Constitutively active Src facilitates NGF-induced phosphorylation of TrkA and causes enhancement of the MAPK signaling in SK-N-MC cells

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Abstract Here we investigated a biological association of constitutively active Src with TrkA in SK-N-MC human neuroblastoma cells. Activation of TrkA and extracellular signal-regulated kinase (ERK) by nerve growth factor (NGF) was inhibited by pretreatment with PP2, an inhibitor of Src family kinases. Moreover, NGF-induced phosphorylation of TrkA and ERK was also attenuated by the transfection with a dominant-negative *src* construct. On the other hand, the transfection with a constitutively active *src* construct enhanced these phosphorylations. In addition, we showed that active Src phosphorylates TrkA directly in vitro, and that Src associates with TrkA through Grb2 after NGF stimulation. These results suggest that constitutively active Src that associates with TrkA through Grb2 after NGF stimulation participates in TrkA phosphorylation and in turn enhances the mitogen-activated protein kinase signaling in SK-N-MC cells.

Key words: Src; TrkA; Grb2; Nerve growth factor; Mitogen-activated protein kinase; SK-N-MC cell

1. Introduction

Src, a non-receptor protein tyrosine kinase, is over-expressed and highly activated in a variety of human cancers including those of the colon, breast, pancreas, and brain [1]. Such an altered Src appears to have an important role in the development, growth, progression and metastasis of these cancers [1,2]. However, the mechanism of Src contribution to the cancer phenotype is not yet known although activated Src has been shown to activate a number of signaling proteins and lead to cell transformation [3–6].

Src has been reported to associate with several receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor and glial cell line-derived neurotrophic factor receptor [7–10]. Particularly, the activation of EGFR by over-expressed Src is intriguing because of biological synergy for mitogenesis in fibroblasts [7]. Moreover, the mutation of PDGFR at Tyr934, phosphorylated by Src, leads to a decrease in mitogenesis in porcine aortic endothelial cells [8]. These results have suggested the possibility that the activation of RTKs by altered Src might contribute to facilitate the malignant progression.

TrkA, a principal receptor of nerve growth factor (NGF), is one of the RTKs [11]. The binding of NGF to TrkA promotes growth, differentiation and survival of several neural cells [11–13]. Recently, Src has been shown to associate with TrkA. Lee et al. [14] demonstrated that the pituitary adenylate cyclase-activating polypeptide, a neuropeptide that acts through G protein-coupled receptors, induces the phosphorylation of TrkA in PC12 pheochromocytoma cells. This phosphorylation of TrkA depends on Src activity, and does not require NGF binding. Moreover, Wooten et al. [15] showed that TrkA is detected in Src immunoprecipitates, which were obtained from PC12 cell lysates in response to NGF treatment. These results have led us to hypothesize that altered Src might activate TrkA and enhance the signaling that could contribute to neuronal cell transformation. In the present study, we therefore investigated whether highly activated Src has influence in the activation of TrkA and its downstream signaling event in neuroblastoma.

To test this, we have used SK-N-MC human neuroblastoma cells that express a high level of constitutively active Src. In addition, we observed the expression of TrkA in these cells. Therefore, SK-N-MC cells could be useful to investigate the interaction between activated Src and TrkA. We demonstrate that the constitutively active Src facilitates NGF-induced phosphorylation of TrkA, causing enhancement of the mitogen-activated protein kinase (MAPK) signaling.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Trypsin-EDTA, penicillin/streptomycin and Lipofectamine™ 2000

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Abbreviations: NGF, nerve growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; SFK, Src family kinase; RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor

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were from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Cansera International (Ontario, Canada). Isogen and Gene *Taq* polymerase chain reaction (PCR) reagents were from Nippon Gene (Tokyo, Japan). Human β-NGF was obtained from Alomone (London, UK). K252a, AG879, PP2, PP3 and U0126 were obtained from Calbiochem (Torrance, CA, USA). Anti-TrkA (763), anti-Trk (C-14), anti-extracellular signal-regulated kinase 1 (ERK1) (K-23), anti-phospho-ERK (E-4), anti-Grb2 (C-23) antibodies, and horseradish peroxidase-conjugated goat antibodies to rabbit or mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-TrkA (Tyr490) and anti-phospho-Src family (Tyr416) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-Src and anti-$p75^{NTR}$ antibodies, active Src, Src kinase reaction buffer and Src manganese/ATP cocktail were from Upstate Biotechnology (Waltham, MA, USA). The First-strand cDNA synthesis kit, protein G-Sepharose, the ECL detection reagent and X-ray film were obtained from Amersham Biosciences (Piscataway, NY, USA).

