Interaction of SH2-Bβ with RET is involved in signaling of GDNF-induced neurite outgrowth

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Summary

RET receptor signalling is essential for glial-cell-line-derived neurotrophic factor (GDNF)-induced survival and differentiation of various neurons such as mesencephalic neurons. To identify proteins that mediate RET-dependent signaling, yeast two-hybrid screening was performed with the intracellular domain of RET as bait. We identified a new interaction between RET and the adapter protein SH2-Bβ. Upon GDNF stimulation of PC12-GFRα1-RET cells (that stably overexpress GDNF receptor α1 and RET), wild-type SH2-Bβ co-immunoprecipitated with RET, whereas the dominant-negative SH2-Bβ mutant R555E did not. RET interacted with endogenous SH2-Bβ both in PC12-GFRα1-RET cells and in rat tissues. Mutagenesis analysis revealed that Tyr981 within the intracellular domain of RET was crucial for the interaction with SH2-Bβ. Morphological evidence showed that SH2-Bβ and RET colocalized in mesencephalic neurons. Furthermore, functional analysis indicated that overexpression of SH2-Bβ facilitated GDNF-induced neurite outgrowth in both PC12-GFRα1-RET cells and cultured mesencephalic neurons, whereas the mutant R555E inhibited the effect. Moreover, inhibition of SH2-Bβ expression by RNA interference caused a significant decrease of GDNF-induced neuronal differentiation in PC12-GFRα1-RET cells. Taken together, our results suggest that SH2-Bβ is a new signaling molecule involved in GDNF-induced neurite outgrowth.

Key words: RET, GDNF, SH2-Bβ, Interaction, Neurite outgrowth

Introduction

Neurotrophic factors play important roles in many biological processes, including survival, proliferation, differentiation and apoptosis of neurons in the nervous system (Baloh et al., 2000a; Airaksinen and Saarma, 2002; Sariola and Saarma, 2003). Glial-cell-line-derived neurotrophic factor (GDNF) family ligands (GFLs) are composed of GDNF (Lin et al., 1993), neurturin (NRTN) (Kotzbauer et al., 1996), artemin (ARTN) (Baloh et al., 1998), and persephin (PSPN) (Milbrandt et al., 1998). They are found to be crucial regulators in neurodevelopment (Lin et al., 1993) and the survival of midbrain dopaminergic and spinal cord neurons both in vitro and in animal disease models (Baloh et al., 1998; Horger et al., 1998; Henderson et al., 1994; Klein et al., 1997; Cao et al., 2004), making them attractive therapeutic candidates for the treatment of neurodegenerative diseases (Grondin and Gash, 1998; Baloh et al., 2000b). Moreover, GDNF is required for inducing branching of ureteric buds during kidney development (Sariola and Sainio, 1997) and also important for the cell-fate decision of undifferentiated spermatogonia in the testis (Meng et al., 2000). All GFLs share the receptor tyrosine kinase RET as their common signaling receptor to exert their biological roles (Airaksinen and Saarma, 2002; Sariola and Saarma, 2003). The ligand-binding specificity of GFLs is determined by GFRα1-4 proteins that have unique binding affinities for each GFL (Airaksinen and Saarma, 2002; Sariola and Saarma, 2003).

Activation of RET can be achieved either by interaction with GFRα and GFLs or by different oncogenic mutations. Upon activation, RET triggers a variety of intracellular signaling pathways, including the Ras-Raf-MEK-ERK, the phosphatidylinositol 3-kinase (PI 3-K)-Akt and the phospholipase C γ (PLCγ) pathways (Sariola and Saarma, 2003; Takahashi, 2001; Manie et al., 2001). Multiple autophosphorylated tyrosine residues in RET, are identified as docking sites for Grb7/Grb10, Src, PLCγ, Shc/Enigma/Frs2/IRS-1/Doks, and Grb2 (Pandey et al., 1995; Encinas et al., 2004; Asai et al., 1996; Borrello et al., 1996; Arighi et al., 1997; Lorenzo et al., 1997; Alberti et al., 1998; Durick et al., 1998; Murakami et al., 2002; Grimm et al., 2001; Crowder et al., 2004; Kurokawa et al., 2001; Melillo et al., 2001a; Melillo et al., 2001b). RET can also activate Rho family GTPases, including Rho, Rac and Cdc42, which are involved in reorganization of the actin cytoskeleton and responsible for cell motility and morphology (van Weering and Bos, 1997; Chiariello et al., 1998; Murakami et al., 1999; Barone et al., 2001). In addition, Grap2 plays a tissue-specific role as an inhibitor of the mitogenic signaling of RET (Ludwig et al., 2003). As a neuronal scaffold protein, and Shank3 mediates signaling and biological function of RET in epithelial cells (Schuetz et al., 2004). In most cases, the studies were carried out in non-neuronal cell lines and constitutively activated RET mutants were used. The role of these signaling molecules in RET-mediated neuronal survival and differentiation remains obscure.

To gain insight into the mechanisms by which GDNF-mediated activation of RET enhances neuronal survival or differentiation, it is of considerable importance to identify new members of the RET downstream signaling pathway that may mediate or contribute to these processes. Therefore, using the intracellular domain of RET as bait, we employed yeast two-hybrid screening on a human brain cDNA library and identified several candidate binding proteins that interact with RET, one of which is the adapter protein Src-homology-2-Bβ (SH2-Bβ).

The isoforms of SH2-B, APS, and Lnk are a family of signaling proteins that have been described as activators, mediators or inhibitors of cytokine and growth factor signaling (Yousaf et al., 2001). At least four splice variants of SH2-B (α, β, γ and δ) have been identified so far, and all of them have identical N-terminal and Src homology 2 (SH2) domains but differ in their C-terminal domains (Yousaf et al., 2001; Osborne et al., 1995; Riedel et al., 1997; Rui et al., 1997). SH2-Bβ was originally identified as a Janus kinase 2 (JAK2)-interacting protein (van Weering and Bos, 1997). It contains several protein-protein interaction motifs, including a PH domain, an SH2 domain and multiple proline-rich regions (Riedel et al., 1997; Rui et al., 1997). SH2-Bβ have been shown to interact with several receptor tyrosine kinases including platelet-derived growth factor receptor (Yousaf et al., 2001), insulin receptor (Riedel et al., 1997), nerve growth factor receptor TrkA (Qian et al., 1998; Rui et al., 1999), brain-derived neurotrophic factor receptor TrkB (Suzuki et al., 2002), as well as fibroblast growth factor receptor FGFR3 (Kong et al., 2003). These studies suggested that SH2-Bβ plays a fundamental role in receptor tyrosine kinase-mediated cellular functions. SH2-Bβ has also been demonstrated to bind to the non-receptor tyrosine kinase growth hormone receptor (Rui et al., 1997; Herrington et al., 2000), to stimulate the kinase activity of JAK2, and to increase tyrosine phosphorylation of STAT3 and STAT5B (Rui et al., 1997; Rui and Carter-Su, 1999).

In this study, we first show by yeast two-hybrid screening that SH2-Bβ is a RET-binding candidate. We then verified that SH2-Bβ co-immunoprecipitates with RET in response to GDNF stimulation after transfection into PC12-GFRα1-RET cells. Furthermore, we show that endogenous SH2-Bβ co-immunoprecipitates with RET not only in PC12-GFRα1-RET cells but also in some homogenates of rat tissues such as spinal cord and mesencephalon. Mutation analysis revealed that Tyr981 within the intracellular domain of RET is crucial for the interaction with SH2-Bβ, and morphological studies showed that RET and SH2-Bβ colocalized in rat mesencephalic neurons. Neurite outgrowth assay demonstrated that overexpression of SH2-Bβ facilitated the GDNF-induced neurite outgrowth in both PC12-GFRα1-RET cells and cultured mesencephalic neurons, whereas the mutant R555E inhibited the effect. Moreover, the neurite outgrowth in the PC12 cells was significantly attenuated when the expression of SH2-Bβ was knocked-down by RNA interference (RNAi). Our results suggest that SH2-Bβ is a new signaling molecule involved in GDNF-induced neurite outgrowth.

