ShcD interacts with TrkB via its PTB and SH2 domains and regulates BDNF-induced MAPK activation

Yuangang You1, Weiqi Li1, Yanhua Gong2, Bin Yin1, Boqin Qiang1, Jiangang Yuan1,* & Xiaozhong Peng1,*

1National Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, 2Department of Biochemistry, Medical College of Chinese People’s Armed Police Force, Tianjin, China

Neurotrophins regulate many aspects of neuronal function through activation of the high affinity Trk receptors. Shc family proteins are implicated in the coupling of RTK to the Ras/mitogen-activated protein kinase signaling cascade. Here we report that the fourth Shc family member, ShcD, associates with TrkB receptor and regulates BDNF-induced MAPK activation. Yeast two-hybrid assay and Co-IP experiments demonstrate ShcD interacts with TrkB in a kinase-activity-dependent manner. Confocal analysis shows ShcD colocalizes well with TrkB in transfected 293T cells. Subsequent mapping experiments and mutational analysis indicate that both PTB and SH2 domains are capable of binding to TrkB and PTB domain binds to TrkB NPQY motif. Furthermore, ShcD is involved in BDNF-induced MAPK activation. In summary, we demonstrate that ShcD is a substrate of TrkB and mediates TrkB downstream signaling pathway. [BMB reports 2010; 43(7): 485-490]

INTRODUCTION

The neurotrophins, a family of polypeptide growth factors, exert their diverse functions in the developing and the mature nervous system, including neuronal survival and death. These molecules include nerve growth factor (NGF), brain-derived growth factor (BDNF), and the NT3, 4/5, 6, and 7 neurotrophins (1). Two different classes of proteins have been identified as receptors for neurotrophin: the high affinity tyrosine kinase Trk receptors including TrkA, TrkB, and TrkC and the low affinity neurotrophin receptor p75 (p75NTR). BDNF is one of the most well characterized neurotrophins. In addition to promotion of cell survival and axonal outgrowth, BDNF and its receptor TrkB regulates the dendritic morphology and synapse formation.

RESULTS

ShcD binds directly to the TrkB receptor in a phosphorylation-dependent manner

The identification of substrates for neurotrophin receptors is fundamental to understanding the function of neurotrophins. Previously we have reported that the PTB-containing adaptor protein Dok5 is a substrate of TrkB/C, but not TrkA (7). The newly revealed ShcD is highly expressed in adult mouse brain, and spot peptide array suggests that ShcD PTB domain is capa-
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Fig. 1. ShcD interacts with TrkB in a kinase-activity-dependent manner. (A) ShcD interacts with the ICD (intracellular domain) of kinase-active TrkB in yeast. The full-length ShcD was cloned into pACT2 vector, then cotransformed with the intracellular domain of TrkB or kinase dead mutant-TrkB1 (K572A) in pAS2-1 into yeasts. Transformants were selected on synthetic media lacking tryptophan and leucine. For negative control, pAS2-1 empty vector and ShcD were also included. (B) 293T cells coexpressing V5-TrkB or V5-TrkB1 (kinase dead mutant) and Flag-ShcD were stimulated with BDNF (100 ng/ml) for 5 min. Cell lysates were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-Flag and anti-V5. Whole cell lysates were simultaneously immunoblotted with anti-V5, anti-Flag or anti-pTyr antibodies. (C) Colocalization of ShcD with TrkB. 293T cells were transiently transfected with pEGFP-ShcD and pmRFP-TrkB or pmRFP-TrkB1 (as a control). 48 h later, the cells were fixed and stained with DAPI, then observed under a fluorescence microscope.

To map the TrkB binding region on ShcD, truncated forms of ShcD containing the CH2, PTB, CH1 or SH2 domain were constructed and yeast two-hybrid assay was performed. In the β-galactosidase activity assays, both the PTB and SH2 domains, but neither the CH2 nor CH1 domain, interacted with TrkB (Fig. 2B). The interaction between ShcD and TrkC was also performed in yeast two-hybrid assay. The results were similar to that between ShcD and TrkB, showing that both PTB and SH2 domains of ShcD were involved in ShcD-TrkC interaction (9). These results were unexpected since the interaction between ShcA/B/C and Trk receptor is mediated by PTB domain but not SH2 domain (10, 11).