2.2. Cell culture

Human neuroblastoma SK-N-MC cells (HTB-10; ATCC) and human neuroblastoma SH-SY5Y cells (CRL-2266; ATCC) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, 5% CO_2 at 37°C.

2.3. Reverse transcription (RT) PCR

Total RNA (1.5 μg) was isolated from SH-SY5Y and SK-N-MC cells using Isogen. After reverse transcription using the First-strand cDNA synthesis kit, cDNA was amplified with Gene *Taq* PCR reagents. For RT-PCR, the following primers were used: TrkA (GenBank accession number (GBN): M23102), 5′-TCTTCACTGAGTTCCTGGAG-3′ and 5′-TTCTCCACCGGGTCTCCAGA-3′; TrkB (GBN: S76473), 5′-TACATCTGTACTAAAATACA-3′ and 5′-GTGTCCCCGATGTCATTCGC-3′; TrkC (GBN: S76475), 5′-CATCCATGTGGAATACTACC-3′ and 5′-TGGGTCACAGTGATAGGAGG-3′; β-actin (GBN: NM_001101), 5′-CCCATGCCATCCTGCGTCTG-3′ and 5′-CGTCATACTCCTGCTTGCTG-3′. The PCR was performed as follows: after 94°C for 4 min for denaturing cDNA, 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min in an automated thermal cycler (Bio-Rad Laboratories). 10 μl of each PCR product was run onto a 2.5% agarose gel and visualized with ethidium bromide.

TrkA primers amplify two fragments of 229 and 247 bp corresponding to two alternatively spliced *trkA* transcripts [16]. TrkB, TrkC and β-actin primers amplify fragments of 515, 543 and 570 bp, respectively.

2.4. Cell treatments, lysis, immunoprecipitation, and Western blotting

SK-N-MC and SH-SY5Y cells (1×10^6) were seeded in 35-mm tissue culture dish (Falcon) and cultured for 3 days. Cells were washed twice with serum-free DMEM and then starved in fresh DMEM without serum for 6 h prior to NGF stimulation. Each culture was incubated with either AG879, K252a, U0126, PP2, PP3, or DMSO (as control) for 20 min before adding NGF.

Cells were washed with cold phosphate-buffered saline (PBS) and solubilized in Nonidet P-40 lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 1% protease inhibitor cocktail).

Precleared cell lysates (1 mg) were incubated with either 2 μg of anti-Trk (C-14) or 4 μg of anti-Src antibody for 16 h at 4°C. Then, immune complexes were precipitated with protein G-Sepharose for 2 h at 4°C.

Whole cell lysates (20 μg or 50 μg for analysis for TrkA) and immunoprecipitates were separated by 7.5 or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then electroblotted onto polyvinylidene fluoride membranes (Immune-Blot PVDF membrane; Bio-Rad Laboratories). The membranes were blocked using 5% non-fat dried milk in PBS, pH 7.2, and primary immunoreactions were performed with anti-ERK1 (1:4000), anti-phospho-ERK (1:2000), anti-Grb2 (1:1000) and anti-$p75^{NTR}$ (1:1000) antibodies for 1 h at room temperature or with anti-TrkA (1:1000), anti-phospho-TrkA (1:1000), anti-Src (1:1000) and anti-phospho-Src (1:1000) antibodies for 18 h at 4°C. The membranes were washed three times with TBST buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and then incubated with secondary antibodies (horseradish peroxidase-conjugated goat antibodies to rabbit or mouse IgG) (1:2000) for 1 h at room temperature. The membranes were washed three times with TBST buffer and developed using an ECL detection reagent prior to exposure to an X-ray film.

