAP gene is recently associated with X-linked lymphoproliferative syndrome (or Duncan disease) (21, 23). X-linked lymphoproliferative syndrome patients are extremely sensitive to Epstein-Barr virus infection, often resulting in fatal infectious mononucleosis and malignant lymphoma (21, 23, 24).

In the current study, we characterized the interaction between SAP and members of the Trk family. We found that SAP associates with TrkA, TrkB, and TrkC, and the interaction requires tyrosine phosphorylation of the receptors. Overexpression of SAP inhibits the tyrosine

[3] The abbreviations used are: NGF, nerve growth factor; aa, amino acid(s); KD, kinase-dead; NT, neurotrophin; PLC, phospholipase C; SAP, SLAM-associated protein; Trk, tropomyosin-related kinase; WT, wild type; ERK, extracellular signal-regulated kinase; MOPS, 4-morpholinepropanesulfonic acid; MBP, myelin basic protein; SH2, Src homology 2.

FIGURE 1. **Interaction of TrkB and SAP in yeast and 293T cells.** *A,* expression of TrkB-WT and -KD in yeast. The intracellular domain of TrkB-WT and -KD receptors were subcloned into pAS2-1 vector and transformed into yeast, Y190. The expression of TrkB-WT and -KD were examined by Western blot analysis. TrkB-WT showed tyrosine phosphorylation (*P-Tyr*) but not TrkB-KD. *B,* interaction of TrkB and SAP in yeast by filter assay. SAP only interacted with the active TrkB-WT but not the TrkB-KD mutant. The binding affinity of SAP-SH2 was weaker than the full-length SAP, and the C-terminal tail alone did not interact with the receptor. *C,* $\beta$-galactosidase activity of TrkB and SAP in yeast. TrkB-WT and SAP showed higher binding affinity than the SAP-SH2 protein, whereas no activity was found between Trk-KD and SAP. *D,* specificity of SAP antibody. SAP antibody recognized SAP proteins in transfected 293T cell lysate and thymus. Preincubation of blocking peptide with SAP antibody abolished the detection of SAP in Western blot analysis. *E,* association of SAP with tyrosine-phosphorylated TrkB in 293T cells. SAP and TrkB-WT or TrkB-KD were co-transfected into 293T cells. The cell lysates were immunoprecipitated (*IP*) with pan-Trk (C-14) antibody and immunoblotted with SAP antibody. The rabbit normal IgG was used as a negative control.
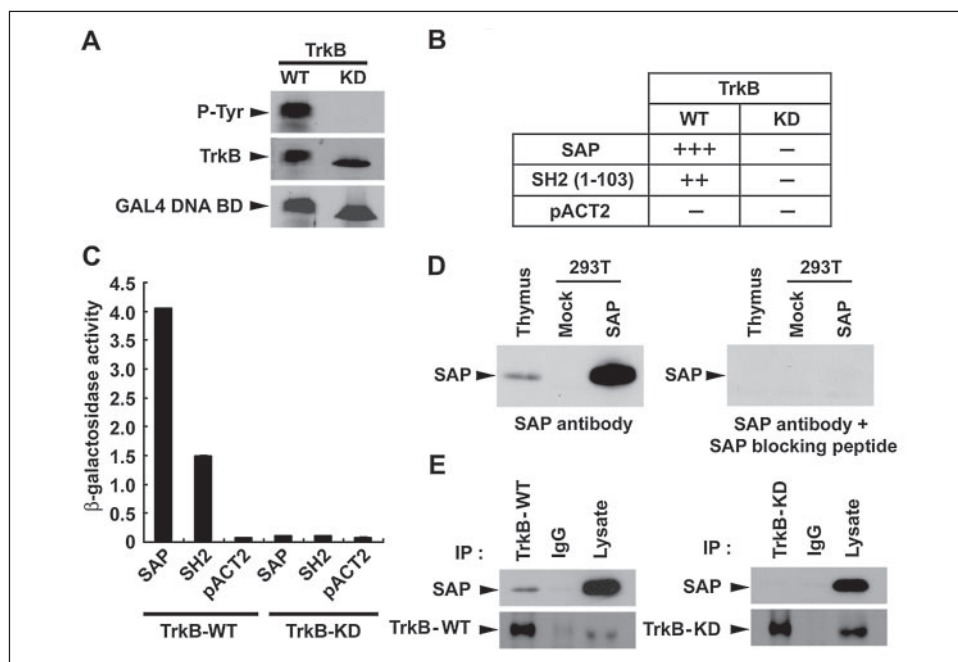


phosphorylation of all three Trk receptors and attenuates binding of Shc and SH2B to TrkB. Furthermore, examination of PC12 cells overexpressing SAP reveals that SAP suppresses the NGF-mediated activation of ERK1/2 and PLCγ and diminishes neurite extension in PC12 cells. Taken together, our studies demonstrate that SAP is a novel Trk-interacting protein, revealing its unexpected role as a negative regulator of Trk signaling and function.

## EXPERIMENTAL PROCEDURES

*Plasmids and Antibodies*—cDNA encoding the intracellular region of TrkB was subcloned into the yeast pAS2–1 vector, which contains the GAL4 binding domain (Clontech). The construct was used as bait in the yeast two-hybrid screen. Five TrkB mutants (Y484F, Y670F, Y674F, Y675F, and Y785F of TrkB) were generated by mutating the particular residue of tyrosine to phenylalanine, precluding phosphorylation at the indicated residues. The kinase-dead mutant of TrkB was obtained by mutating the lysine 540 at the ATP binding pocket to alanine using the overlapping PCR technique. The intracellular domains of the mutants were reamplified and subcloned into pAS2-1 vector. The full-length and the truncated mutant of SAP mouse cDNA (encoding amino acids 1–126, 1–103, or 104–126 of SAP) were subcloned into the yeast pACT2 vector (Clontech), which contains the GAL4 transcriptional activation domain. All yeast constructs were used for mapping the binding region of TrkB and SAP. In addition, full-length rat TrkA, TrkB, and TrkC, as well as the five full-length TrkB tyrosine mutants, were amplified and subcloned into the mammalian expression vector pCDNA3 (Invitrogen) for overexpression experiments. GST-SAP and His-tagged SAP constructs were generated by subcloning the full-length SAP sequence into pGEX-6P-1 (Amersham Biosciences) and pET14b (Novagen) vectors, respectively. Protein purification was performed according to the manufacturer's protocol.

The rabbit polyclonal antibody against Trk (C-14) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The monoclonal antibodies against GAL4 DNA binding domain (GAL4 DNA-BD) and FLAG tag were purchased from Clontech and Sigma, respectively. The antibodies against TrkB and SH2B were purchased from BD Biosciences. The polyclonal antibodies recognizing TrkA, phospho-TrkA (Tyr[490]), phospho-TrkA (Tyr[674/675]), p44/42 mitogen-activated protein

kinase, phospho-p44/42 mitogen-activated protein kinase, AKT, phospho-AKT (Ser[473]), PLCγ-1, phospho-PLCγ-1, and the monoclonal antibodies recognizing hemagglutinin tag and phosphotyrosine were purchased from Cell Signaling Inc. Rabbit SAP antibody was raised by His-tagged SAP and purified by GST-SAP using AminoLink Kit (Pierce).