To further define the binding ability of ShcD PTB and SH2 domains to TrkB receptor, we employed GST pull-down assay. The results are consistent with those obtained in yeast two-hybrid assay, showing that ShcD PTB and SH2 domains can bind to the activated TrkB receptors (Fig. 2C). Collectively, these data indicate that TrkB binds to both the PTB and SH2 domains of ShcD.

The PTB domain of ShcD associates with TrkB at the juxtamembrane NPQY motif
It is well documented that the classical NPQY sequence of Trk
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Fig. 2. Both PTB and SH2 domains of ShcD are sufficient to bind TrkB receptor. (A) Schematic diagram shows the four domains of ShcD. (B) ShcD PTB and SH2 domains bind to TrkB in yeast two-hybrid assay. Truncated forms of ShcD containing CH2 domain, PTB domain, CH1 domain and SH2 domain were individually cotransformed with TrkB ICD (intracellular domain) into yeast cells. Then the β-galactosidase activity assays were performed. (C) ShcD PTB and SH2 domains bind to active TrkB receptor in vitro. Lysates from BDNF-treated 293T cells transiently transfected with wild-type TrkB or kinase inactive mutant TrkBM1 were incubated with GST, GST-ShcD-PTB and GST-ShcD-SH2. Then the agarose-bound proteins were subjected to anti-V5 antibody. Whole cell lysates were examined by anti-V5 or anti-pTyr antibody to detect the expression and phosphorylation level of TrkB receptor (second and third panel).

Fig. 3. ShcD PTB domain binds to NPQY motif of TrkB. (A) Schematic diagram shows the locations of one key lysine residue and five phosphotyrosine residues of the TrkB ICD. (B) Association of ShcD PTB domain with TrkB mutants in yeast two-hybrid assay. ShcD and wild type TrkB or different single point TrkB mutants (BM1 (K572A), BM2 (Y516F), BM3 (Y702A), BM4 (Y706D), BM5 (Y707E) and BM6 (Y817F)) were cotransformed into yeast SFY526, and β-galactosidase activity was measured to detect their association. (C) ShcD PTB domain binds specifically to TrkB Y516 in vitro. Cell lysates prepared from 293T cells expressing TrkB variants as indicated were assayed for binding to GST-ShcD-PTB.

receptors serves as specific binding site for PTB domain of downstream substrates. So we propose that the juxtamembrane Y516 of TrkB located in the conserved NPQY motif, is also the binding site for the PTB domain of ShcD protein. To verify this possibility, wild type TrkB or a series of single point TrkB mutants (shown in Fig. 3A) were assayed in an yeast two-hybrid assay. ShcD and wild type TrkB or different single point TrkB mutants (BM1 (K572A), BM2 (Y516F), BM3 (Y702A), BM4 (Y706D), BM5 (Y707E) and BM6 (Y817F)) were cotransformed into yeast SFY526, and β-galactosidase activity was measured to detect their association. (C) ShcD PTB domain binds specifically to TrkB Y516 in vitro. Cell lysates prepared from 293T cells expressing TrkB variants as indicated were assayed for binding to GST-ShcD-PTB.
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**Fig. 4.** Participation of ShcD in BDNF mediated activation of MAPK in 293T cells. 293T cells cotransfected with 3.1 Flag empty vector or Flag-ShcD and V5-TrkB were stimulated with BDNF (100 ng/ml) for the indicated times. Cell extracts were analyzed by immunoblotting with anti-V5, anti-Flag, anti-Erk, or anti-phospho-Erk antibodies.

**ShcD mediates MAPK activation**

Next, we examined the effects of ShcD on MAPK signaling pathway. Western blot analysis in Fig. 4 showed a slight increased and prolonged activation of MAPK in cells transfected with ShcD compared to those transfected with control empty vector. These observations suggest that ShcD serves as a link between ligand-activated TrkB receptor and MAPK signaling pathway.