Results

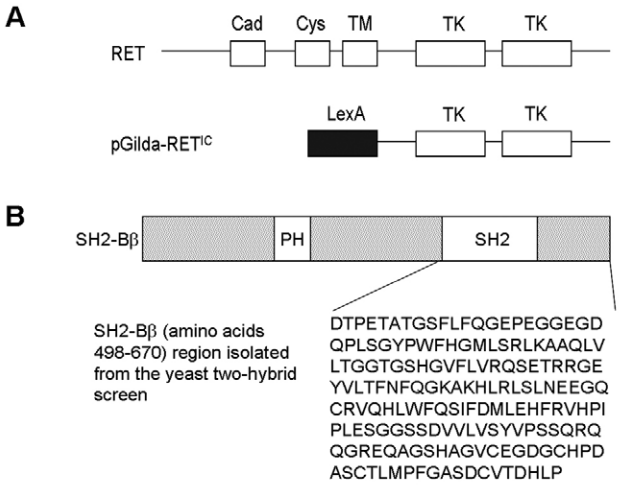
Association of the intracellular domain of RET and SH2-Bβ in yeast

In an attempt to identify proteins involved in the regulation or signaling of RET receptors, a yeast two-hybrid screen of a LexA human brain cDNA library was performed with the intracellular domain (amino acids residues 658-1114) of RET as bait (Fig. 1A). In the screen, 274 potential positive clones were isolated and of these, 68 clones scored positive based on the β-galactosidase filter-lift assay. False positives were further eliminated by testing against LexA-lamin, and 21 clones remained positive and were subjected to sequencing. One of the positive clones was identified harboring a 519 bp cDNA fragment that corresponds to amino acid residues 498-670 of SH2-Bβ. The region includes the entire SH2 domain plus part of the flanking sequences as shown in Fig. 1B.

Furthermore, yeast two-hybrid assays by β-galactosidase activity analysis were used to test the interaction between full-length wild-type SH2-Bβ and other receptors, and to determine which region in SH2-Bβ interacts with RET (Fig. 1C,D). Both RET and TrkA were found to interact with SH2-Bβ, whereas EGF receptor was not. TrkA was used as a positive control here because it was shown to bind to SH2-B (Qian et al., 1998; Rui et al., 1999). SH2-Bβ contains multiple protein-protein interaction domains including a PH domain and an SH2 domain. To determine whether the SH2 domain of SH2-Bβ is involved in its interaction with RET, the essential Arg555 within the SH2 domain of SH2-Bβ was replaced with Glu and the dominant-negative mutant form of SH2-Bβ was named for R555E, as previously reported by others (Rui et al., 1999). Our results also indicated that only the SH2 domain of SH2-Bβ is sufficient and is required for the interaction of SH2-Bβ with RET, because no binding was found between RET and the PH domain of SH2-Bβ or R555E. Thus, we demonstrated that the intracellular domain of RET can interact with SH2-Bβ in yeast and that this interaction was mediated by the SH2 domain of SH2-Bβ.

GDNF is required for interaction of SH2-Bβ with RET in PC12-GFRα1-RET cells

The ability of SH2-Bβ to interact with RET was also examined by immunoprecipitation in PC12 cells that had been co-transfected with SH2-Bβ and RET. Since there is no endogenous GFRα1 and little RET in wild PC12 cells, we used our previously established PC12-GFRα1-RET cell line (Wang et al., 2004a), PC12 cells that stably overexpress GFRα1 and RET. Myc-epitope-tagged derivatives of SH2-Bβ were constructed as mentioned in Materials and Methods. To confirm whether wild-type SH2-Bβ or SH2-Bβ mutant R555E interact with RET in mammalian cells, PC12-GFRα1-RET cells were transiently mock transfected with empty pcDNA3 vector as a control, and with pcDNA3-myc-SH2-Bβ or pcDNA3-myc-R555E as SH2-Bβ dominant-negative mutants. In the absence or presence of GDNF stimulation, cell lysates were immunoprecipitated with either anti-RET antibody or anti-myc antibody, then resolved by 10% SDS-PAGE, and immunoblotted with anti-myc or anti-RET antibodies. As shown in Fig. 2A, myc-SH2-Bβ proteins were expressed in the PC12-GFRα1-RET cells that had been transiently transfected with pcDNA3-myc-SH2-Bβ or with pcDNA3-myc-R555E, but not in cells that had been mock transfected with pcDNA3 vector. However, binding of RET to myc-SH2-Bβ was only detected in GDNF-treated cells transfected with pcDNA3-myc-SH2-Bβ. These results suggest that activation of RET by stimulation with GDNF is required for interaction of RET with SH2-Bβ in PC12-GFRα1-RET cells.



C

Bait	Prey	Filter Assay
pGilda-RETIC	pB42AD-SH2-Bβ	+
pGilda-RETIC	pB42AD-SH2-Bβ-SH2	+
pGilda-RETIC	pB42AD-SH2-Bβ-PH	-
pGilda-RETIC	pB42AD-R555E	-
pGilda-RETIC	pB42AD	-
pGilda-EGFRIC	pB42AD-SH2-Bβ	-
pGilda-TrkAIC	pB42AD-SH2-Bβ	+
pGilda-53	pB42AD-T	+
pGilda	pB42AD	-

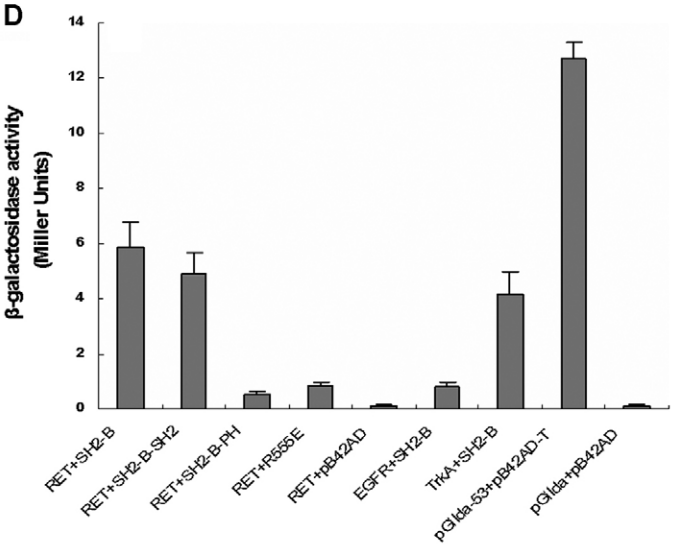


Fig. 1. RET interacts with SH2-Bβ in a two-hybrid screen. (A) Schematic representation of the pGilda-RET^{IC} bait. Cad, Cadherin domain; Cys, cysteine-rich domain; TM, transmembrane domain; TK, tyrosine kinase domain; LexA, LexA fusion vector pGilda. (B) The human SH2-Bβ region (amino acids 498-670) containing the SH2 domain was isolated from the two-hybrid screen. PH, pleckstrin homology domain; SH2, Src homology 2 domain. (C,D) Filter assay and liquid culture assay using o-nitrophenyl-D-galactoside (ONPG) was performed for β-galactosidase activity analysis. Full-length wild-type SH2-Bβ was co-transformed into yeast reporter strain EGY48 with the bait encoding the intracellular domain of RET, TrkA or EGF receptor. To determine the binding domain of the interaction between SH2-Bβ and RET, RET^{IC} bait was transformed with the SH2 domain, the PH domain of SH2-Bβ or R555E (SH2-Bβ dominant-negative mutant). Positive and negative controls are described in Materials and Methods.

Recently, Wang et al. reported that the endogenous SH2-Bβ isoform is the primary isoform of SH2-B expressed in wild-type PC12 cells (Wang et al., 2004b). Therefore, we next examined whether endogenous SH2-Bβ was able to associate with RET in PC12-GFRα1-RET cells. We found that endogenous SH2-Bβ co-immunoprecipitates with RET in PC12-GFRα1-RET cells that had been stimulated with GDNF (Fig. 2B). We could not detect the interaction in wild-type PC12 cells treated with GDNF or in PC12-GFRα1-RET cells without GDNF treatment. These results further confirmed that activation of RET is essential for the interaction with adapter protein SH2-Bβ.