*Yeast Two-hybrid Screen*—A yeast two-hybrid screen was performed according to the Matchmaker two-hybrid screen protocol (Clontech). The pAS2-1-TrkB was used as bait to screen a mouse postnatal day 12 muscle cDNA library. Yeast strain Y190 was transformed with bait and library plasmids, and the transformants were selected on SD-Trp-Leu-His agar plates. The His[+] colonies were assayed by lift filter assay. An *o*-nitrophenyl-$\beta$-D-galactopyranoside-based $\beta$-galactosidase activity assay was performed according to the manufacturer's instructions (Clontech).

*Cell Culture and Transfection*—293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum plus penicillin (50 units/ml) and streptomycin (100 $\mu$g/ml; Invitrogen) antibiotics. PC12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with heat-inactivated horse serum (6%, v/v), heat-inactivated fetal bovine serum (6%, v/v), penicillin (50 units/ml), and streptomycin (100 $\mu$g/ml). Cells were normally cultured on 100-mm diameter tissue culture dishes (Corning) at 37 °C in humidified incubators with 5% $CO_2$ for 293T cells. For PC12 cells, they were cultured on collagen-coated 100-mm diameter tissue culture dishes at 37 °C in humidified incubators with 7.5% $CO_2$. Medium was changed every 3 days.

Transient transfection was performed using Lipofectamine PLUS (Invitrogen) reagents according to the manufacturer's instructions. For stable transfection, PC12 cells were cultured in the standard culture medium for 48 h after transfection. G418 selection at 400 $\mu$g/ml was applied after 20-fold dilution of the cells. G418 selection continued for 3 weeks until single colonies were formed. The isolated clones were characterized by Western blot analysis to verify protein expression. Positive clones were maintained in the culture medium with G418 at 200 $\mu$g/ml.

For differentiation of PC12 cells, a density of $5 \times 10^3$ cells/35-mm plate was plated and incubated for 1 day before induction of differenti-

ation with 50 ng/ml NGF (Alomone Laboratories). Serum concentration of the medium was changed to 1% heat-inactivated horse serum and fetal bovine serum during differentiation.

Primary cortical neuron cultures were prepared from fetal mice at embryonic day 18. Cortices were dissected in Dulbecco's modified Eagle's medium, dissociated in the same medium, and plated on poly-D-lysine-coated culture plates. Cells were cultured in neurobasal medium containing B27 supplement, 0.5 mM glutamine, penicillin (50 units/ml), and streptomycin (100 mg/ml). Cultures were incubated at 37 °C in a humidified atmosphere with 5% $CO_2$. Cytosine arabinoside (10 mM) was added after 3 days *in vitro* to inhibit nonneuronal proliferation.

*Preparation of Cell Extracts and Western Blot Analysis*—Cells were harvested and lysed with Tris lysis buffer containing 137 mM NaCl, 1 mM EDTA, 0.1% (v/v) glycerol, 1% (v/v) Nonidet P-40, 2 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 1 mM sodium orthovanadate (NaoVa), and 10 mg/ml soybean trypsin inhibitor (Sigma) in 50 mM Tris-HCl buffer (pH 8.0). Protein lysates were analyzed by SDS-PAGE and subsequently transferred onto a nitrocellulose membrane (Micron). The membrane was further blocked with 0.1% (v/v) Tween 20 and 5% (w/v) nonfat milk in Tris-buffered saline at room temperature for 1 h. The primary antibody (1:500 or 1:1000) was incubated at 4 °C overnight followed by horseradish peroxidase-conjugated secondary antibody (New England Biolabs) (1:5000) for 1 h. Protein amount on the membrane was then detected using the ECL Western blot system (Amersham Biosciences).

*Fusion Protein and Pull-down Assay*—GST-SAP was overexpressed in BL21(DE3) bacterial strain and purified using glutathione-Sepharose 4B according to the Amersham Biosciences protocol. For the binding assay, 2 μg of GST or GST-SAP was incubated with 2 mg of adult rat brain lysate homogenized with Tris lysis buffer at 4 °C overnight. Thirty μl of glutathione-Sepharose beads were then mixed with the lysates at 4 °C for 1 h, washed three times with lysis buffer, and then resuspended in SDS loading buffer. The proteins were resolved by SDS-PAGE and analyzed by Western blot analysis.

*In Vitro Phosphorylation Assay*—TrkB kinase assay was performed at 30 °C for 30 min in kinase buffer (20 mM MOPS, pH 7.4, 15 mM $MgCl_2$, 100 μM ATP) containing 1 μCi of $[\gamma\text{-}^{32}P]$ATP. Two pmol of recombinant TrkB (Upstate Biotechnology, Inc., Lake Placid, NY) were incubated at a 1:1 molar ratio with GST, GST-SAP, His-thioredoxin, or His-SAP in the presence of myelin basic protein (MBP; 5 μg) as substrate for the kinase assay. Phosphorylated MBP proteins were separated on SDS-PAGE and visualized by autoradiography.

*Co-immunoprecipitation*—For co-immunoprecipitation, the transfected 293T cell lysates, the adult rat brain and thymus tissue lysates were prepared using Tris lysis buffer. One mg of 293T lysates or 2 mg of tissue extracts were incubated with 1 μg of primary antibody at 4 °C overnight followed by mixing 30 μl of protein G-Sepharose at 4 °C for 1 h. The samples were washed two times with Tris buffer and resuspended in SDS loading buffer. The proteins were analyzed by SDS-PAGE and Western blot analysis.

*Southern Blot Analysis*—For Southern blot analysis, cDNAs were reverse-transcribed from 5 μg of different rat tissue RNA using oligo(dT) random hexamer primers and Superscript II reverse transcriptase (Invitrogen). After RNase H treatment, the cDNAs were amplified by PCR using primers 5′-ATGGATGCAGTGACT-3′ and 5′-CAGTATCCAGTTGAA-3′, corresponding to bp 1–15 and bp 294–309 of the SAP SH2 domain. PCR products were electrophoresed on a 1% agarose gel and then transferred to a nylon plus membrane (Osmonics Inc.). A radioactive, $^{32}P$-labeled DNA probe representing
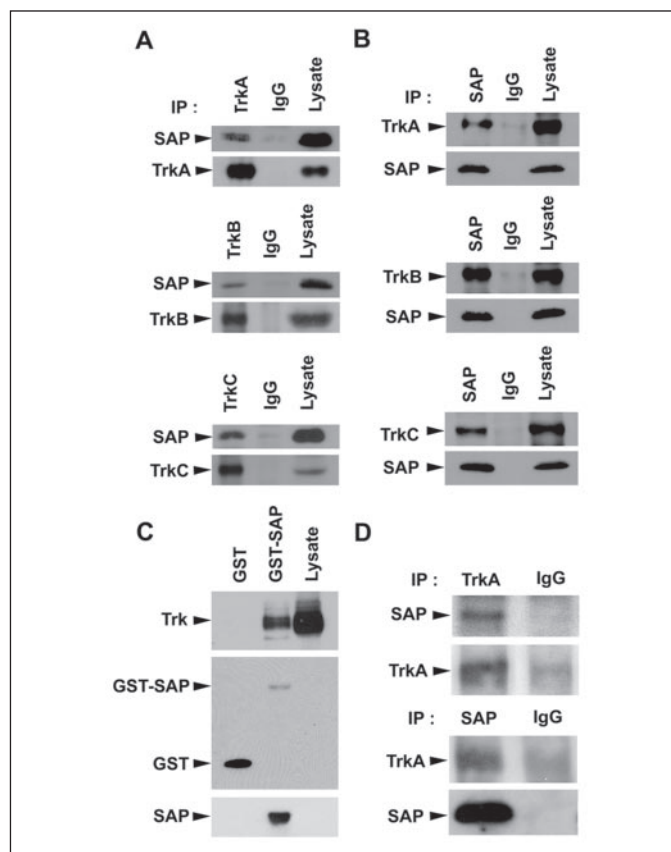