It was reported that ShcA is involved in MAPK activation through formation of Shc-Grb2 complex. Once activated by cell-surface receptors, ShcA becomes phosphorylated at three Tyr sites of the central CH1 region, resulting in recruitment of the Grb2-Sos complex and subsequent ras activation. Unlike ShcA, ShcD possesses six Tyr residues in its CH1 domain. We then further determined whether ShcD binds Grb2 when phosphorylated by TrkB (see supplemental material Fig. S2). The Y6F mutations within CH1 domain were introduced into the ShcD gene by site-directed mutagenesis and this mutant was designated as ShcD 6F (Fig. S2A). As indicated in Fig. S2B, ShcD could be phosphorylated by TrkB, and additional BDNF stimulation resulted in enhanced ShcD phosphorylation and increased binding to TrkB, further demonstrating that their interaction is dependent on receptor activation and autophosphorylation. Interestingly, the ShcD 6F mutant retained the ability to associate with TrkB although its tyrosine phosphorylation was almost undetectable even in the presence of BDNF. Moreover, ShcD-Grb2 interaction could be detected only in immunoprecipitates prepared from 293T cells stimulated with BDNF but not from nonstimulated lysates; in contrast, mutation of the six tyrosine residues in CH1 domain abolished ShcD binding to Grb2 upon BDNF stimulation. These data indicate that the six tyrosine residues contained within the ShcD CH1 region is required for ShcD phosphorylation by TrkB and subsequent binding to Grb2.

**DISCUSSION**

In this study, we described the identification and characterization of a substrate of TrkB, ShcD. Our experiments demonstrated direct interaction between ShcD and TrkB, both the PTB and SH2 domains of ShcD are involved in this interaction and the interaction is dependent on receptor tyrosine kinase activity. In contrast, the interaction between ShcA/B/C and Trk receptors is mediated solely through the PTB domain, suggesting differences in how these Shc adapters interact with Trk receptors. We also detected the interaction of ShcD with TrkA or TrkC in yeast two-hybrid assay and found that full-length ShcD bound comparably to the intracellular domains of these three Trk receptors, suggesting that ShcD is a general substrate of Trk family members. This is unlike Dok5, which is a substrate of TrkB/C, but not TrkA (7).

Mapping experiments indicate that ShcD PTB domain interacts with Tyr516 (within the conserved NPQY motif) of human TrkB in a phosphorylation-dependent fashion. However, we failed to identify the binding sites of ShcD SH2 domain on TrkB because single point mutation of tyrosine residue on TrkB ICD does not affect SH2 binding in yeast two-hybrid assay (data not shown), raising the possibility that SH2 binds at multiple phosphotyrosines on TrkB. This is not unexpected since several PTB or SH2 domain containing adaptors bind to multiple tyrosine residues on certain receptor tyrosine kinase. For example, Grb2 binds three tyrosine residues on TrkA through its SH2 domain, and Shc binds at five tyrosine sites on the PDGFRβ receptor (12-14).

Till now all of the four Shc proteins are capable of binding to the three Trk receptors. Upon neurotrophin stimulation, Shc...
adaptors are tyrosine phosphorylated and associate with Grb2, leading to MAPK activation. There are obvious differences in the temporal and spatial expression of Shc family members during neural development. They may function nonredundantly, contributing differently to the Trk signaling. Besides Shc proteins, there are many other Trk receptor-binding partners, such as Grb2, Numb, Dok5, etc. Due to their differential expression pattern and different binding affinity, apparently, Trk receptor-mediated interactions with downstream adaptor proteins is far more complex than currently appreciated (15). These adaptors certainly compete with each other for receptor binding in some cases. The detailed mechanism and biological significance of this interaction remains to be clarified. The mechanism by which Trk receptors signal after neurotrophin activation is incompletely understood.