Identification of a binding site crucial for the interaction of RET with SH2-Bβ

It has been assumed that there are five tyrosine phosphorylation sites (Y905, Y981, Y1015, Y1062 and Y1096) within the intracellular domain of RET, which mainly serve as potential docking sites for signaling molecules (Kodama et al., 2005). For mapping which residue is involved in the interaction with SH2-Bβ, five RET mutants (Y905F, Y981F, Y1015F, Y1062F and Y1096F) were constructed by mutating the tyrosine residue to phenylalanine. Moreover, to strengthen the importance of tyrosine phosphorylation to the interaction of RET with SH2-Bβ, we changed Lys758 of the ATP binding site within the kinase domain of RET to Met, assuming that this mutation could render a catalytically inactive receptor (Hanks et al., 1988). Subsequently, wild-type RET or the above point mutants were co-transfected into HEK293T cells together with GFRα1 and SH2-Bβ, and then stimulated with GDNF. As expected, the interaction was abolished by the Lys758Met mutation of RET, which resulted in a kinase-dead receptor, confirming that association of the SH2-Bβ SH2 domain with RET depends on an intact kinase activity of the receptor (Fig. 3). Change of Tyr981 to Phe significantly attenuated the interaction between RET and SH2-Bβ, whereas none of other four tyrosine residues was dominantly relevant for SH2-Bβ binding, because mutation of them did not obviously impair interaction (Fig. 3). These results demonstrated that Y981 was crucial for the interaction of RET with SH2-Bβ.

RET forms a co-precipitable protein complex with SH2-Bβ from tissue homogenates

To examine whether RET and SH2-Bβ form a natural complex in mammalian tissues, we performed immunoprecipitation assays with homogenates from different tissues of adult rat, including mesencephalon, spinal cord, kidney and muscle. Proteins from the homogenates were immunoprecipitated with anti-RET antibody and immunoblotted with anti-SH2-Bβ or anti-RET antibodies. As shown in Fig. 4, RET co-immunoprecipitates with SH2-Bβ in mesencephalon and spinal cord but not in kidney, although RET was expressed in all three types of tissues. These results indicated that RET can

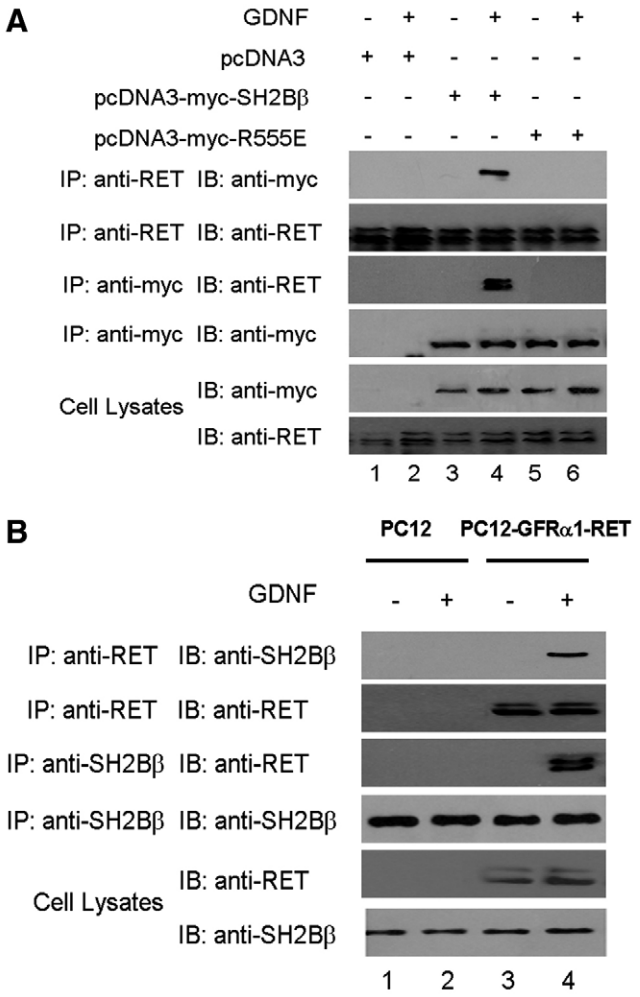


Fig. 2. GDNF stimulates interaction of RET with SH2-Bβ in PC12-GFRα1-RET cells. (A) PC12-GFRα1-RET cells were transfected with pcDNA3, pcDNA3-myc-SH2-Bβ, or pcDNA3-myc-R555E respectively. Cells were treated or not with 100 ng/ml GDNF for 10 minutes before lysed on ice and collected. Then, cell lysates were immunoprecipitated (IP) with anti-myc or anti-RET antibodies, followed by immunoblotting (IB) with anti-RET or anti-myc antibodies. Cell lysates were also subjected to immunoblotting with anti-RET or anti-myc antibodies. (B) Cultured PC12 cells or PC12-GFRα1-RET cells were treated or not with 100 ng/ml GDNF for 10 minutes before they were lysed and collected. Cell lysates were immunoprecipitated with anti-RET or anti-SH2-Bβ antibodies, followed by immunoblotting with anti-SH2-Bβ or anti-RET antibodies. Expression of RET and SH2-Bβ in cells was determined by immunoblotting with anti-RET or anti-SH2-Bβ antibodies. IP, immunoprecipitation; IB, immunoblot.

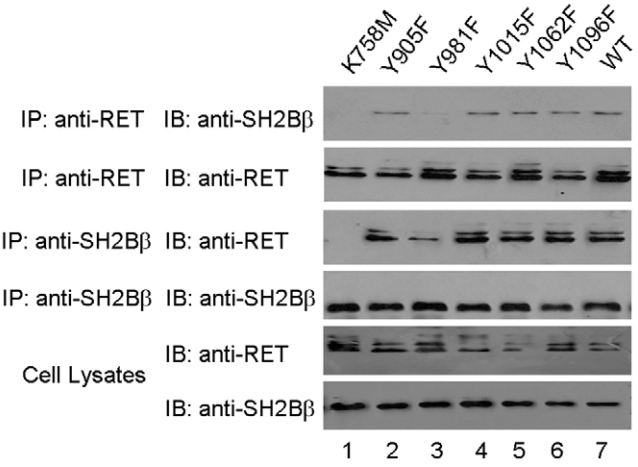


Fig. 3. Identification of the binding site in RET crucial for interaction with SH2-Bβ. HEK293T cells were co-transfected with wild-type RET or the indicated RET mutants, together with GFRα1 and SH2-Bβ; cells were then stimulated with 100 ng/ml GDNF for 10 minutes. Cell lysates were immunoprecipitated with anti-RET or anti-SH2-Bβ antibodies, followed by immunoblotting with anti-SH2-Bβ or anti-RET antibodies. Mutation of Tyr981 to Phe severely attenuates the interaction between RET and SH2-Bβ. WT, wild type; K, lysine; M, methionine; Y, tyrosine; F, phenylalanine.