FIGURE 2. **Association of Trk receptors and SAP *in vitro* and *in vivo*.** *A*, interaction of SAP with TrkA, TrkB, and TrkC in 293T cells. SAP and TrkA, TrkB, and TrkC were co-transfected into 293T cells. The cell lysates were immunoprecipitated (*IP*) with pan-Trk antibody and immunoblotted with SAP antibody. *B*, the cell lysates were also immunoprecipitated with SAP antibody and immunoblotted with pan-Trk antibody. *C*, SAP interacted with Trk receptors *in vitro*. Purified SAP protein, but not GST, interacted with the Trk receptors in the brain lysates. *D*, SAP interacted with TrkA in thymus. SAP was immunoprecipitated with Trk antibody in thymus lysates (*top*). Reverse co-immunoprecipitation using SAP antibody also demonstrated association between SAP and Trk in thymus (*bottom*).

the SH2 domain of SAP was hybridized with the membrane, and the blot was exposed to the film at $-80$ °C for 3 h.

*Immunocytochemical Staining*—PC12 cells were fixed with 4% paraformaldehyde for 30 min and blocked with 4% fetal bovine serum in phosphate-buffered saline containing 0.4% Triton X-100 at room temperature for 20 min. Cells were subsequently washed three times with phosphate-buffered saline and incubated with rhodamine-conjugated phalloidin (1:500) for 1 h to stain F-actin and 4′,6-diamidino-2-phenylindole (1:10,000) for 15 min to stain the nucleus. The cells were then washed three times with phosphate-buffered saline and mounted with Mowiol. The prepared slides were analyzed with an Olympus confocal microscope. Primary cortical neurons (*in vitro* day 7) were blocked and permeabilized with 0.1% Triton X-100, 4% fetal bovine serum, and 1% bovine serum albumin for 20 min followed by incubation with TrkB (1:1000) and SAP (1:5000) antibodies with or without preincubation of SAP blocking peptide at 4 °C overnight. The cells were then incubated with fluorescein isothiocyanate- or Rh-conjugated goat anti-mouse or rabbit IgG at room temperature for 1 h. After washing three times with phosphate-buffered saline, the cells were mounted with Mowiol and examined under a Leica fluorescent microscope.

*Quantification of Neurite Outgrowth*—To study the differentiation morphology of PC12 cells when SAP was overexpressed, the length of the longest neurite and total neurite length of the SAP- or mock-transfected stable cell line was quantified. The length of neurites was

FIGURE 3. **Expression of SAP transcripts in brain and neuronal cells.** *A*, SAP transcripts were detected in rat brain during development. RNA from rat brain and muscle at embryonic day 16, postnatal day 1, postnatal day 15, and adult (*Ad*) stages were used for reverse transcriptase-PCR (*left*). The relative levels of SAP transcript in thymus and brain of adult rat was also investigated (*right*). SAP cDNAs were reamplified by PCR and the expression of SAP transcripts was detected by Southern blot analysis using a DNA probe against the SH2 domain of SAP. SAP was expressed during development in brain and muscle. *B*, SAP transcripts were detected in cortical neurons (*CN*; *in vitro* day 7) and PC12 cells. Samples without the addition of reverse-transcriptase (*No RT*) served as controls to preclude amplification of SAP from genomic DNA contamination. Thymus served as a positive control for the experiments. *C*, co-localization of TrkB and SAP in culture cortical neuron (*in vitro* day 7). Both TrkB and SAP immunoreactivities were localized to the cell soma and neurites (*arrowheads*). Preincubation of SAP antibody with specific blocking peptide abolished the SAP staining, suggesting that SAP antibody is specific for immunostaining. *Scale bar*, 20 μm. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *DAPI*, 4′,6-diamidino-2-phenylindole.
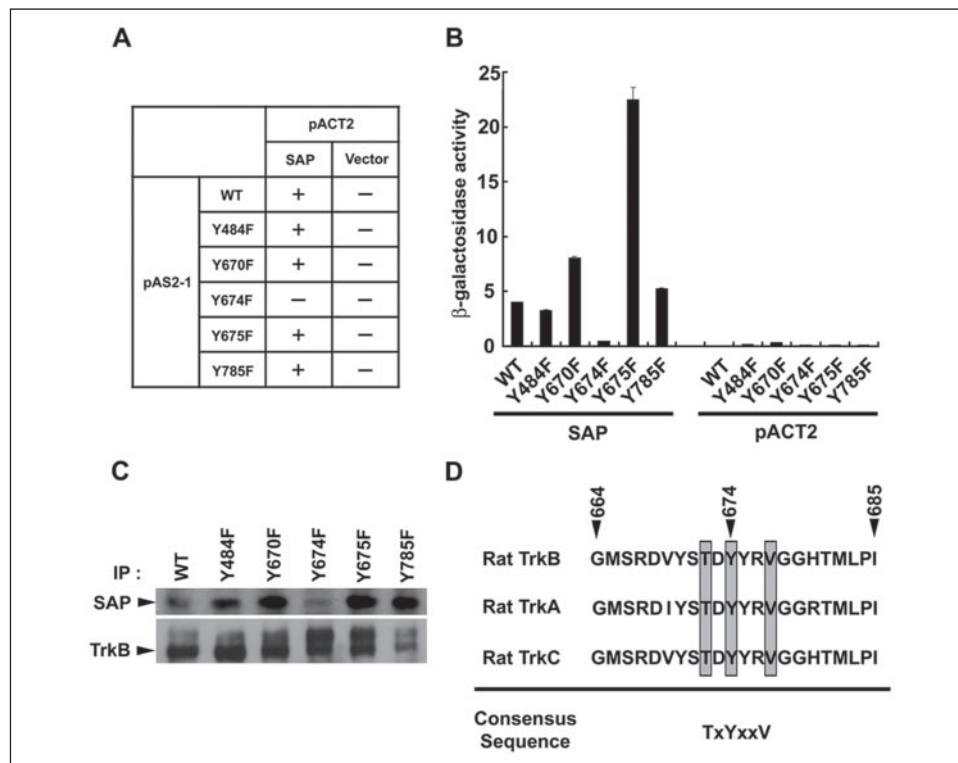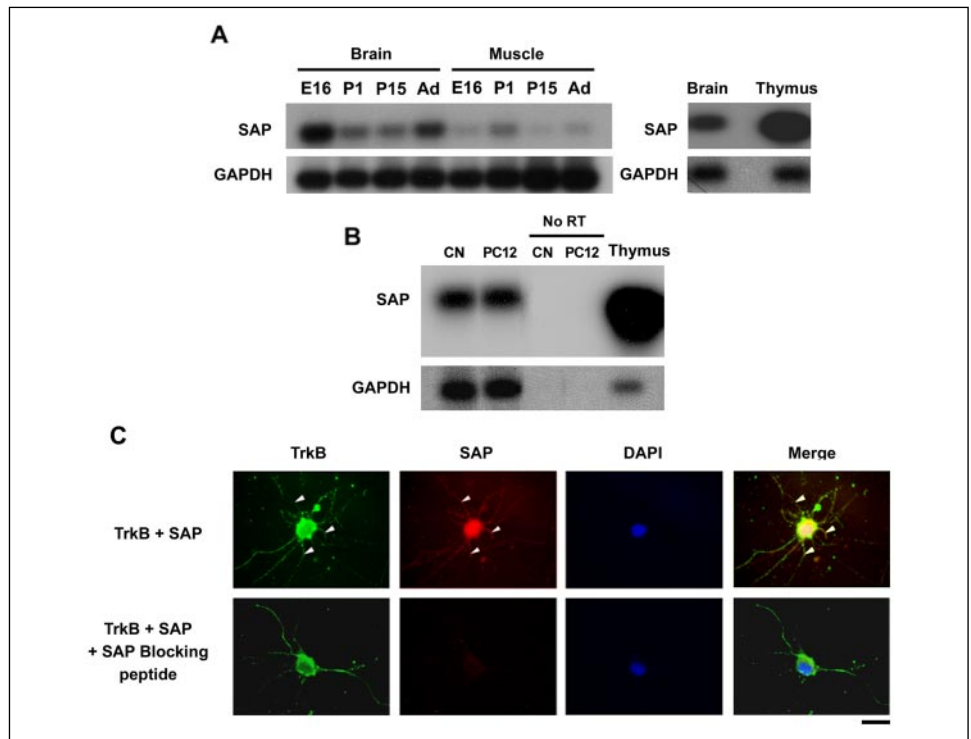
FIGURE 4. **Tyrosine 674 of TrkB receptor is required for the interaction.** *A*, association of SAP with TrkB mutants in yeast by filter assay. SAP interacted with all TrkB mutants except Y674F. *B*, β-galactosidase activity of SAP with TrkB mutants in yeast. All TrkB mutants showed galactosidase activity with SAP except Y674F. *C*, interactions of SAP with TrkB mutants in 293T cells. SAP and different TrkB mutants were co-transfected into 293T cells. The cell lysates were immunoprecipitated (*IP*) with pan-Trk antibody and immunoblotted with SAP antibody. Mutation of tyrosine 674 disrupted the binding of SAP to TrkB. *D*, alignment of the activation loop sequence of TrkA, TrkB, and TrkC receptors. The *schematic diagram* shows that TrkA, TrkB, and TrkC contain a potential SAP binding sequence (T*XYXXV*).

