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Evidence that truncated TrkB isoform, TrkB-Shc can regulate phosphorylated TrkB protein levels

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ABSTRACT

Tropomyosin receptor kinase B (TrkB) is best known as the receptor for brain-derived neurotrophic factor (BDNF). In humans, three major isoforms of TrkB, the full-length receptor (TrkB-TK+) and two C-terminal truncated receptors (TrkB-TK− and TrkB-Shc) are expressed in various tissues. In comparison to TrkB-TK+ and TrkB-TK−, TrkB-Shc is less well characterized. In this study, we analyzed the biological function of the TrkB-Shc receptor in response to exogenous BDNF treatment. In experiments transiently overexpressing TrkB-Shc in CHOK1 cells, we found that TrkB-Shc protein levels were rapidly decreased when cells were exposed to exogenous BDNF. When we assessed the functional impact of TrkB-Shc on TrkB-TK+ activity, we found that phosphorylated TrkB-TK+ protein levels were significantly decreased in the presence of TrkB-Shc and moreso following BDNF exposure. Interestingly, while the reduction of phosphorylated TrkB-TK+ protein was more pronounced in the presence of TrkB-Shc following BDNF exposure, the stability of TrkB-Shc protein itself was increased. Our findings suggest that cells may increase TrkB-Shc protein levels in response to exogenous BDNF exposure to regulate TrkB-TK+ activity by increasing degradation of activated receptor complexes as a means to prevent overactivation or inappropriate temporal and spatial activation of BDNF/TrkB-TK+ signaling.

1. Introduction

Tropomyosin receptor kinase B (TrkB) is best known as the receptor for brain-derived neurotrophic factor (BDNF), although it has also been demonstrated to bind neurotrophin-4/5 and neurotrophin-3 [1,2]. TrkB is a member of the Trk receptor family of type III receptor tyrosine kinases that is linked to various cell signaling cascades including Akt, ERK1/2, and PLCγ. The mammalian full-length TrkB receptor (TrkB-TK+) was discovered more than two decades ago and since then at least 36 possible alternative transcript variants and protein isoforms have been identified [3]. In humans, three major isoforms of TrkB, the full-length and two C-terminal truncated receptors are expressed.

The full-length TrkB receptor, TrkB-TK+, consists of an N-terminal signal sequence followed by numerous domains including a cysteine- and leucine-rich sequence followed by a second cysteine-rich domain, 2 immunoglobulin (Ig)-like domains that also include the BDNF-binding region, a transmembrane domain, an Shc-binding motif, a catalytic tyrosine kinase domain near the C-terminus that is necessary for activating second messenger signaling [1,4], and a C-terminal PLCγ-docking site. TrkB-TK+ is the principal mediator of the neurotrophic effects of BDNF. Upon ligand binding, monomeric TrkB-TK+ homodimerizes and undergoes trans-phosphorylation at key tyrosine residues in the C-terminal domain that couple it to downstream signaling pathways.

The two truncated TrkB receptor isoforms include TrkB-TK− and TrkB-Shc. Both truncated isoforms are generated from alternatively spliced transcripts and are truncated at the C-terminus, thus lacking the tyrosine kinase domain [5,6]. However, the TrkB-TK− and TrkB-Shc receptors differ in that each contain unique amino acid sequences at their C-terminus. The TrkB-Shc isoform includes the Shc binding domain that is absent in TrkB-TK− [6].

Both truncated TrkB receptors show differential tissue and cell-type expression. TrkB-TK− is expressed in multiple tissues including brain, heart, lung, skeletal muscle, kidney, and pancreas [6]. In the brain, TrkB-TK− is expressed by both neurons and glia [7–9]. In contrast, TrkB-Shc is expressed in the spinal cord and multiple brain regions including the cerebellum, cortex, pons, hind brain, and diencephalon [6]. Interestingly, TrkB-Shc mRNA is detected in neurons but not in astrocytes [6].

Abbreviations: BDNF, brain-derived neurotrophic factor; CHOK1, Chinese hamster ovary K1; CHX, cycloheximide; TrkB, tropomyosin receptor kinase B; TrkB-TK+, full-length TrkB.

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In comparison to TrkB-TK−, the function of TrkB-Shc is not fully elucidated. *In vitro*, TrkB-TK− has been demonstrated to inhibit neurotrophin signaling either by sequestering or trapping neurotrophins (when expressed in glia) and thereby preventing binding and signal transduction via TrkB-TK+ homodimers [10,11] and/or act as a dominant-negative receptor by forming inactive heterodimers with TrkB-TK+ and hence, preventing neurotrophin signaling [12,13]. To date, similar co-transfection studies *in vitro* using TrkB-TK+ and TrkB-Shc have demonstrated that TrkB-Shc cannot be tyrosine phosphorylated [6] also suggesting a dominant negative function. However, Haapasalo et al. [14] demonstrated that co-expression of TrkB-Shc leads to increased cell surface expression levels of the TrkB-TK+ receptor in N2a neuronal cells and primary hippocampal neurons but did not investigate the phosphorylation state of TrkB.

In this study, we further characterize the biological function of the TrkB-Shc receptor in response to brief BDNF exposure. We report that TrkB-Shc protein levels are regulated by exogenous BDNF and that binding results in the regulation of phosphorylated TrkB-TK+ protein levels.