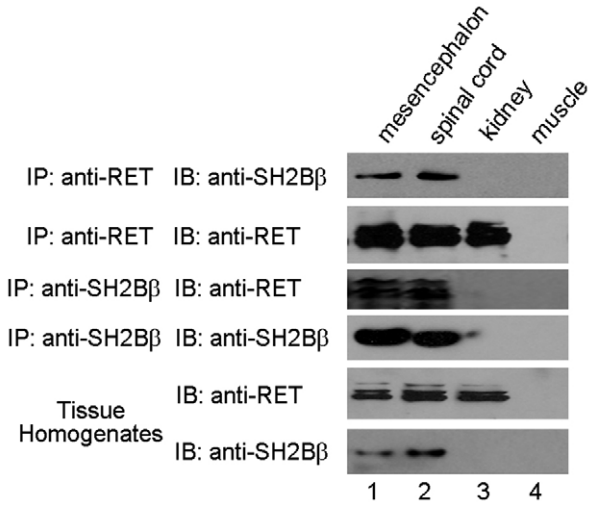


Fig. 4. Endogenous SH2-Bβ interacts with RET in rat-tissue homogenates. Different tissue homogenates from mesencephalon, spinal cord, kidney and muscle were prepared as described in Materials and Methods, and then immunoprecipitated with anti-RET or anti-SH2-Bβ antibodies, followed by immunoblotting with anti-SH2-Bβ or anti-RET antibodies. Protein expressions in tissues were visualized by immunoblotting with anti-SH2-Bβ or anti-RET antibodies.

be co-precipitated in a complex with SH2-Bβ from the neural tissue homogenates.

RET colocalizes with SH2-Bβ in rat mesencephalic neurons

Before studying the possible biological function of the interaction between RET and SH2-Bβ, we needed to obtain direct morphological evidence that RET and SH2-Bβ colocalized in the same cells. Therefore, we next carried out immunohistochemistry analysis on their cellular localization. The slices from the adult rat spinal cord or mesencephalon were processed for immunofluorescence double-labeling. As shown in Fig. 5A, immunofluorescences for SH2-Bβ (green) and RET (red) are in the red nucleus of rat mesencephalon. The merged images show that SH2-Bβ and RET colocalized in neurons of red nucleus (Fig. 5A). We also observed that SH2-

Bβ and RET colocalized in neurons of adult rat spinal cord (data not shown). Therefore, these results demonstrate that SH2-Bβ and RET colocalize in neuronal cells.

Furthermore, to clarify the sub-cellular localization of SH2-Bβ, we explored immunostaining of cultured mesencephalic neurons with anti-SH2-Bβ antibody. Distribution of SH2-Bβ was determined by double-immunostaining either with the dendrite marker MAP-2 or with axon marker Tau-1 (Fig. 5B). Our data demonstrates that distribution of SH2B-β is both somatodendritic and axonal.

SH2-Bβ mediated GDNF induction of neuronal differentiation in PC12-GFRα1-RET cells

To identify a possible physiological role of SH2-Bβ in the downstream signaling of RET, we first performed the neurite-outgrowth assay in PC12-GFRα1-RET cells, which represents the hallmark of the differentiated cells. As expected, neurite outgrowth was significantly increased in wild-type PC12 cells treated with 100 ng/ml NGF, or in PC12-GFRα1-RET cells treated with 100 ng/ml GDNF, but not in wild-type PC12 cells with the GDNF treatment (Wang et al., 2004a; data not shown). Thus, the stable PC12-GFRα1-RET cells were co-transfected with pEGFP-N2, encoding green fluorescent protein (GFP), at the ratio of 1:10 together with wild-type pcDNA3-myc-SH2-Bβ, with pcDNA3-myc-R555E as dominant-negative mutant of SH2-Bβ, or with pcDNA3 as control. In the presence of GDNF, cells co-transfected with pcDNA3/GFP enhanced neurite outgrowth in a manner similar to wild-type PC12 cells (Fig. 6A and data not shown). Strikingly, transfection of SH2-Bβ/GFP promoted neurite outgrowth induced by GDNF, whereas transfection of R555E/GFP inhibited the effect (Fig. 6A). Upon GDNF stimulation, neurite outgrowth was significantly enhanced in cells transfected with SH2-Bβ, and significantly inhibited in cells transfected with R555E compared with that of controls (Fig. 6B). However, in the absence of GDNF, overexpression of SH2-Bβ or R555E did not significantly influence the ratio of neurite-bearing cells, suggesting that SH2-Bβ is involved in signaling activated by GDNF to mediate neurite outgrowth.

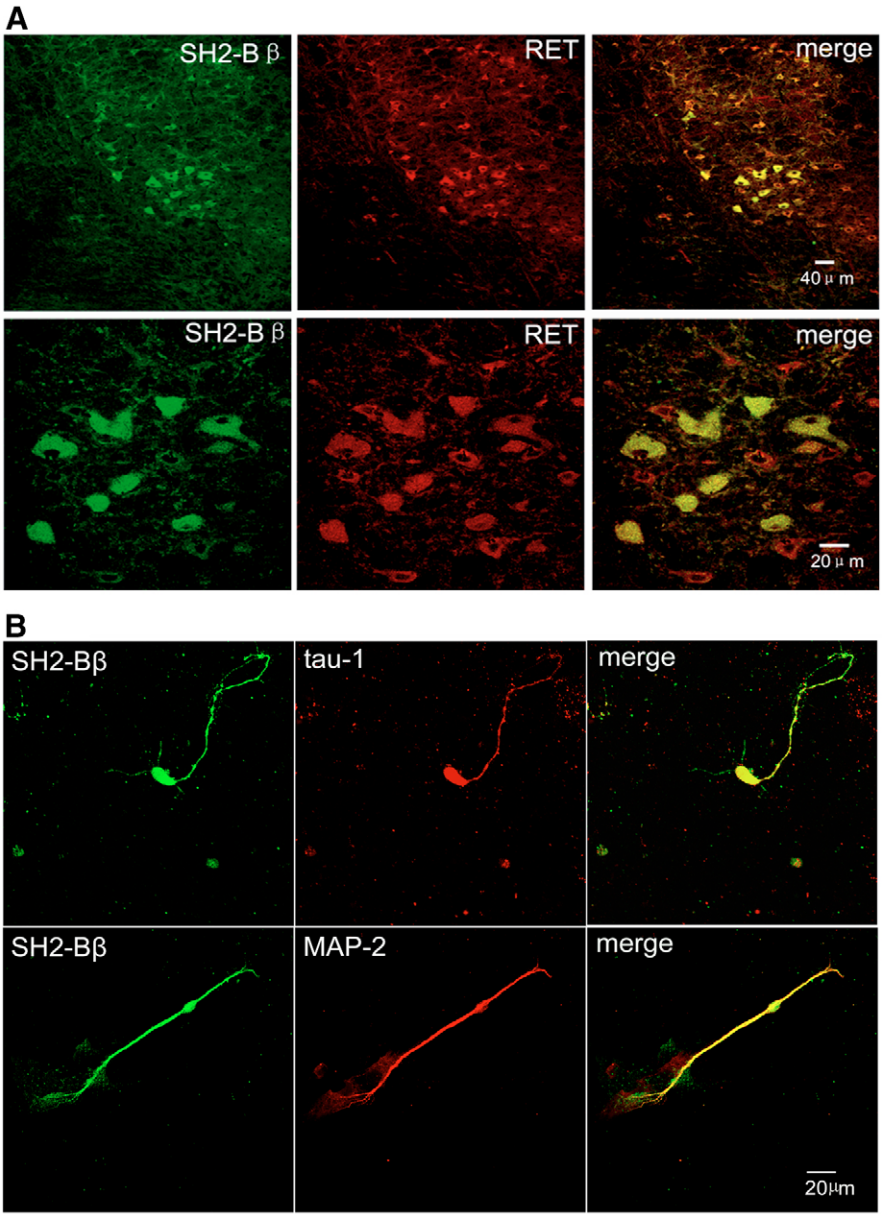


Fig. 5. SH2-Bβ and RET are colocolized in rat mesencephalic neurons. (A) The tissue slices from rat mesencephalon were first immunostained with anti-SH2-Bβ antibodies (green) and then immunostained with anti-RET antibodies (red). The merged images indicate that SH2-Bβ and RET can colocolize in red nucleus neurons (yellow) of rat mesencephalon. Bars, 40 μm (top), 20 μm (bottom). (B) Subcellular localization of SH2-Bβ in primary cultured rat mesencephalic neurons. The distribution of SH2-Bβ (green) is shown by immunofluorescence, its colocalization with either the dendrite marker MAP-2 or the axon marker tau-1 (red) is shown in the merged images (yellow). Bar, 20 μm.