2.5. Transient transfection

SK-N-MC cells were grown to 80% confluence in 35-mm tissue culture dishes. The culture medium was replaced with 2 ml of fresh DMEM supplemented with 10% FBS and the cells were further cultured for another 24 h. A mixture containing 3 μg of an expression vector containing *src* cDNA mutant (dominant-negative *src* K296R/Y528F or constitutively active *src* Y529F; Upstate Biotechnology) and 10 μl of Lipofectamine™ 2000 reagents was carefully added to each culture and incubated in 5% CO_2 at 37°C for 48 h. The transfection efficiency was verified by Western blot analysis of Src expression.

2.6. In vitro Src kinase assay

TrkA was prepared by immunoprecipitation with 2 μg of anti-Trk antibody for 16 h at 4°C from 2 mg of SK-N-MC cell lysates. TrkA immunoprecipitates were incubated for 10 min at 30°C in reaction mix containing 10 μl of active Src, 10 μl of Src kinase reaction buffer and 10 μl of Src manganese/ATP cocktail. The reaction was stopped with the addition of Laemmli sample buffer.

3. Results and discussion

3.1. Expression of constitutively active Src and neurotrophin receptors in SH-SY5Y and SK-N-MC cells

We selected a useful neuroblastoma cell line for studying the interaction between constitutively active Src and TrkA. SH-SY5Y cells appear to express activated Src and neurotrophin receptors, including TrkA, TrB, TrkC and $p75^{NTR}$ [17–19]. On the other hand, it has been reported that SK-N-MC cells that express activated Src also express $p75^{NTR}$ [17,20].

We verified the expression of constitutively active Src and neurotrophin receptors in these neuroblastoma cells by RT-PCR and Western blotting. SH-SY5Y cells express protein level of TrkA and constitutively active Src, which was demonstrated by the phosphorylation of Src at Tyr416 [21,22] (Fig. 1B). In addition, RT-PCR products for TrkA, TrkB and TrkC were detected in these cells (Fig. 1A). Similarly, SK-N-MC cells express constitutively active Src and neurotrophin receptors except for TrkC (Fig. 1A,B). In contrast to a previous report that showed SK-N-MC cells express $p75^{NTR}$ but not TrkA [20], we could detect both receptors in these cells. A high level of $p75^{NTR}$ expression was detected in SK-N-MC cells. However, the expression of TrkA was sufficient to investigate a signaling event via this receptor.

The expression level of constitutively active Src in SK-N-MC cells was higher than that in SH-SY5Y cells although both cells express equal levels of TrkA. These results prompted us to investigate the biological association of activated Src with TrkA in SK-N-MC cells.

3.2. NGF-induced signaling in SK-N-MC cells

The binding of NGF to TrkA activates its cytoplasmic kinase, resulting in autophosphorylation of specific tyrosine residues within the intracellular domain [4,5]. Phosphorylated tyrosines serve as protein interaction sites for several signaling molecules, such as Shc, phospholipase Cγ, FRS-2 and SH2-B, and activate several defined signaling cascades, including Raf/ERK and phosphatidylinositol 3-kinase/Akt [13]. Therefore, we investigated whether these signaling pathways are activated after NGF stimulation in SK-N-MC cells.