2. Materials and methods

2.1. Materials

Chemicals and reagents used are listed below with the supplier. From Life Technologies: Dulbecco's modified Eagle's medium/Ham's F-12 medium (DF12 1:1 mixture); fetal bovine serum; glutamax, Lipofectamine 2000; myc antibody. From Sigma: bovine-serum albumin fraction V; BDNF (Sigma; recombinant protein Cat # B3795); cycloheximide; protease inhibitor cocktail; β-actin antibody. Other reagents were bicinchoninic acid assay and phosphatase inhibitor cocktail (Pierce); phospho-TrkB antibody (Epitomics); mouse and rabbit peroxidase-conjugated affinity purified secondary antibodies (Dako); Immobilon-P enhanced chemiluminescence reagent (Millipore).

2.2. Cell culture, transfections and treatments

Chinese Hamster Ovary K1 (CHOK1) cells were obtained from the American Type Culture Collection and grown at 37 °C in a 5% CO_2 atmosphere. CHOK1 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DF12 1:1 mixture) containing 10% (v/v) FBS supplemented with 2 mM glutamax (Life Technologies).

2.3. Western blotting

CHOK1 cells were plated at a density of 5×10^5 in 12 well plates and transfected with overexpression plasmids as indicated in the figures and figure legends [pcDNA3.1 (empty vector), pcDNA3.1-TrkB-Shc-myc and/or pcDNA3.1-TrkB-TK+ (500 ng/well)] for 24 h using Lipofectamine 2000 (2 μl/well). Cells were treated with 15 ng of BDNF (Abcam; recombinant protein Cat # AB9794) post-transfection in 0.1% BSA-DF12 medium for 15 min and then harvested or switched to 0.1% BSA-DF12 containing 100 μg/ml cycloheximde (without BDNF) and incubated for an additional 3 h before harvest. Cells were harvested for total protein using RIPA buffer supplemented with protease inhibitors (2 mM AEBSF, 0.015 mM aprotinin, 0.038 mM leupeptin, 0.030 mM pepstatin A, 0.028 mM E-64, 0.08 mM bestatin) (Sigma) and phosphatase inhibitors (Pierce). Protein concentrations were determined by the bicinchoninic acid method. Fifteen or twenty micrograms of each sample were mixed with 5× SDS loading buffer (containing β-mercaptoethanol), boiled at 95 °C for 5 min, and separated on 10% SDS–PAGE gels. Proteins were transferred onto nitrocellulose membranes and blocked using 5% (w/v) non-fat milk, 0.1% (v/v) Tween-20 in TBS (TBST) at room temperature for 1 h. Membranes were incubated with primary antibodies overnight at 4 °C: phospho-TrkB (1:5000) (Epitomics); myc (1:5000) (Invitrogen) and β-actin (1:10,000) (Sigma). Membranes were washed 3 × 10 min with TBST and incubated with mouse or rabbit peroxidase-conjugated affinity purified secondary antibody for 1 h (Dako). After further washing, bound antibodies were detected with Immobilon-P enhanced chemiluminescence reagent (Millipore) and visualized by autoradiography. Immunoreactive species were quantitated by densitometry using Image J (version 1.37v) (National Institutes of Health USA). The brightness/contrast of images have been adjusted using Adobe Photoshop CS (version 8).

2.4. Data presentation and statistics

All results shown are representative of 2–4 separate experiments as detailed in the figure legends. Data are presented as mean + standard error of the mean (SEM). Where appropriate, statistical differences were determined by student *t*-tests using STATISTICA 7 (StatSoft Inc., 2000, STATISTICA for Windows). A p-value $\leqslant 0.05$ (two-tailed) was considered statistically significant.

3. Results

3.1. TrkB-Shc protein expression is regulated by BDNF

Previously, cell surface expression of TrkB-TK+ was shown to be reduced by concentrations of BDNF that could stimulate TrkB-TK+ phosphorylation [14]. Since TrkB-Shc is also capable of binding BDNF, we determined whether its protein expression could be modulated by BDNF exposure. Using CHOK1 cells transiently transfected with myc-tagged TrkB-Shc, we found that expression of TrkB-Shc protein levels was significantly reduced when cells were briefly incubated with 15 ng of BDNF ($t = -2.95$, df = 4, $p = 0.04$) (Fig. 1A, B). Treatment of cells with BDNF did not affect β-actin protein levels (Fig. 1A).

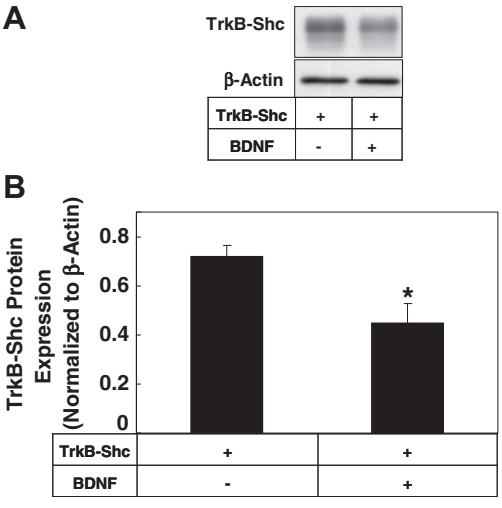

Fig. 1. Effect of BDNF on TrkB-Shc protein levels. CHOK1 cells were transfected with TrkB-Shc-myc for 24 h and treated with 15 ng BDNF for 15 min before harvest. Proteins were separated by SDS–PAGE and immunoprobed. (A) Representative western blot image. (B) Bands were quantitated by densitometry and presented as protein expression normalized to β-actin + SEM. $^*p = 0.04$. Representative of $n = 4$ independent experiments.