recorded using MetaMorph version 5.0r1 software, and more than 200 individual cells were counted from randomly selected fields in three separate trials ($n = 3$).

## RESULTS

*SAP Identified as a TrkB-interacting Protein in a Yeast Two-hybrid Screen*—To study the postsynaptic function of TrkB, we have attempted to screen for TrkB-interacting protein using a postnatal day 12 mouse muscle cDNA library in a yeast two-hybrid screen. The intracellular region of TrkB was used as bait. To verify whether TrkB expressed in yeast resulted in autophosphorylation of the receptor, wild type (WT) and the kinase-dead (KD) TrkB transformed yeast cells were collected and subjected to Western blot analysis. Our results showed that TrkB was tyrosine-phosphorylated only in the WT transformants and not in the KD transformants (Fig. 1*A*). This finding indicated that the bait used was expressed in an active conformation in yeast.

Results from the yeast two-hybrid screen identified SAP as a TrkB-interacting protein in yeast. SAP encodes a 126-amino acid protein, which is comprised of a single SH2 domain (aa 1–103) and a short C-terminal tail (aa 104–126). The library clone of SAP encoded the full-length SAP protein (126 aa) with a short extra N-terminal sequence before the start codon. Deletion of the 5' extra sequence did not interfere with TrkB and SAP association (data not shown), suggesting that full-length SAP can interact with TrkB in yeast.

To map the TrkB binding region on SAP, truncated forms of SAP containing only the SH2 domain (aa 1–103) or the C-terminal tail (aa 104–126) were constructed. In the lift filter and the β-galactosidase activity assays, both the full-length SAP and the SH2 domain, but not the C-terminal tail, interacted with TrkB. In addition, SAP failed to associate with the KD mutant of TrkB in yeast (Fig. 1*C*). These observations suggest that the SH2 domain of SAP and tyrosine phosphorylation of TrkB were both required for the association between TrkB and SAP in yeast.

We next proceeded to verify if the association between SAP and TrkB was also observed in mammalian cells. First of all, the purity of our in house SAP antibody was examined. We found that incubation of SAP antibody with lysates from thymus and SAP-overexpressing 293T cells resulted in a single band of ~15 kDa in size, corresponding to the size of SAP. This band was not detected in mock-transfected PC12 cells (Fig. 1*D*). In addition, preincubation of blocking peptide with SAP antibody abolished the detection of SAP in Western blot analysis (Fig. 1*D*). These observations collectively suggest that our SAP antibody specifically detected SAP. To examine whether SAP and TrkB association occurs in mammalian cells, TrkB-WT or -KD was co-transfected with SAP into 293T cells, and the lysates were used for co-immunoprecipitation. Western blot analysis showed that only TrkB-WT, and not TrkB-KD, co-immunoprecipitated with SAP (Fig. 1*E*). Our observations therefore showed that in agreement with the yeast binding results, SAP interacted preferentially with phosphorylated TrkB.

*SAP Interacted with TrkA, TrkB, and TrkC in 293T Cells*—To examine whether SAP interacted with other Trk family receptors, SAP was co-transfected with TrkA, TrkB, or TrkC in 293T cells, and the lysates were analyzed by co-immunoprecipitation assays. Interestingly, SAP was found to co-immunoprecipitate with TrkA, TrkB, or TrkC when immunoprecipitation was performed with either pan-Trk antibody (Fig. 2*A*) or anti-SAP antibody (Fig. 2*B*). The interaction between SAP and Trk receptors was further verified by GST pull-down assays. GST or GST-SAP proteins were incubated with adult rat brain lysates. Only GST-SAP, but not GST, pulled down the endogenous Trk receptors from the lysates (Fig. 2*C*). Taken together, our findings demonstrated that SAP interacted with all three members of the Trk family.

To examine whether the association between SAP and Trk receptors occur *in vivo*, co-immunoprecipitation assays were performed using thymus lysate due to its abundant expression of both SAP and TrkA. Immunoprecipitation with either pan-Trk antibody (Fig. 2*D*, *top*) or anti-SAP antibody (Fig. 2*D*, *bottom*) both revealed that SAP co-immunoprecipitated with TrkA in the thymus. These analyses therefore demonstrated that SAP interacted with Trk receptors both *in vitro* and *in vivo*.