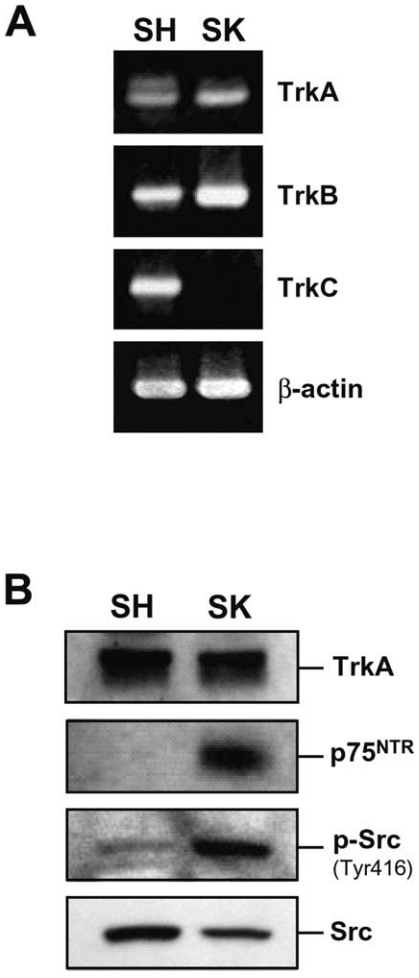

Fig. 1. Expression of constitutively active Src and neurotrophin receptors in SH-SY5Y and SK-N-MC cells. A: RT-PCR analysis of neurotrophin receptors. Total RNA from SH-SY5Y and SK-N-MC cells was extracted and reverse-transcribed to cDNA. PCR was performed using gene-specific primers for TrkA, TrkB and TrkC with β-actin as a control (see Section 2). B: Western blot analysis of TrkA, $p75^{NTR}$ and constitutively active Src (p-Src). Cells were lysed, and then total lysates were resolved by SDS–PAGE and probed with anti-TrkA, anti-$p75^{NTR}$, anti-phospho-Src (Tyr416) and anti-Src antibodies.

Exposure of serum-starved SK-N-MC cells to NGF induced the phosphorylation of TrkA, which peaked 3 min after NGF stimulation and then declined (Fig. 2A). We also observed a transient phosphorylation of ERK1/2, which lasted 1–10 min after NGF stimulation (Fig. 2A). The NGF-induced phosphorylation of TrkA and ERK1/2 was enhanced in a dose-dependent manner (Fig. 2B). In contrast, phospho-Akt was undetectable (data not shown).

The NGF-induced phosphorylation of ERK1/2 was completely blocked by pretreatment with 30 μM U0126 that inhibits the ERK upstream kinase MAPK kinase [23] (Fig. 2C). Moreover, ERK phosphorylation was also inhibited by 30 μM AG879 and 200 nM K252a, inhibitors of TrkA phosphorylation [24,25] (Fig. 2C). These results suggest that NGF activates the MAPK pathway via TrkA in SK-N-MC cells.

3.3. *Participation of constitutively active Src in NGF-induced signal transduction*

To clarify the involvement of constitutively active Src in NGF-induced signal transduction, we investigated the inhibitory effect of PP2, a selective inhibitor of Src family kinases (SFKs) [26]. As shown in Fig. 3, PP2 but not PP3 (negative control) decreased the amount of phospho-Src (Tyr416) in a