To further confirm the effect of SH2-Bβ on PC12 cell differentiation, the RNAi approach was employed. Thus, 21-nucleotide long small interference RNA (siRNA) duplexes directed against SH2-Bβ were co-transfected with pEGFP-N2 into PC12-GFRα1-RET cells to suppress expression of endogenous SH2-Bβ. Two days after transfection, cells were analyzed for SH2-Bβ expression by western blot. As shown in Fig. 6E, expression of SH2-Bβ in SH2-Bβ-siRNA-transfected cells was significantly decreased in comparison with missense RNAi-transfected cells or control. Moreover, in agreement with results of SH2-Bβ overexpression experiments, inhibition of SH2-Bβ expression by SH2-Bβ-siRNA significantly attenuated GDNF-induced differentiation in PC12-GFRα1-RET cells (Fig. 6C,D).

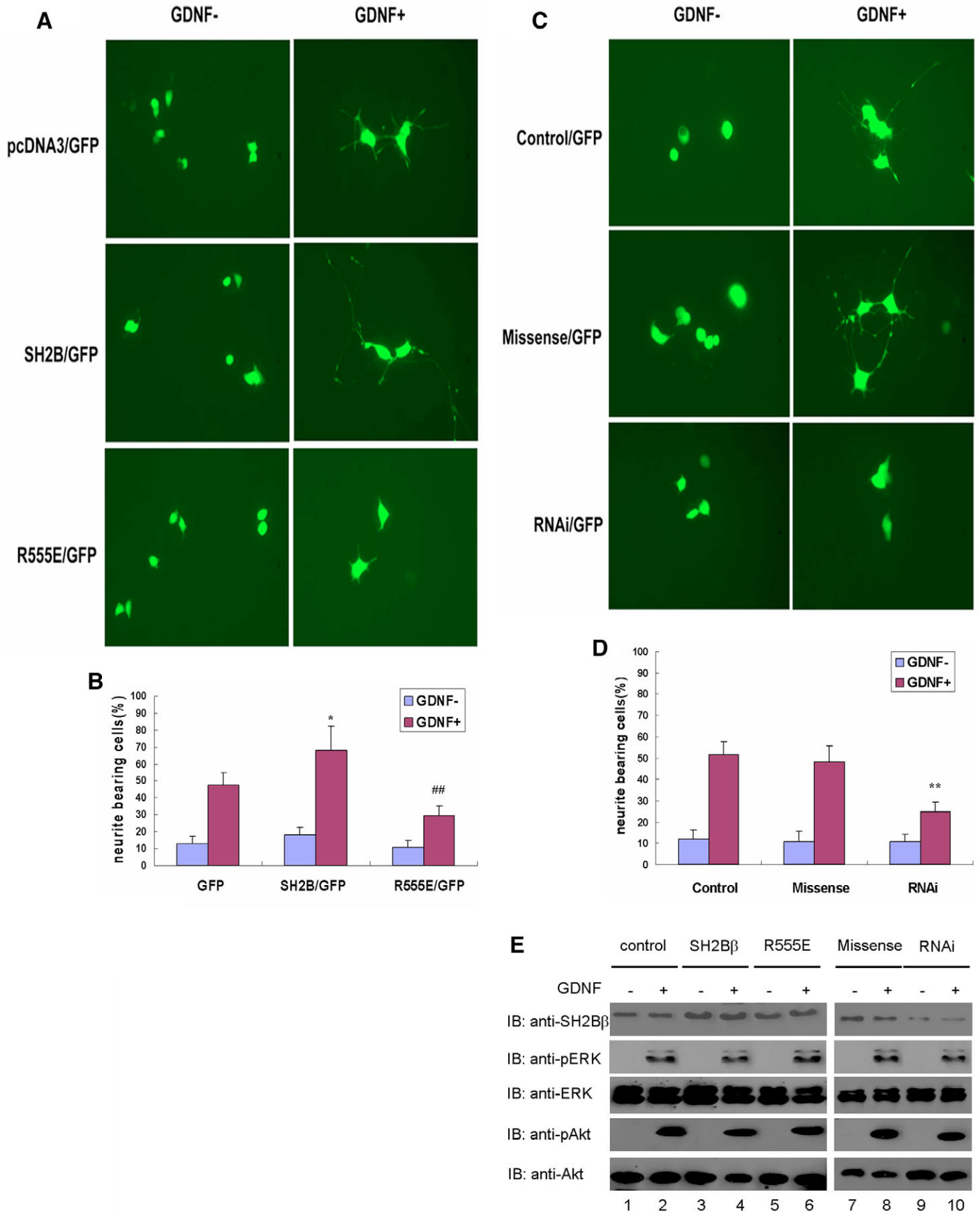


Fig. 6. See next page for legend.

Fig. 6. SH2-Bβ is involved in GDNF-induced neuronal differentiation of PC12-GFRα1-RET cells. (A) PC12-GFRα1-RET cells were co-transfected with pEGFP-N2 (encoding GFP) in the ratio of 1:10 with pcDNA3 as control, pcDNA3-myc-SH2-Bβ or R555E. The cells were visualized using fluorescence microscopy (magnification 20×) based on the expression of the GFP. (B) Quantification of neuronal differentiation of PC12-GFRα1-RET cells. Values are the mean ± s.d. of three independent experiments performed in triplicate culture wells. Three fields were examined from each well. *, significant difference between SH2-Bβ/GFP and pcDNA3/GFP control ($P<0.05$, factorial ANOVA); ##, significant difference between R555E/GFP and control ($P<0.01$, factorial ANOVA). (C) Decrease in GDNF-induced differentiation of PC12-GFRα1-RET cells by SH2-Bβ-siRNA. PC12-GFRα1-RET cells cultured on 24-well plates were transfected with pEGFP-N2 alone or together with missense RNA (20 μM) as control, or with SH2-Bβ-siRNA (20 μM). (D) Quantification of neuronal differentiation of PC12-GFRα1-RET cells. Values are the mean ± s.d. of three independent experiments. **, significant difference between RNAi group and missense RNA group ($P<0.01$, factorial ANOVA). (E) Effect of overexpression or knock-down of SH2-Bβ on GDNF-induced activation of ERK and Akt in PC12-GFRα1-RET cells. PC12-GFRα1-RET cells were transfected with plasmids pcDNA3 (control), pcDNA3-myc-SH2-Bβ, pcDNA3-myc-R555E, missense RNA or SH2-Bβ-siRNA. Cells were stimulated with or without 100 ng/ml GDNF for 10 minutes and then lysed. Equal amounts of total protein of cell lysates were immunoblotted with anti-SH2-Bβ, anti-pERK, anti-ERK, anti-pAkt or anti-Akt antibody.

GDNF-induced activation of ERK and Akt is not affected by overexpression or knock-down of SH2-Bβ in PC12-GFRα1-RET cells

The differentiation of PC12 cells has been shown to be mainly associated with the activation of both Ras-Raf-MEK-ERK and PI 3-K-Akt-kinase signaling (Kaplan and Miller, 2000; Vaudry et al., 2002). Thus, we examined whether GDNF-induced activation of ERK or Akt is affected by overexpression or knock-down of SH2-Bβ in PC12-GFRα1-RET cells. Overexpression of SH2-Bβ was achieved by transfection of pcDNA3-myc-SH2-Bβ into PC12-GFRα1-RET cells, whereas knock-down of endogenous SH2-Bβ expression was achieved by transfection of SH2-Bβ-siRNA into cells or by overexpression of dominant-negative SH2-Bβ to block the endogenous SH2-Bβ signaling. After stimulation or not with 100 ng/ml GDNF for 10 minutes, cells lysates were collected and subjected to western blot analysis with anti-SH2-Bβ, anti-pERK, anti-ERK, anti-pAkt or anti-Akt antibodies. As shown in Fig. 6E, the levels of both pERK and pAkt induced by GDNF were not markedly changed in cells that overexpressed SH2-Bβ or R555E or experienced a knock-down of SH2-Bβ, which indicates that SH2-Bβ might not be involved in the activation of the Ras-Raf-MEK-ERK or the PI 3-K-Akt kinase cascades in PC12-GFRα1-RET cells. These results suggest that SH2-Bβ probably initiates a new pathway that is required for RET-induced neurite outgrowth stimulated by GNDF.