*SAP Transcripts Were Expressed in Brain and Muscle during Development*—Since Trk receptors were observed to associate with SAP, we were interested to examine whether SAP was expressed in the tissues where Trk receptors are abundantly expressed. Among the Trk receptors, TrkB is highly expressed in the brain and also has been recently demonstrated to be expressed in muscles (25, 26). Using semiquantitative reverse transcription-PCR, we found that SAP was also
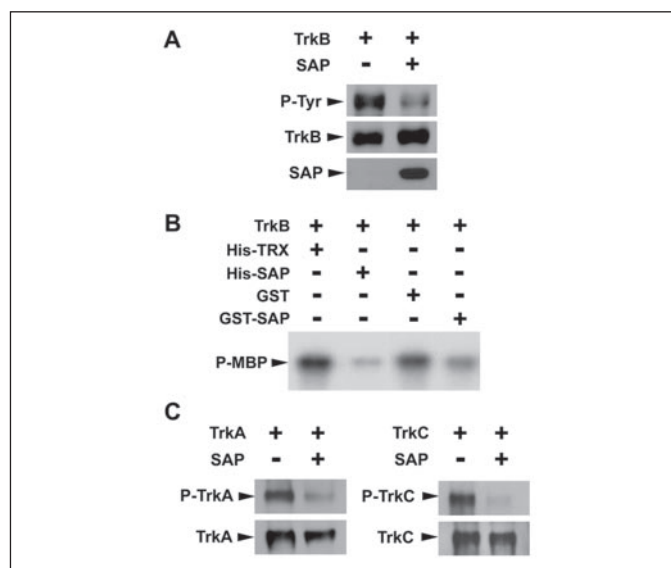


FIGURE 5. **Inhibition of tyrosine phosphorylation of Trk receptors by SAP.** *A*, inhibition of tyrosine phosphorylation of TrkB receptor by SAP. TrkB and SAP were co-transfected into 293T cells. The cell lysates were immunoprecipitated with pan-Trk antibody and immunoblotted with phosphotyrosine antibody. In the presence of SAP, phosphorylation of tyrosine residues on TrkB was inhibited. *B*, attenuation of TrkB kinase activity by SAP in *in vitro* kinase assay. Purified TrkB was incubated with MBP as substrate in the presence of His-thioredoxin (*His-TRX*), His-SAP, GST, or GST-SAP in an *in vitro* kinase assay. The addition of both GST-SAP and His-SAP markedly attenuated TrkB-mediated phosphorylation of MBP. *C*, total tyrosine phosphorylation of TrkA and TrkC was inhibited when SAP was co-expressed in 293T cells. SAP was co-transfected with TrkA and TrkC into 293T cells. The cell lysates were immunoprecipitated with pan-Trk antibody and tyrosine phosphorylation of the receptor was recognized with phosphotyrosine antibody. SAP suppressed the tyrosine phosphorylation of TrkA and TrkC.

detected in the brain and muscle, although levels of expression were much lower in the brain compared with that in thymus (Fig. 3*A*). Moreover, SAP transcripts were detected throughout development in the brain, whereas expression of SAP was strongest in the muscle at postnatal day 1 (Fig. 3*A*). The detection of SAP expression in the brain prompted us to further explore whether SAP was detected in neurons. Using cortical neuronal cultures, we verified that SAP expression in the brain was at least partially attributable to SAP expression in neurons. In addition, we found that SAP was expressed in PC12 cells, although SAP transcript level was also much lower in PC12 cells and cortical neurons compared with that in thymus (Fig. 3*B*). To further explore whether SAP expression co-localized with TrkB, cortical neurons were double-stained against SAP and TrkB. We found that both TrkB and SAP immunoreactivities were observed in the cell body and neurites of the neurons, indicating that TrkB and SAP may co-localize in neurons (Fig. 3*C*). Taken together, these observations indicate that in addition to the immune system, SAP was also expressed at a lower level in muscle and neurons.

*SAP Interacted with Tyrosine 674 of the TrkB Receptor*—Having verified that TrkB associated with SAP, we next proceeded to characterize the site of interaction on TrkB for SAP binding. Since tyrosine phosphorylation of TrkB was required for its association with SAP (Fig. 1), we speculated that the phosphorylated tyrosine residues may serve as docking sites for SAP. To examine this possibility, and to delineate the tyrosine residue for the interaction, five TrkB phosphorylation mutants (Y484F, Y670F, Y674F, Y675F, and Y785F) corresponding to the five major tyrosine phosphorylation sites were constructed by mutating the tyrosine residue to phenylalanine. In yeast, TrkB-WT and all TrkB mutants (Y484F, Y670F, Y675F, and Y785F) interacted with SAP, except for the Y674F mutant of TrkB (Fig. 4, *A* and *B*). Similarly, association between SAP and TrkB was maintained for all TrkB mutants in 293T