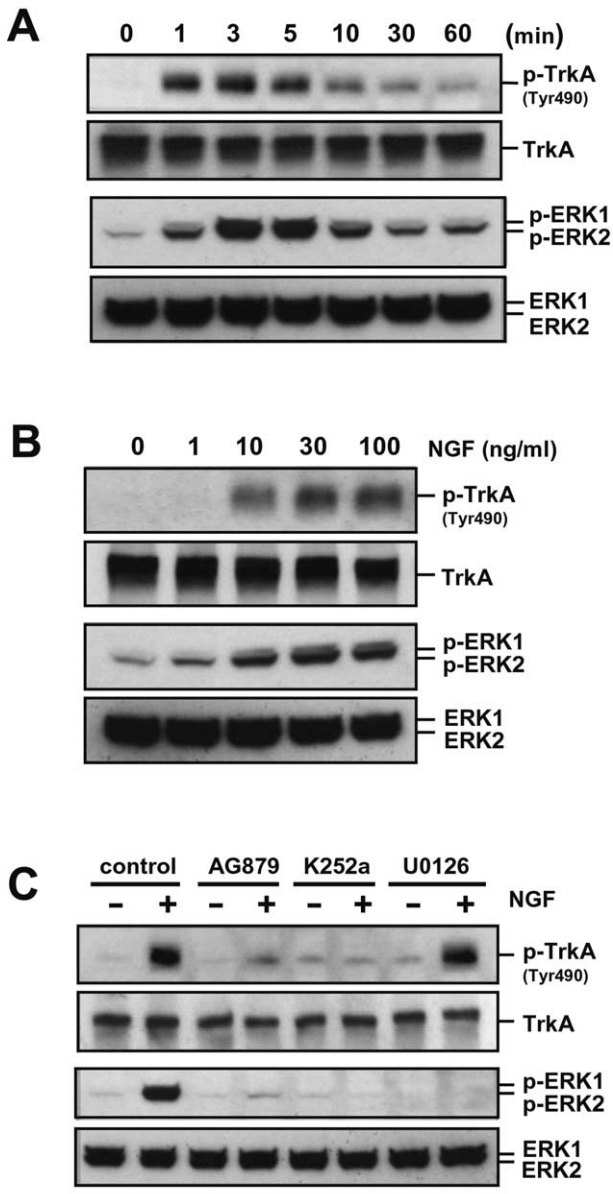

Fig. 2. NGF-induced signaling in SK-N-MC cells. SK-N-MC cells were starved for 6 h and then treated with NGF. A: Time course of NGF-induced phosphorylation of TrkA and ERK. Cells were treated with 100 ng/ml NGF and lysed at the indicated time. B: Dose response of NGF-induced phosphorylation of TrkA and ERK. Cells were incubated in the presence of NGF at the indicated concentration for 3 min prior to cell lysis. C: Inhibition of NGF-induced ERK phosphorylation by treatment with inhibitors of TrkA and ERK phosphorylations. Cells were preincubated in the presence of 30 μM AG879, 200 nM K252a and 30 μM U0126 for 20 min, and stimulated with 100 ng/ml NGF for 3 min. TrkA and ERK activations were analyzed by Western blotting with anti-phospho-TrkA (Tyr490) and anti-phospho-ERK antibodies, respectively. After stripping, TrkA and ERK expressions were verified by reprobing the same blots with anti-TrkA and anti-ERK1 antibodies (lower panels). The positions of phosphorylated $p42^{ERK2}$ and $p44^{ERK1}$ are shown.

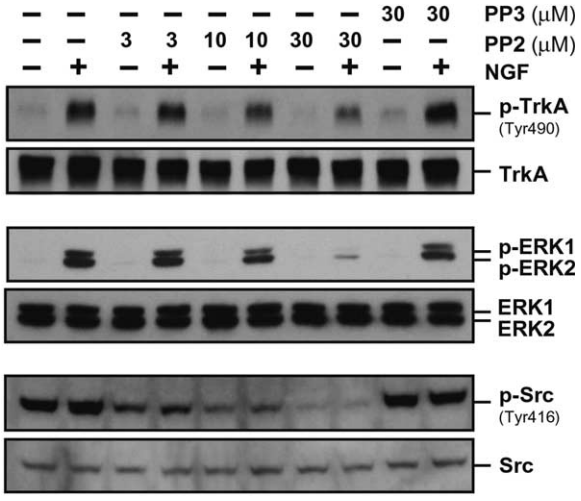

Fig. 3. Inhibition of NGF-induced phosphorylation of TrkA and ERK by pretreatment with PP2, a SFK inhibitor. SK-N-MC cells were serum-starved for 6 h. Cells were preincubated in the presence of PP2 or PP3 at the indicated concentration for 20 min, and stimulated with 100 ng/ml NGF for 3 min. Cells were lysed, and then TrkA, ERK and Src activations were analyzed by Western blotting with anti-phospho-TrkA (Tyr490), anti-phospho-ERK and anti-phospho-Src (Tyr416) antibodies, respectively. After stripping, loading of equal amounts of TrkA, ERK and Src was verified by reprobing the same blots with anti-TrkA, anti-ERK1 and anti-Src antibodies (lower panels).

dose-dependent manner suggesting that PP2 inhibits Src activity. Pretreatment of SK-N-MC cells with PP2 attenuated NGF-induced phosphorylation of TrkA and ERK1/2.