In our study, the over-expression data showed that ShcD interacts with TrkB and enhances BDNF-induced MAPK activation. TrkB is expressed in both the developing and mature nervous system. Very few TrkB molecules are localized in neuronal cells and most of these TrkB molecules are localized in synapses. ShcD is mainly expressed in mature nervous system, however, its distribution in neuronal cells is not precisely determined. If ShcD is distributed on synapses of neuronal cells, then ShcD may play a direct role in neural system via BDNF-TrkB signaling pathway. Certainly, in this case, other adaptors may also act as downstream effectors of TrkB. If ShcD is not distributed on synapses, then ShcD will not function downstream of TrkB in mature nervous system. Further studies will be needed to elucidate the precise function of ShcD interaction with TrkB in nervous system.

In conclusion, we have identified ShcD as the substrate of TrkB receptor and found that it participates in MAPK activation induced by BDNF. This result provides new insights on ShcD function and that ShcD may play an important role in Trk signaling. Ongoing studies will be directed at determining the role of ShcD in primary cultured neurons and in mouse model.

MATERIALS AND METHODS

Reagents, antibodies and cells
Human recombinant BDNF was purchased from Peprotech. The anti-V5 polyclonal antibody was purchased from Novus. The anti-Flag monoclonal antibody was from Sigma. The anti-Erk, anti-phospho-p44/42Erk MAP kinase and anti-pTyr (PY99) antibody were purchased from Santa Cruz Biotechnology. The anti-Flag monoclonal antibody was from Sigma. The anti-V5 polyclonal antibody was purchased from Novus. 

Plasmid construction
All the constructs encoding wild type TrkB and TrkB mutants have been described previously (7). Full-length human ShcD cDNA was isolated from fetal brain cDNA library (Clontech) and cloned into pcDNA3.1-Flag vector. Then the full-length ShcD and truncated forms of ShcD (CH2 domain, PTB domain, CH1 domain and SH2 domain) were subcloned into pACT2 vector, which contains the GAL4 transcriptional activation domain (Clontech). The PTB (aa 180 to 351) and SH2 (aa 524 to 603) domains of ShcD were subcloned into pGEX-6P-1 (Amersham Biosciences) to generate glutathione S-transferase (GST) fusion proteins. pcDNA3.1Flag-ShcD 6F (Y374F, Y403F, Y413F, Y424F, Y465F) were generated by using Quick-Change Site-Directed Mutagenesis Kit (Stratagene). For image analysis, ShcD cDNA were subcloned into pcEGFP-N1 vector.

Yeast two-hybrid assay
The yeast two-hybrid assay was described previously (7).

GST pull-down assay
pGEX6P-1, pGEX6P-ShcD-PTB and pGEX6P-ShcD-SH2 were transformed into E. coli strain Rosetta (DE3). The fusion proteins were expressed and purified according to the manufacturer’s instructions. For GST pull-down assays, 293T cells were transiently transfected with pcDNA3.1V5-TrkB/BM1 plasmids. 24 h later, the cells were treated with 100 ng/ml BDNF for 10 min and lysed. The cell lysates were cleared and incubated with the same amount of GST, GST-ShcD-PTB, GST-ShcD-SH2 fusion proteins at 4°C for 2 h. The beads were washed and boiled in the presence of SDS-PAGE loading buffer. Protein samples were analyzed by SDS-PAGE and Western blot.

Co-immunoprecipitation
293T cells were transiently co-transfected with pcDNA3.1V5-TrkB and pcDNA3.1Flag-ShcD or pcDNA3.1V5-TrkB/BM1 and pcDNA3.1Flag-ShcD, then stimulated with 100 ng/ml BDNF for 10 min. The cells were lysed in 750 μl lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 120 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM NaVO₃, 25 mM NaF) containing protease inhibitors. Cell lysates were precipitated with anti-Flag antibody and analyzed by Western blotting with the indicated antibodies.

Fluorescence microscopy
Transfected 293T cells were fixed with 4% paraformaldehyde for 20 min at room temperature, then washed with phosphate-buffered saline (PBS) for 3 times and stained with DAPI. Coverslips were mounted and observed under Leica confocal microscope.

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