FIGURE 6. **Inhibition of tyrosine phosphorylation of TrkB by SAP and regulation of SH2B and Shc binding.** *A*, schematic diagram showing the locations of the phosphotyrosine residues of the TrkB receptor and the adaptor proteins recruited to the specific tyrosine residues. *B*, overexpression of SAP inhibited phosphorylation of TrkB at Tyr[484] and Tyr[674/675]. SAP was overexpressed together with TrkB in 293T cells. The cell lysates were immunoprecipitated (*IP*) with pan-Trk antibody and immunoblotted with phospho-TrkB-specific antibodies. SAP overexpression suppressed tyrosine phosphorylation at Tyr[484] and Tyr[674/675] of TrkB. *C*, SAP attenuated binding of SH2B to the activation loop of TrkB and inhibited the activation of ERK1/2. SAP and SH2B were co-transfected with TrkB receptor into 293T cells. TrkB was immunoprecipitated with pan-Trk antibody in the cell lysates. SAP inhibited the tyrosine phosphorylation of TrkB and activation of ERK1/2. Overexpression of SH2B enhanced the tyrosine phosphorylation of TrkB and activation of ERK1/2. In the presence of SAP, SH2B-triggered TrkB and ERK1/2 phosphorylation was suppressed, and the binding affinity of SH2B to TrkB was reduced. *D*, SAP inhibited TrkB phosphorylation and Shc binding to TrkB receptor. SAP and Shc were co-transfected into 293T cells. The cell lysates were immunoprecipitated with pan-Trk antibody. SAP inhibited the tyrosine phosphorylation of TrkB and suppressed the binding of Shc to the TrkB receptor.
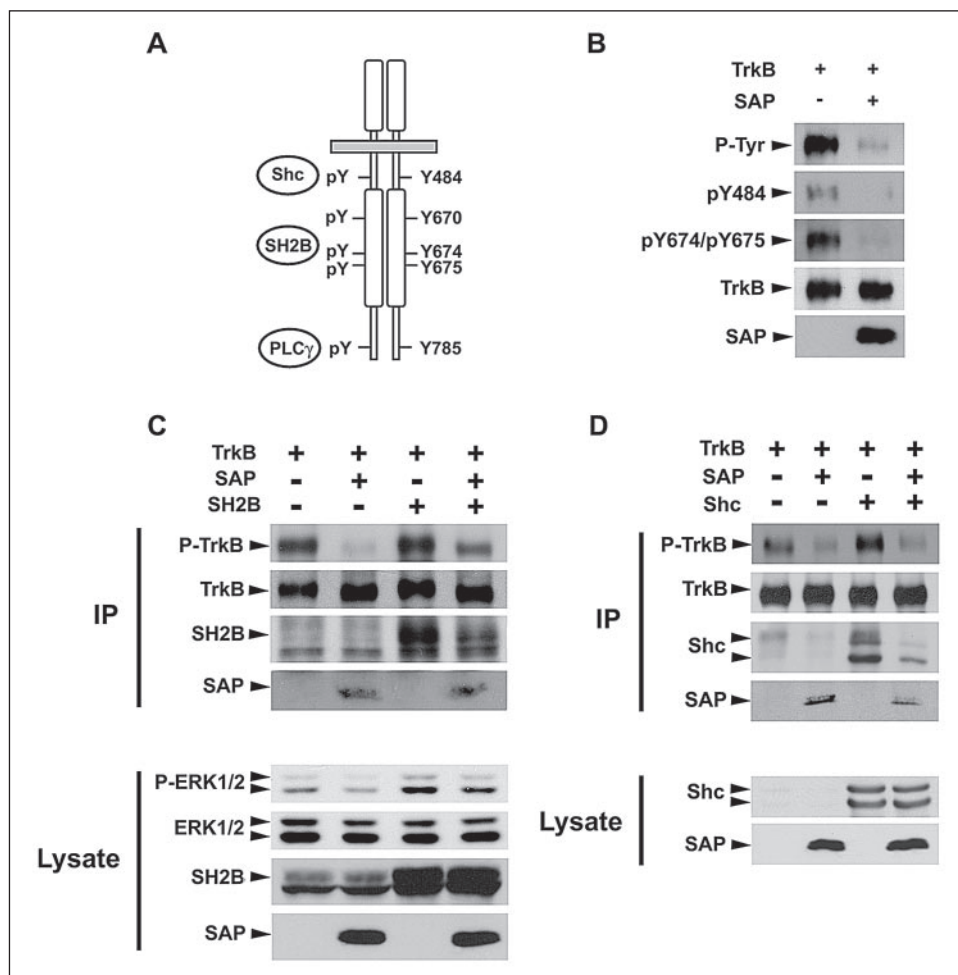
cells except when Tyr[674] was mutated to phenylalanine (Fig. 4*C*). The requirement of Tyr[674] of TrkB for its association with SAP prompted us to examine the amino acid sequence of the activation loop. A search for the consensus binding motif for SAP (TIpY*XX*(V/I), where pY represents phosphotyrosine) (27) revealed that a potential SAP binding motif (T*X*pY*XX*V) was found in TrkB (Fig. 4*D*). Alignment of the activation loop for TrkA, TrkB, and TrkC revealed that TrkA and TrkC similarly contained the consensus binding motif of SAP (Fig. 4*D*). This observation corroborates with our demonstration that SAP interacted with TrkB via Tyr[674] of TrkB and may account for the observed interaction between SAP and all TrkA, TrkB, and TrkC receptors in the binding assays (Fig. 2, *C* and *D*).

*SAP Inhibited Tyrosine Phosphorylation of Trk Receptors*—Recruitment of adaptor molecules such as SH2B and rAPS to the activation loop of Trk receptors has been shown to modulate the phosphorylation of the receptors in addition to regulating the activation of downstream signal transduction (5, 10–12). Since SAP associated with TrkB via Tyr[674] in the activation loop, binding of SAP to TrkB may also affect its phosphorylation and activation. To examine whether SAP can regulate the phosphorylation of TrkB, SAP and TrkB were overexpressed in 293T cells, and the tyrosine phosphorylation of TrkB was examined. Interestingly, when SAP was co-transfected with TrkB, tyrosine phosphorylation of the receptor was markedly reduced (Fig. 5*A*). This observation indicates that binding of SAP to TrkB attenuated phosphorylation of TrkB. To further explore whether SAP modulates the kinase activity of TrkB, the effect of SAP on TrkB-mediated phosphorylation of MBP was examined. MBP is typically used as a substrate for serine/

threonine kinase and Src kinase and, recently, receptor tyrosine kinases such as ErbB (28). Consistent with the ability of SAP to attenuate TrkB autophosphorylation, incubation with SAP, but not GST and negative control thioredoxin, markedly reduced TrkB-mediated tyrosine phosphorylation of MBP in an *in vitro* kinase assay (Fig. 5*B*). Our observations therefore suggest that SAP attenuated both the autophosphorylation and kinase activity of TrkB.

Since SAP associated with all three members of the Trk family, we wanted to examine whether SAP can similarly attenuate the activation of TrkA and TrkC. Importantly, we found that overexpression of SAP also markedly reduced the phosphorylation of TrkA and TrkC in 293T cells (Fig. 5*C*). These findings suggest that binding of SAP to the activation loop of Trk receptors may affect the autophosphorylation of TrkA, TrkB, and TrkC.

*SAP Inhibited Binding of SH2B and Shc to TrkB*—Among the adaptor molecules recruited to activated TrkB, Shc associates with TrkB by direct binding to Tyr[484], whereas SH2B interacts with TrkB via the tyrosine residues in the activation loop (Fig. 6*A*). Association of these adaptor molecules with TrkB requires tyrosine phosphorylation of TrkB (11, 29, 30). Since SAP overexpression inhibited total tyrosine phosphorylation of TrkB, we were interested to know if SAP binding of TrkB can inhibit the association of other adaptor molecules with TrkB. First of all, the effect of SAP on the phosphorylation of specific tyrosine residues on TrkB was characterized. Using phosphospecific antibodies, we found that SAP markedly inhibited phosphorylation at tyrosine 484 and tyrosine 674/675 of TrkB, which are crucial for the respective association of Shc and SH2B with TrkB (Fig. 6*B*).

In accordance with the inhibition of tyrosine phosphorylation at Tyr$^{674/675}$, we found that overexpression of SAP markedly attenuated the binding affinity of SH2B to TrkB in 293T cells, but the association of SAP with TrkB was apparently unaffected by SH2B overexpression (Fig. 6C). Reduction in SH2B binding to TrkB was accompanied by reduced levels of phospho-TrkB, and slight decrease in SH2B-induced ERK1/2 activation. It is noted that SAP also slightly inhibited ERK1/2 activation in the absence of SH2B. Our observations suggest that SAP attenuated binding of SH2B to TrkB, and resulted in a decrease in SH2B-induced mitogen-activated protein kinase activation.

We next examined whether SAP also regulated association of Shc with TrkB. Similar to what was observed with SH2B, SAP overexpression reduced the binding of Shc to TrkB (Fig. 6D). In agreement with Fig. 5, tyrosine phosphorylation of TrkB was concomitantly suppressed when SAP was overexpressed. Taken together, our observations demonstrated that SAP may negatively regulate TrkB activation by attenuating association of other adaptor molecules with TrkB.