SH2-Bβ promotes GDNF-induced neurite outgrowth in cultured mesencephalic neurons

Since in ventral mesencephalic brain RET is expressed in abundance (Trupp et al., 1997), we separated and cultured such neurons from middle brain of embryonic day 14 (E14) rats. Seven days later, cultured neurons were co-transfected with plasmid pEGFP-N2 and wild-type pcDNA3-SH2-Bβ, pcDNA3-R555E (as dominat-negative mutant of SH2-Bβ) or pcDNA3 (as a mock transfection) at a ratio of 1:10. The length of the longest neurite of neurons in each group was assayed with Metamorph Image Processing Software. As shown in Fig. 7, after GDNF stimulation, neurites of neurons transfected together with SH2-Bβ/GFP were longer than those of neurons transfected together with pcDNA3/GFP (mock), whereas the neurites of neurons co-transfected with R555E/GFP were shorter than those of the controls. These results suggested that SH2-Bβ promotes GDNF-induced neurite outgrowth of cultured mesencephalic neurons.

Discussion

Several findings make the biology of GDNF unexpectedly complicated (Pozas and Ibanez, 2005), including the recently identified RET-independent new GFLs receptor NCAM (Paratcha et al., 2003), as well as crosstalk between GDNF-GFRα1-MET (Popsueva et al., 2003) and NGF-TrkA-RET (Tsui-Pierchala et al., 2002), even as a potential additional unknown transmembrane effectors for GDNF promoted differentiation of cortical GABAergic neurons. Albeit all of above mentioned, the physiological significance of functions or roles mediated through GDNF-GFRα1-RET pathway were reinforced by the fact that RET-independent GFRα1 is dispensable for organogenesis and nerve regeneration in vivo, which indicates that trans-signaling and GFRα-dependent NCAM signaling play physiologically a minor role (Enomoto et al., 2004). Thus, given the importance of the RET-mediated signaling in the nervous system, not only for the survival and differentiation of neurons but for those human cancers where excessive activation of RET has been observed, we wished to identify new RET-interacting proteins that might represent important substrates or regulators for its downstream signaling to facilitate a better understanding of the role of RET in neuronal survival and differentiation. Using the intracellular domain of RET as bait, we employed a yeast two-hybrid screen and identified the adapter protein SH2-Bβ as a RET-binding partner candidate.

It is well-known that phosphotyrosine sites formed by the actions of receptor tyrosine kinases can bind downstream effectors with phosphotyrosine recognition domains that mainly include SH2 or phosphotyrosine-binding (PTB) domain (Pawson and Nash, 2003). In our yeast two-hybrid screen, a fragment of the SH2-Bβ including the entire SH2 domain was identified. Our further investigations with the approach of deletion analysis and site-directed mutation confirmed that the SH2 domain of SH2-Bβ alone was sufficient and required for mediating the interaction of SH2-Bβ with RET. These results are consistent with the reports that SH2-Bβ binds to several receptors through its SH2 domain (Rui et al., 1999; Kong et al., 2003). We also found that EGF receptor can not bind to SH2-Bβ, indicating SH2-Bβ binds selectively to receptor tyrosine kinases.

The intracellular domain of RET contains 14 tyrosine residues in total. It is assumed that there are five tyrosine phosphorylation sites among them, mainly serving as potential docking sites for signaling molecules (Kodama et al., 2005). In detail, phosphorylated Tyr1062 represents a binding site for a variety of adaptor proteins including Shc, Frs2, Dok1/4/5/6,

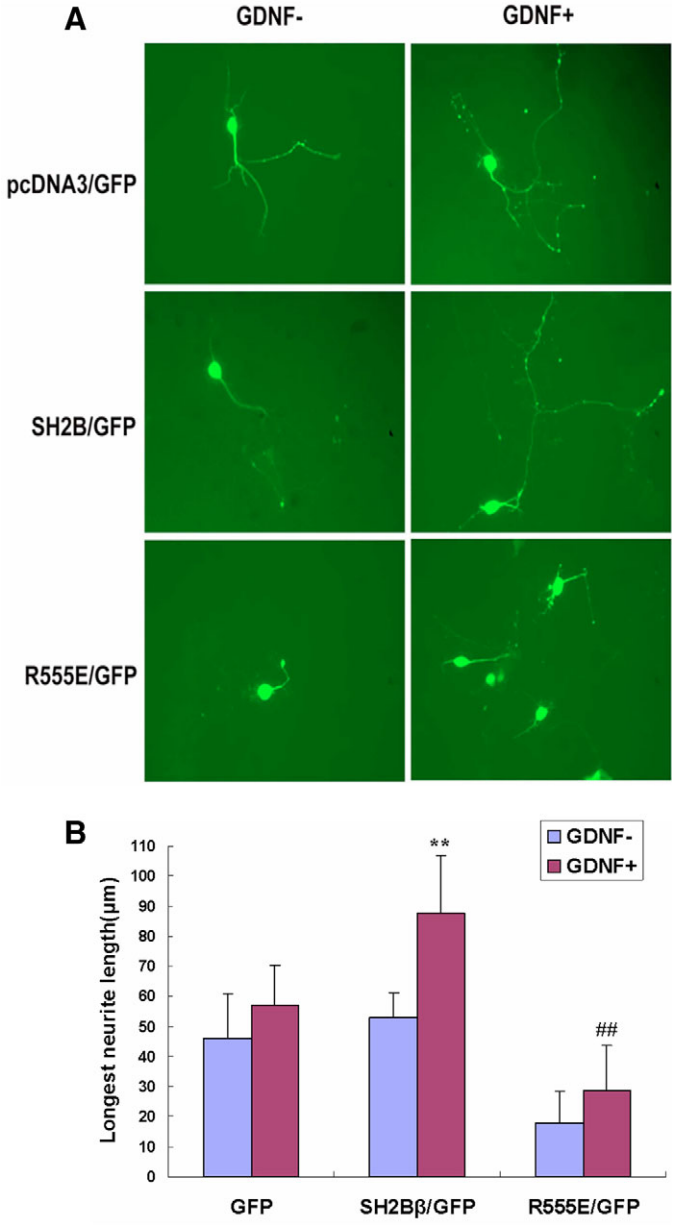


Fig. 7. SH2-Bβ promotes neurite outgrowth in cultured mesencephalic neurons. Cultured mesencephalic neurons were co-transfected with pEGFP-N2 in the ratio of 1:10 together with pcDNA3 (control), pcDNA3-myc-SH2-Bβ or R555E. Images were taken under fluorescence microscopy (magnification 20×) to identify GFP-positve neurons. (B) Quantification of neurite outgrowth in cultured mesencephalic neurons. Metamorph image-analysis software was used to quantify the length of the longest neurite. Data are presented as the mean ± s.d. of three independent experiments performed in triplicate experiments. **, significant difference between SH2-Bβ/GFP and pcDNA3/GFP control (P<0.01, factorial ANOVA); ##, significant difference between R555E/GFP and control (P<0.01, factorial ANOVA).

IRS-1/2 and Enigma. This tyrosine is important not only for the transforming ability of mutant RET, but also for a crucial role in organogenesis, such as the development of the enteric nervous system and the kidney. In addition, it was found that Tyr905 binds to Grb7/10, Tyr981 to Src, Tyr1015 to PLCγ, and Tyr1096 to Grb2. After screening of a phosphopeptide library, Songyang et al. have reported that the optimal consensus sequence for the SH2 domain is probably pYEEI (Songyang et al., 1993). Since none of the 14 tyrosine residues fits this optimal SH2 domain-binding sequence, we decided to analyze the five major tyrosine phosphorylation sites. Thus, we generated Tyr to Phe point mutants of residues 905, 981, 1015, 1062 and 1096 in RET. In addition, we used the catalytically compromised Lys758Met kinase-dead mutant, to reconfirm the phosphorylation-dependent binding of RET to SH2-Bβ. Finally, our results demonstrated that Tyr981 is crucial for the interaction of RET with SH2-Bβ.