PP2 has been reported to be a selective inhibitor of SFKs [26]. However, to rule out the possibility that PP2 might directly inhibit the phosphorylation of TrkA and ERK, we verified the Src activity inhibition by the transfection with a dominant-negative *src* construct. The transfection with the dominant-negative *src* construct allows for the expression of mutant Src (K297R/Y528F), which appears without phosphotyrosine activity in the presence of normal binding activity of adapter proteins [27,28]. Consistent with the effect of PP2, the transfection with the dominant-negative *src* construct inhibited NGF-induced phosphorylation of TrkA and ERK1/2 when compared with cells transfected with the empty vector (Fig. 4A). In addition, the transfection with a constitutively active *src* construct enhanced NGF-induced phosphorylation of TrkA and ERK1/2 (Fig. 4B).

In an in vitro Src kinase assay, TrkA was phosphorylated by active Src directly (Fig. 5). Src also appears to activate ERK by phosphorylating signaling molecules, such as Shc or focal adhesion kinase (FAK), generating binding sites for Grb2 [29]. In SK-N-MC cells, however, these signaling molecules would be little affected by constitutively active Src because the phosphorylation of ERK is detected only at a low level in the absence of NGF stimulation. Moreover, there was no change in the expression of phospho-Src (Tyr416) under NGF stimulation conditions (Fig. 3), supporting the possibility that constitutively active Src also did not affect the signaling molecules in the presence of NGF stimulation. Thus, our results suggest that constitutively active Src contributes partly to phosphorylation of TrkA, resulting in enhancement of MAPK signaling in SK-N-MC cells.

3.4. Association of Src with TrkA

We investigated the association of Src with TrkA. In anti-Src or anti-Trk immunoprecipitation study, co-immunoprecipitates of Src with TrkA were not detected in the absence or presence of NGF stimulation (Fig. 6). Moreover, constitutively active Src had little effect on TrkA phosphorylation in the absence of NGF stimulation (Fig. 3). These results have led us to hypothesize that the association of Src with TrkA may be mediated by adapter molecules that associate with both TrkA and Src.