*Activation of TrkA, ERK1/2, and PLCγ Was Attenuated in PC12 Cells Overexpressing SAP*—Since SAP suppressed the autophosphorylation of Trk receptors in 293T cells, we proceeded to examine whether SAP could negatively regulate the NGF-induced activation of various signaling pathways in PC12 cells. We stably transfected SAP into PC12 cells. As shown in Fig. 7A, SAP was expressed in the SAP-overexpressing line (PC12-SAP) but not the mock-transfected line (PC12-Mock). In accordance with the effect of SAP on TrkA phosphorylation following transient transfection, NGF-induced activation of TrkA phosphorylation was reduced in SAP-overexpressing PC12 cells (Fig. 7B). In addition, NGF-induced activation of ERK1/2 and PLCγ was also attenuated in PC12-SAP cells, although the time frame within which SAP attenuated the signaling pathways varied. Reduction in ERK1/2 phosphorylation was not evident until at least 15 min of NGF treatment, whereas PLCγ phosphorylation was slightly suppressed throughout NGF treatment in PC12-SAP cells. NGF-induced Akt phosphorylation, on the other hand, was only weakly affected by SAP overexpression (Fig. 7B). Finally, we investigated whether SAP could also affect NGF-triggered transcription of immediately early gene *egr-1*. As shown in Fig. 7B, induction of Egr-1 expression was attenuated in PC12-SAP cells. Taken together, findings reported here demonstrated that in addition to inhibiting TrkA phosphorylation and activation, SAP also attenuated the initiation of various signaling pathways downstream of TrkA activation.

*NGF-induced Neurite Outgrowth Was Inhibited in PC12-SAP Cells*—To examine whether SAP-mediated attenuation of NGF-induced signaling resulted in morphological changes in PC12 cells, the effect of SAP on NGF-dependent neurite outgrowth in PC12 cells was also investigated. Neurite length was characterized in PC12-Mock and PC12-SAP cells following 0, 1, and 4 days of NGF treatment. Prior to NGF treatment (day 0), the neurite morphology of SAP and mock-transfected PC12 cells was similar. After NGF treatment for a day, the neurite length of SAP-expressing cells was already shorter compared with that of mock-transfected cells. By day 4, SAP-expressing cells were already exhibiting appreciably shorter neurites compared with that of mock-transfected cells (Fig. 8A). Total neurite length and the longest neurite length for both PC12-Mock and PC12-SAP were quantified (Fig. 8, B and C). The results indicated that NGF-induced neurite outgrowth of PC12 cells was attenuated in the presence of SAP, and significantly reduced after 4 days of NGF treatment. Similar results were obtained in another stable clone as well as following transient transfection of SAP in PC12 cells (data not shown).
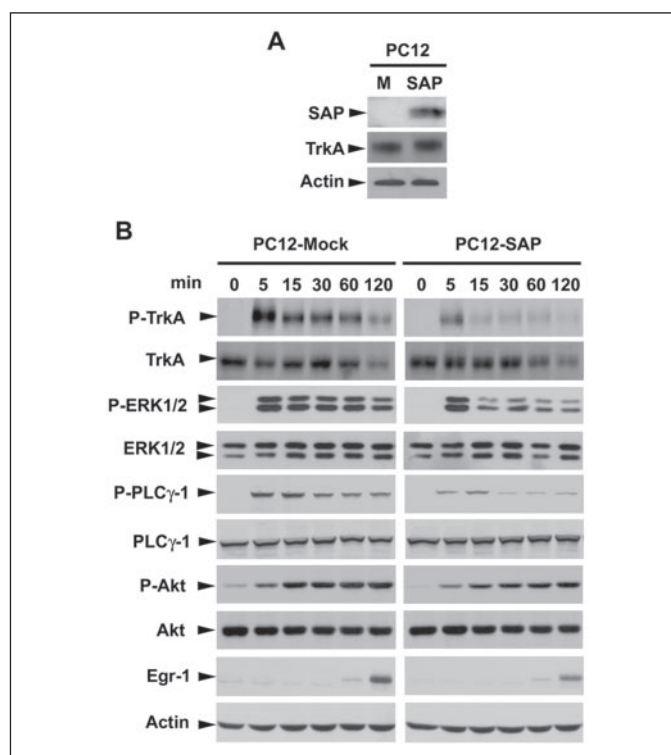


FIGURE 7. **Inhibition of NGF-induced signaling in PC12 cells overexpressing SAP.** *A*, expressions of SAP and TrkA in mock-transfected or SAP-expressing stable cell lines were analyzed. Actin served as an equal loading control. *B*, SAP suppressed the phosphorylation of TrkA, ERK1/2, and PLCγ-1 following NGF treatment. PC12-Mock and PC12-SAP stable cells were treated with 50 ng/ml NGF for 5, 15, 30, 60, and 120 min. Cell lysates were collected and subjected to Western blot analysis. In PC12 cells overexpressing SAP, phosphorylation of TrkA, ERK1/2, and PLCγ-1 was down-regulated compared with that in PC12-Mock control. The expression of transcription factor, Egr-1, was also suppressed by SAP in PC12 cells.

## DISCUSSION

Recent characterization of the adaptor molecules that are recruited to Trk receptors upon receptor activation revealed the essential regulatory role of these adaptor molecules on Trk receptor activation, and on the initiation of downstream signaling cascades (1, 8, 9). Here we report the identification of a novel TrkB-interacting protein, SAP, which acted as a negative regulator of Trk signaling upon association with the receptor. We found that SAP interacted with all three Trk receptors, TrkA, TrkB, and TrkC. Association of TrkB with SAP occurred via Tyr$^{674}$ on TrkB and required tyrosine phosphorylation of the receptor. In addition, SAP binding with TrkB not only resulted in marked reduction of TrkB autophosphorylation and kinase activity, but also attenuated association of SH2B and Shc with TrkB, thereby limiting the downstream initiation of mitogen-activated protein kinase kinase. Finally, we demonstrated that inhibition of signaling downstream of TrkA by SAP resulted in the reduction of neurite outgrowth in PC12 cells following NGF treatment.