The PC12 cell line derived from rat pheochromocytoma, a tumor arising from chromaffin cells of the adrenal medulla, is a widely used and well-known model system to study neuronal signaling (Vaudry et al., 2002). Taking the advantage of an established PC12-GFRα1-RET cell line that does not only overexpress GFRα1 and RET receptor but also represent neuronal differentiation after treatment with GDNF (Wang et al., 2004a), we evaluated the binding and function of overexpressed SH2-Bβ and its dominant-mutant R555E (Rui et al., 1999). Not only did we confirm their interaction in vivo by immunoprecipitation results, but we also found that wild-type SH2-Bβ promotes RET-mediated neurite outgrowth of PC12 cells. The interaction and its function required the SH2 domain of SH2-Bβ, because its dominant mutant R555E did not convey a similar effect. The conclusion that SH2-Bβ mediates neuritogenesis via RET signaling was further strengthened by RNAi experiments. Furthermore, we found that endogenous SH2-Bβ co-immunoprecipitates with RET both in PC12-GFRα1-RET cells and in rat tissues lysates, such as mesencephalon and spinal cord. Morphological evidence was given because SH2-Bβ and RET colocalized in neurons of both rat spinal cord and mesencephalic neurons, indicating that the physical interaction between the two proteins occurs in these neurons. Our data thus demonstrate that SH2-Bβ can interact with RET both in vitro and in vivo, strongly suggesting that SH2-Bβ has important biological functions to regulate RET-dependent processes in vivo.

It is well documented that GDNF is a potent trophic factor, that it has a strong effect on neuronal differentiation and that it promotes survival and sprouting of ventral mesencephalic dopaminergic neurons in primary cultures (Lin et al., 1993; Akerud et al., 2002). Therefore, we assessed the functional activity of SH2-Bβ with respect to RET in primary cultured mesencephalic neurons that express endogenous RET and GFRα1. The wild-type SH2-Bβ was found to enhance GDNF-RET-mediated neurite outgrowth in mesencephalic neurons, whereas R555E had the opposite effect. These results demonstrate that the interaction between SH2-Bβ and RET receptor is required for GDNF-induced neurite outgrowth in mesencephalic neurons. In addition to promotion of neurite growth and sprouting, RET was reported to activate several intracellular signaling cascades that regulate cell survival, proliferation, migration, chemotaxis, branching morphogenesis and synaptic plasticity (Airaksinen and Saarma, 2002; Sariola and Saarma, 2003). But can the interaction of SH2-Bβ with RET be also involved in other RET-induced biological effects? Further functional analysis should be carried out. Since we did not observe the binding of SH2-Bβ with RET in kidney, and expression of SH2-Bβ has

not been detected in kidney (Yousaf et al., 2001), it is unlikely that SH2-Bβ have a role in RET-mediated kidnogenesis.

SH2-Bβ is a positive regulator of nerve growth factor-mediated activation of the Akt/Forkhead pathway (Wang et al., 2004b). It has also been reported that, in response to leptin, SH2-Bβ promotes the activation of the PI 3-K pathway by insulin-receptor substrate 1 and 2 (Duan et al., 2004). We can therefore assume that either Ras-ERK or PI 3-K-Akt is important for mediating the signaling by interaction with RET-SH2-Bβ. However, our results demonstrate that GDNF-induced activation of ERK and Akt are not markedly affected by overexpression or knock-down of SH2-Bβ in PC12-GFRα1-RET cells. Recently, a very interesting finding was that SH2-Bβ can shuttle constitutively between the nucleus and cytoplasm (Chen and Carter-Su, 2004). It was shown that SH2-Bβ needs continuous access to the cytoplasm and/or plasma membrane to participate in NGF-induced neurite outgrowth. These results by Chen and Carter-Su suggest that the stimulatory effect of SH2-Bβ on NGF-induced neurite outgrowth of PC12 cells is either downstream of MAPK or via some other pathway yet to be identified. It has been well studied that Ras-ERK and PI 3-K-Akt pathways are activated mainly through Tyr1062 and Tyr1096 of RET (Kodama et al., 2005), whereas little is known about the downstream effects of Tyr981, except its binding partner Src. Our results demonstrated that Tyr981 is crucial for the interaction between RET and SH2-Bβ. Taken together, SH2-Bβ probably initiates a new signaling pathway by binding with activated RET through a Tyr981 site.

SH2-Bβ was originally identified as a JAK2-interacting protein that can stimulate kinase activity of JAK2, thereby increasing tyrosine phosphorylation of STAT3 and STAT5B, and promote the translocation of phosphorylated STATs from the cytoplasm to the nucleus (van Weering and Bos, 1997; Rui et al., 1997; Kong et al., 2003; Rui et al., 1999; Rui and Carter-Su, 1999). STAT3 has recently been reported to be constitutively activated by autophosphorylation of the RET Met918Thr mutation (Yuan et al., 2004). We have examined the translocation of STAT5 in HEK293T cells and in primary cultured mesencephalic neurons by immunocytochemistry. However, we failed to observe SH2-Bβ promoting the translocation of STAT5 into nucleus upon RET activation (data not shown). The mechanism underlying the signaling through RET-SH2-Bβ interaction remains to be determined.

In summary, using a yeast two-hybrid strategy, we have identified SH2-Bβ as a new RET-binding protein. We have provided substantial evidence that SH2-Bβ forms a complex with RET not only in PC12-GFRα1-RET cells but also in rat tissue homogenates. Morphological data demonstrated that RET and SH2-Bβ are colocalized in some neurons. Biochemical analysis revealed that TYR981 in RET is crucial for the interaction with SH2-Bβ. Moreover, functional analysis demonstrated that SH2-Bβ promotes GDNF-induced neurite outgrowth in both PC12-GFRα1-RET cells and cultured mesencephalic neurons. Our results thus suggest that SH2-Bβ is a new signaling molecule involved in GDNF-induced neurite outgrowth.

Materials and Methods

Plasmid constructs

Full-length RET constructs were generous gifts from Carlos F. Ibanez. The intracellular domain of the human RET was generated by PCR and cloned in-frame into the LexA fusion vector pGilda (Clontech) as bait pGilda-RET^{IC}. Full-length SH2-Bβ was cloned by RT-PCR from a rat-brain cDNA library. To construct the myc-epitope-tagged SH2-Bβ pcDNA3-myc-SH2-Bβ, we performed PCR and subcloned full-length SH2-Bβ into pcDNA3 vector from Invitrogen. The mutants for SH2-Bβ and RET were generated by applying the QuickChange site-directed mutagenesis method from Stratagene. All of the constructs were fully sequenced before used for transformation or transfection.

Yeast two-hybrid screen and assay

Yeast two-hybrid screening was performed according to the manufacturer's protocols (Clontech). The yeast strain EGY48 (MATa, his3, trp1, ura3, LEU2::plexAop6-LEU2) of *Saccharomyces cerevisiae*, the LexA yeast two-hybrid system and MatchMaker human brain cDNA library were from Clontech. The pGilda-RET^{IC} construct was co-transformed with human brain cDNA library fused to pB42AD into the EGY48 strain with PEG/LiAc solution. The co-transformants were plated on SD-Gal Ura-His-Trp-Leu drop-out galactose induction medium for 3-4 days at 30°C to induce expression of reporter proteins fused with the activation-domain. Filter-lift color assays and liquid-culture assays with o-nitrophenyl-D-galactoside (ONPG) was performed following the Clontech Yeast Protocols Handbook for β-galactosidase activity analysis as described before (Zhang et al., 2004). During the analysis, pGilda-53 co-transformed with pB42AD-T was used as a positive control, whereas pGilda co-transformed with pB42AD was used as a negative control. Potential positive clones were selected, and prey-plasmids containing library cDNA inserts were isolated and shuttled into *Escherichia coli* KC8 cells. Positive colonies were further confirmed by testing pB42AD-cDNA against LexA-lamin to eliminate false positives, and then sequenced.