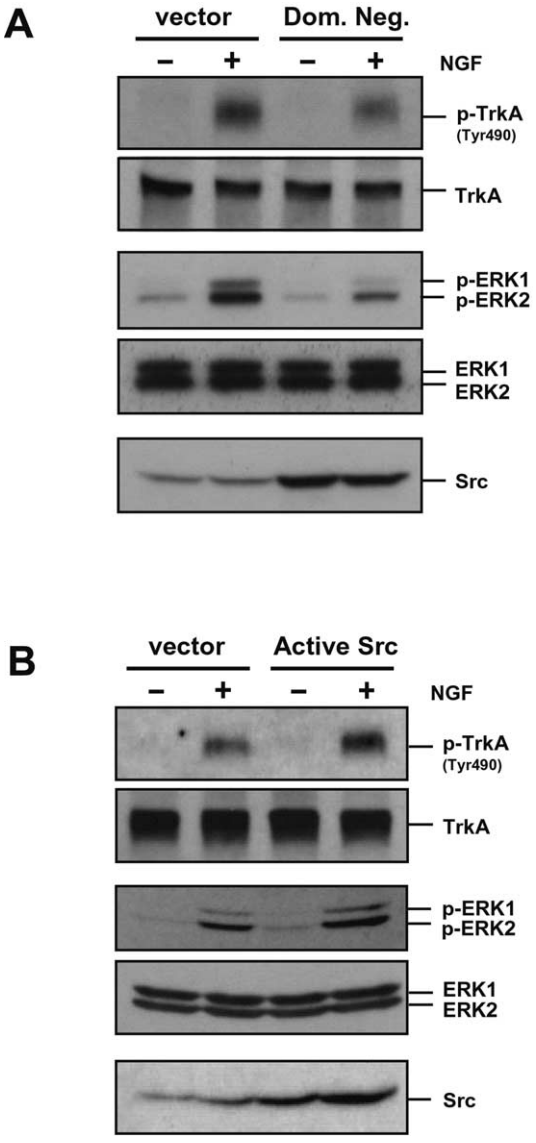

Fig. 4. Effect of dominant-negative Src or over-expressed constitutively active Src on NGF-induced phosphorylation of TrkA and ERK. SK-N-MC cells were transiently transfected with a dominant-negative *src* construct (A) or a constitutively active *src* construct (B) by a Lipofectamine method as described in Section 2. Transfected cells were starved in DMEM without serum for 6 h, and stimulated with 100 ng/ml NGF for 3 min. Cells were lysed, and then TrkA and ERK activations were assessed by Western blotting with anti-phospho-TrkA (Tyr490) and anti-phospho-ERK antibodies, respectively. After stripping, loading of equal amounts of TrkA and ERK was verified by reprobing the same blots with anti-TrkA and anti-ERK1 antibodies (lower panels). The transfection efficiency was verified by Western blotting with anti-Src antibody.

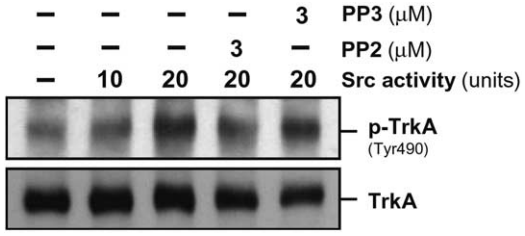

Fig. 5. In vitro phosphorylation of TrkA by active Src. TrkA was prepared by immunoprecipitation with anti-Trk antibody from SK-N-MC cell lysates. The TrkA immunoprecipitates were incubated in the presence of active Src at the indicated activity for 10 min at 30°C. TrkA phosphorylation was assessed by Western blotting with anti-phospho-TrkA (Tyr490) antibody. After stripping, loading of equal amounts of TrkA was verified by reprobing the same blots with anti-TrkA antibody (lower panels).

Grb2 has been reported to associate with TrkA or FAK, a SFK [30,31]. Therefore, we investigated the link between Grb2 and TrkA or Src. Analysis of anti-Trk or anti-Src immunoprecipitates indicated that Grb2 is co-immunoprecipitated with both TrkA and Src (Fig. 6). The amount of co-immunoprecipitates of Grb2 with Src was not affected by NGF stimulation. On the other hand, the amount of co-immunoprecipitates of Grb2 with TrkA was found to be increased by NGF stimulation. These results suggest that constitutively active Src associates with TrkA through Grb2 after NGF stimulation. Although Shc also appears to associate with both TrkA and Src [32,33], co-immunoprecipitates of Shc with Src were not detected in SK-N-MC cell lysates (data not shown).

In conclusion, our results suggest that constitutively active Src that associates with TrkA through Grb2 after NGF stimulation facilitates phosphorylation of TrkA and in turn activates the MAPK pathway. Src can increase the transcription of cyclin D1 in NIH3T3 and MCF7 cells [34,35]. This effect of Src requires activation of MAPK signaling. Src-activated MAPK signaling also induces Rat-1 cells to enter the S phase of the cell cycle [36]. Moreover, the cells that express constitutively active MAPK kinase have been reported to form transformed foci, grow efficiently in soft agar, and be highly tumorigenic in nude mice [37]. These results suggest that MAPK signaling plays an important role in cell transformation. Therefore, the enhancement of MAPK signaling mediated by interaction between altered Src and TrkA may contribute to neuronal cell transformation.

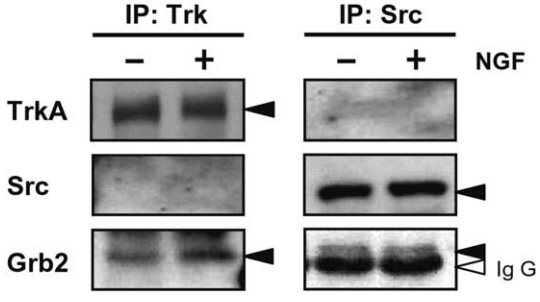

Fig. 6. Co-immunoprecipitation of Grb2 with TrkA or Src. SK-N-MC cells were serum-starved for 6 h, and stimulated with 100 ng/ml NGF for 3 min. Cell lysates were immunoprecipitated with either anti-Trk or anti-Src antibody, and analyzed by Western blotting with anti-TrkA, anti-Src and anti-Grb2 antibodies.

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