Initiation of signaling cascades downstream of Trk activation requires binding of SH2- or phosphotyrosine binding-containing molecules. Adaptor molecules such as Shc, PLCγ, or SH2B directly interact with specific phosphorylated tyrosine residues on the receptor via their SH2 or phosphotyrosine binding domain (6, 11, 32). Our mapping experiments and the co-immunoprecipitation assays demonstrated a similar phenomenon where the SH2 domain of SAP was required for the interaction, and the binding required tyrosine phosphorylation of TrkB. Contrary to most adaptor molecules, which usually augments or instigates multiple signaling pathways upon association with Trk receptors, our data showed that SAP inhibited Trk-induced signaling pathways.
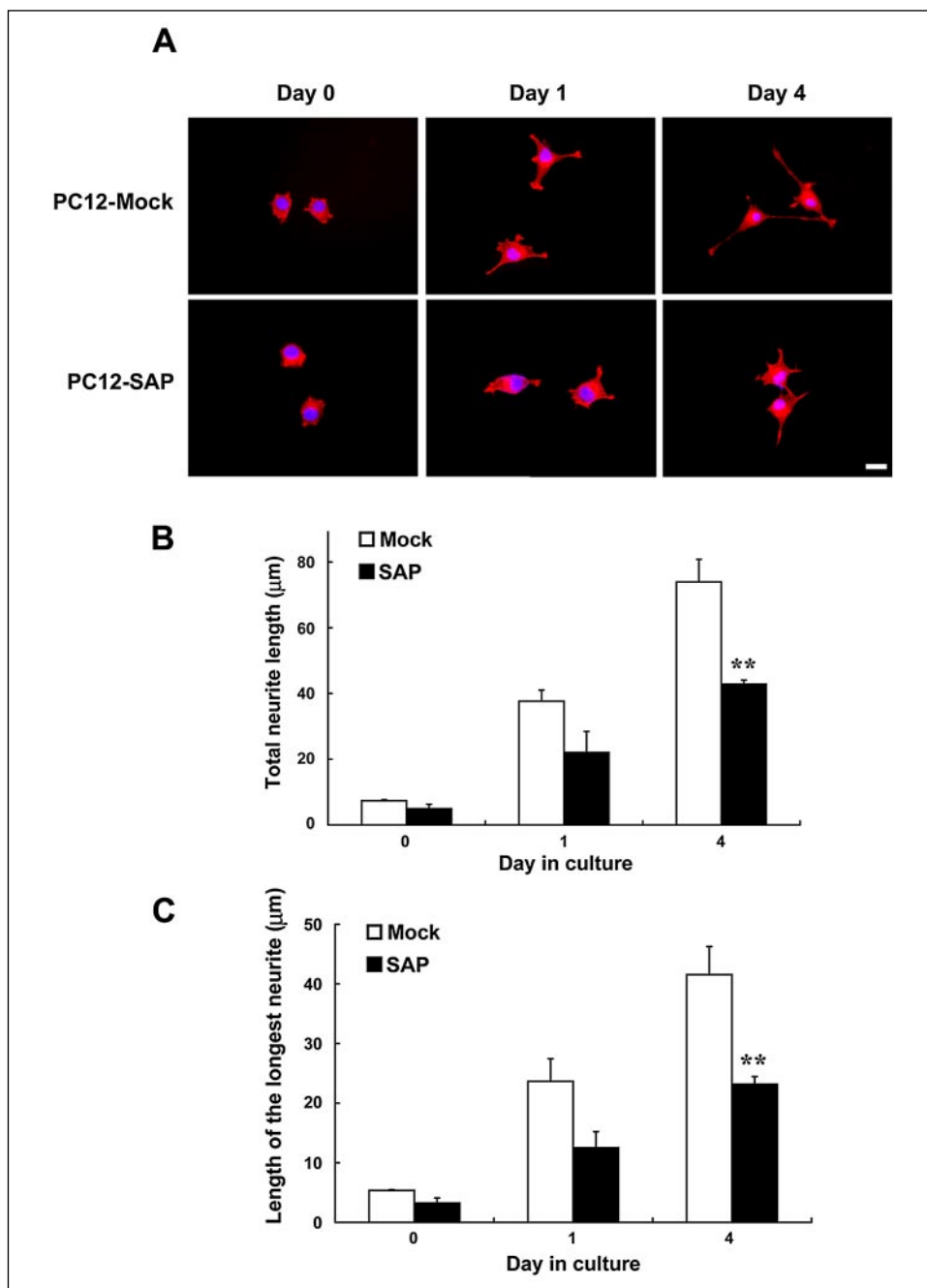
FIGURE 8. **NGF-induced neurite outgrowth was attenuated in SAP-expressing PC12 cells.** *A*, stable mock-transfected or SAP-expressing PC12 cells were treated with 50 ng/ml NGF. Cells were fixed after 0, 1, and 4 days of NGF treatment, actin was stained with phalloidin (*red*), and nucleus was stained with 4′,6-diamidino-2-phenylindole (*blue*). SAP overexpression resulted in reduced neurite length following NGF treatment. Shown is quantitation of the total neurite length (*B*) and the longest neurite length (*C*) of PC12-Mock or PC12-SAP cells at days 0, 1, and 4. *Scale bar*, 20 μm.

Identification of SAP as a negative regulator of Trk signaling places SAP in the list of molecules that have been shown to attenuate Trk signaling. Phosphatase SHP-1, for example, was demonstrated to associate with phosphorylated Tyr[490] of TrkA to directly dephosphorylate TrkA, limiting its activation (33, 34). On the other hand, overexpression of another phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) also reduces NGF-induced TrkA activation, markedly decreasing neurite outgrowth in PC12 cells (35).

How does association of SAP with TrkB result in inhibition of Trk activation and signaling? Findings reported here provided a few clues. First of all, whereas association between SAP and TrkB required only Tyr[674], interaction between SAP and TrkB almost abrogated phosphorylation at multiple tyrosine residues, including Tyr[484] and Tyr[674/675]. How association of SAP inhibited phosphorylation at these residues remained unclear, but recent evidence revealed that phosphorylation of tyrosine residues in the activation loop of the human insulin receptors stabilizes the conformation of the activation loop, thereby facilitating entry of ATP and protein substrate to the catalytic site of the kinase domain (31). Binding of SAP inhibited phosphorylation of the tyrosine residues in the activation loop and may hence destabilize the conformation of the activation loop to reduce activation of the receptor. On the other hand, our data demonstrated that SAP inhibited Trk-induced signaling by reducing association of other adaptor molecules with TrkB, such as SH2B and Shc. Indeed, SAP was initially identified as a negative regulator of interferon-γ production in cytotoxic T cells by inhibiting recruitment of SHP-2 to SLAM receptors (21). Our data demonstrated a striking similarity between the inhibition of SLAM receptor signaling and Trk receptor signaling by SAP, suggesting that SAP may emerge as a common negative regulator of signal transduction downstream of receptor activation.

## SAP in Trk Signaling

Despite its well characterized functions in the immune cells, SAP is ubiquitously expressed in various tissues, although the most abundant expression is detected in thymus and spleen (23). Here we showed that SAP transcripts were detected in the brain and muscle during development, coinciding temporally with the expression of Trk receptors in these tissues. In addition, detection of SAP expression in cortical neurons and PC12 cells suggests that SAP may serve as a negative regulator of Trk signaling in neurons. Indeed, overexpression of SAP in PC12 cells attenuated NGF-induced neurite outgrowth, suggesting that SAP interaction with TrkA may take part in modulating neuronal function. Although the precise function of SAP interaction with TrkB in muscle has not been examined, the concomitant expression of SAP and TrkB in muscle during the postnatal stages suggests that SAP may play a physiological role in TrkB signaling in muscle. Further experiments will be required to examine this possibility.

Taken together, our present study demonstrates that SAP serves as novel regulator of Trk receptor, and negatively modulates the tyrosine phosphorylation of the receptor as well as the activation of downstream signaling pathways. Whereas dysfunction of SAP in the immune system may result in X-linked lymphoproliferative syndrome, our study provides new insights on SAP function and the possible interaction of SAP and TrkB in the nervous system.

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