Cell culture and transfection

Wild-type PC12 cells and our previously established stably transfected PC12-GFRα1-RET cells (Wang et al., 2004a) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL) supplemented with 5% fetal bovine serum (FBS) and 5% heat-inactivated horse serum (HyClone). Hygromycin and G418 were needed to maintain the extraneous protein expression in PC12-GFRα1-RET. Human embryonic kidney (HEK) 293T cells were grown in DMEM supplemented with 10% FBS. All cells were cultured at 37°C, 5% CO_2. Before transfection, PC12 cells were harvested at the logarithmic growth stage (3-5×10^5 cells/ml) and seeded at 2-6×10^4 cells/ml per well on a 24-well plate coated with poly-L-lysine, whereas HEK293T cells were seeded at 1-3×10^5 cells/ml per 10 cm dish. Lipofectamine reagent (Invitrogen) and calcium phosphate reagent (Promega) were used to transfect PC12 and HEK293T cells as advised in the manufacturer's instructions. pEGFP-N2 and indicated plasmids were co-transfected into PC12 cells. RET mutants, GFRα1 and SH2-Bβ were co-transfected into HEK293T cells. Fourty-eight hours after transfection, all the cells were starved for 5 hours and subsequently stimulated with 100 ng/ml GDNF for 10 minutes at 37°C. Cells were lysed for immunoprecipitation assays, SDS-PAGE and western blotting. Antibodies including anti-RET, anti-myc, anti-SH2-Bβ, anti-ERK and anti-Akt were all from Santa Cruz. Differentiation assay of PC12 cells was performed as described before (Wang et al., 2004a), and the cells possessing one or more neurites of a length more than twice the diameter of the cell body were scored as positive.

Inhibition of SH2-Bβ expression by RNA interference

The target region of siRNA was 1724-1742 nucleotides downstream of the start codon (at position –344 in rat SH2-Bβ cDNA), containing a G/C content of 47.62%. The 21-bp nucleotide sequence was 5′-GCACCUGCGUUUGUCACUAdTdT-3′ and showed no match with other sequences. The RNAs were chemically synthesized, deprotected and gel-purified by GENECHEM. To demonstrate the silencing effect of endogenous SH2-Bβ expression by siRNA, cells in a 24-well culture plate were co-transfected with vector pEGFP-N2 and with siRNA duplex using Transmessenger (Qiagen). For testing the inhibition of SH2-Bβ expression, cells were lysed 48 hours after transfection and lysates were subjected to immunoblotting analysis. To test the inhibition of differentiation, cells stimulated with GDNF were scored for differentiation under the fluorescence microscope as described before (Wang et al., 2004a).

Preparation of tissue homogenates

Different tissues such as mesencephalon, spinal cord, kidney and muscle were dissected and separated from adult male Sprague Dawley rats. Each tissue was homogenized in a pestle tissue grinder at slow speed in solubilization buffer (25 mM HEPES-NaOH, pH 7.4, 125 mM K-acetate, 5 mM $MgCl_2$, 0.32 M sucrose, and 1% Triton X-100). Proteins solubilized from each rat tissue homogenate were quantified according to the Bradford method. Then, equal amounts of protein were incubated with corresponding antibodies (Santa Cruz) for 3 hours at 4°C under mild agitation; bound proteins were analyzed by immunoblotting with corresponding antibodies as described.

Immunoprecipitation and immunoblotting

Harvested cells were washed twice with cold phosphate-buffered saline, solubilized with ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5% Na-deoxycholate, 0.02% Na-azide, 1 mM NaF, 1 mM Na-vanadate, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 1 mM dithiothreitol, 0.1% SDS,

2 μg/ml pepstatin, 2 μg/ml leupeptin and 2 μg/ml aprotinin) (Sigma) and incubated on ice for 20 minutes. Lysates were clarified by centrifugation at 11,200 ***g*** for 10 minutes at 4°C. Protein concentration of the supernatants was determined by the Bradford method. For immunoblotting, 10 μl of supernatant was subjected to SDS-PAGE, immunoblotted and visualized with enhanced chemiluminescence (ECL, Pierce). For immunoprecipitation, 300-500 μl of supernatant was incubated with 5 μl corresponding antibody (Santa Cruz) for 3 hours at 4°C. Protein G-agarose beads (Roche) were then added for 3 hours; immunoprecipitated samples were then washed three times with lysis buffer, boiled 3-5 minutes in sample-loading buffer and then subjected to western blotting analysis.

Primary culture and neurite outgrowth assay of mesencephalic neurons

Two-thirds of the ventral mesencephalon were dissected from E14 Sprague Dawley rat embryos. Tissue sections (1 mm^3) were pooled in ice-cold Hanks-buffed salt solution. Tissue sections were dissociated by a consecutive treatment with 0.25% trypsin, followed by careful trituration at 37°C for 30 minutes. Subsequent to centrifugation, the supernatant was discarded and the pellet was dispersed in a 1:1 mixture of DMEM:F12 supplemented with 10% FBS. The cell suspension was seeded in the centre of 12-well plates coated with poly-L-lysine at a density of 1×10^5 cells/ml. The cultures were kept in an atmosphere of 5% CO_2, 95% air at 37°C for 7 days. Medium was changed every 3 days. Cultured neurons were transfected using the calcium phosphate transfection system according to manufacturer's instructions (Promega). The ratio of target gene and GFP was 10:1. Transfected cells were washed twice with PBS (pH 7.4) and then supplied with new primary culture medium supplemented with 10 ng/ml GDNF or without GDNF (control). Neuritogenic effects of GDNF on GFP-positive neurons were observed under a fluorescent microscope (Olympus, excitation 454 nm) 3 days later and the length of the longest neurite of GFP-positive neurons was quantified by MetaMorph image analysis software. The data were analyzed by factorial ANOVA and each value was the mean ± s.d. sampled from three independent experiments.

Immunocytochemistry

After 7 days culture, cultured primary mesencephalic neurons that had been cultured on cover-glasses were fixed in 4% paraformaldehyde with 0.2% saturated picric acid in 0.1 M phosphate buffer (PB) for 15 minutes at room temperature. After one wash with 0.01 M PBS, cells were incubated with primary antibodies (goat anti-SH2Bβ 1:200, SantaCruz; mouse anti-MAP-2 1:400; mouse anti-Tau-1 1:200, both Chemicon) in PBS containing 3% BSA and 0.3% Triton X-100 overnight at 4°C. Following three washes in 0.01 M PBS, cells were incubated with FITC-conjugated donkey anti-goat (1:100) and rhodamine-conjugated donkey anti-mouse antibody (1:100) both Jackson ImmunoResearch Laboratory for 30 minutes at room temperature. Subsequently, cover-glasses were washed in 0.01 M PBS, placed on slides and then examined under a Leica SP2 confocal microscope.

Immuohistochemistry

Five adult male Sprague Dawley rats, weighing 200-250 g, were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 0.1 M PB pH 7.4, followed by perfusion with 4% paraformaldehyde with 0.2% saturated picric acid in 0.1 M PB. The brains and spinal cords were removed, kept for 1.5 hours in the same fixative at 4°C, and then cryoprotected overnight at 4°C in 0.01 M PBS pH 7.4, containing 20% sucrose. The tissues were then sliced at 14 μm and the sections were mounted on glass slide on a Leica 1900 cryostat. After that, sections were washed in PBS, incubated with the primary antibodies (goat anti-SH2Bβ 1:200; rabbit anti-RET 1:100, both SantaCruz) in PBS containing 3% BSA and 0.3% Triton X-100 at 4°C for 48 hours. After three washes in PBS, sections were incubated with FITC-conjugated donkey anti-goat (1:100) and rhodamine-conjugated donkey anti-rabbit (1:100; Jackson ImmunoResearch Laboratory) antibodies. Sections were washed, placed on coverslips and examined with a Leica SP2 confocal microscope.

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