

Neurotrophic receptor tyrosine kinase B induces c-fos-associated cell survival

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Abstract. The neurotrophic receptor tyrosine kinase B (TrkB) is a cell surface receptor for brain-derived neurotrophic factor (BDNF) with kinase activity. This protein is expressed in various tumors and thought to participate in various cellular processes. In this study, we established 293T cells stably expressing human TrkB to elucidate its intracellular functions. Using this cell system, we examined the biological roles of TrkB and its downstream target molecules. The TrkB expressing cells showed an increased survival rate through increased c-fos mRNA expression by BDNF, which were completely suppressed by TrkB inhibitor. Moreover, the combination of inhibitors of mitogen-activated protein kinase (MEK) and phosphatidylinositol 3-kinase (PI3K) partially reduced both the cell survival rate and c-fos mRNA expression, whereas monotreatment of these reagents could not affect cell survival nor c-fos mRNA expression. These results suggested that TrkB could play a role in c-fos-associated cell survival through both MEK and PI3K pathway. It is conceivable that activation of TrkB has a significant impact on tumorigenesis and metastasis.

Introduction

Trk family is composed of three related proteins, TrkA, TrkB and TrkC, to which different neurotrophins can be bound (1). In spite of their structural similarity, only the activation of TrkB leads to unfavorable prognosis in various tumors, suggesting that TrkB might be a potential prognostic factor of malignant diseases (2,3).

TrkB is a 145 kDa cell surface receptor of BDNF. Following BDNF binding, TrkB forms homodimers resulting in autophosphorylation at tyrosine residue and kinase acti-

vation, which leads to the activation of downstream molecules, including MEK and PI3K/Akt (4). As these signaling pathways were known to play important roles in cell proliferation, differentiation and survival, TrkB may also participate in various cellular processes.

Recent reports showed that both TrkB and BDNF were frequently overexpressed in various cancers, such as pancreatic carcinoma, prostate carcinoma, Wilms' tumor and neuroblastomas, particularly those with aggressive behavior and poor prognosis (5-8). It was also reported that the activation of TrkB induced malignant transformation of the epithelial cells through MEK and PI3K/Akt signaling (9). It is conceivable that TrkB plays some role in cellular processes, since MAP kinase and PI3K pathways were known to be correlated with cell survival through tumor-associated gene expression. Furthermore, BDNF has also been reported to induce the expression of tumor-associated genes (5,10).

In this study, we investigated biological roles of TrkB in cellular processes to clarify the relationship between the expression of TrkB and poor prognosis of the tumor.

Material and methods

Reagents. Recombinant human BDNF protein was purchased from PeproTech Inc. (London, UK). Tyrosine kinase inhibitor K252a, MEK inhibitor PD98059, and PI3K inhibitor LY294002 were obtained from Sigma Chemicals (St. Louis, MO). Anti-β-actin (C-2), anti-human TrkB and anti-phospho Trk (E-6) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and R&D Systems (Minneapolis, MN), respectively.

Establishment of a cell line stably expressing TrkB. To obtain 293T cells (American Type Culture Collection, Manassas, VA) with overexpression of TrkB, human TrkB cDNA was cloned and inserted into the expression pcDNA Myc-His vectors (Invitrogen, Carlsbad, CA). 293T cells were transfected using Superfect reagent (Qiagen, Hilden, Germany) and selected using blasticidin (50 mg/ml; Invitrogen). TrkB was stably expressing in human 293T cells (293T-TrkB) and empty-vector was transfected as a control (293T-mock). Expression levels of TrkB mRNA were analyzed by real-time PCR and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression of TrkB protein was

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Table I. Oligonucleotides for real-time and RT-PCR.

TrkB-RQ-F	5'- CGTGTACAGCACTGACTACTACAGG -3'
TrkB-RQ-R	5'- GTACATGATGCTCTCTGGAGGC -3'
TrkB-RQ-probe	5'- TGGCCACACAATGCTGCCCATT -3'
RQ-uPAR-F	5'- ATCACCAGCCTTACCGAGGTTG -3'
RQ-uPAR-R	5'- ATCCTTTGGACGCCCTTCTTC -3'
RQ-uPAR-probe	5'- TTCCCGAAGCCGTTACCTCGAATG -3'
RQ-uPA-F	5'- CCCACTACTACGGCTCTGAAGTCA -3'
RQ-uPA-R	5'- AGTGTGAGACTCTCGTGTAGACGC -3'
RQ-uPA-probe	5'- TCCAAGGCCGCATGACTTTGACTG -3'
RQ-GAPDH-F	5'- GAAGGTGAAGGTCGGAGTC -3'
RQ-GAPDH-R	5'- GAAGATGGTGATGGGATTTC -3'
c-fos F	5'- GGATAGCCTCTCTTACTACCAC -3'
c-fos R	5'- TCCTGTCATGGTCTTCACAACG -3'
β -actin F	5'- AGCAAGAGAGGCATCCTCACCCTGAAGTACC -3'
β -actin R	5'- CAGATTCTCCTTAATGTACGCACGATTTC -3'

examined by Western blot analysis. All experiments were performed in triplicate.

Cell culture and treatment. 293T cells were maintained in RPMI-1640 media (Sigma Chemical) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin (Sigma Chemical) at 37°C under 5% CO₂.

After cells were grown to 70% confluency, they were incubated in serum-free media for 16 h and subsequently treated with 10 ng/ml of BDNF. In antagonist experiments, cells were incubated with 50 μ M of PD98059, 25 μ M of LY294002 or 200 nM of K252a for 1 h before BDNF treatment. Cells were harvested at 0, 8, 12, 16, 18 and 24 h after BDNF treatment to examine cell number and the expression levels of several related genes.

Quantitative real-time reverse-transcriptase PCR. The mRNA levels of TrkB, urokinase-type plasminogen activator receptor (uPAR), urokinase-type plasminogen activator (uPA) and GAPDH were quantified by real-time PCR with SYBR-Green Dye using ABI PRISM 7700 (Perkin-Elmer Applied Biosystems, Foster City, CA). Briefly, total RNA was extracted from cells using RNeasy (Qiagen) according to manufacturer's instructions and cDNAs were generated by reverse transcriptase reaction. cDNA was added to the PCR mixture containing 10 mM Tris (pH 8.0), 50 mM KCl, 10 mM EDTA, 5 mM MgCl₂, 100 μ M each of dATP, dCTP, dGTP and dTTP, 0.2 μ M each of forward and reverse primers, 0.1 M fluorogenic probe, and 1.25 U AmpliTaq Gold (PE Applied Biosystems). Following activation of AmpliTaq Gold for 10 min at 95°C, 50 cycles of 15 sec at 95°C and 1 min at 60°C were carried out in a model 7700 Sequence Detector (PE Applied Biosystems). The primers used for amplification are listed in Table I.

Semi-quantitative reverse transcriptase-PCR (RT-PCR). The RT-PCR was carried out as follows: 96°C for 10 min, followed by 32 cycles (94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec) and 3 min at 72°C. PCR products were

separated in 2% agarose gel and visualized by ethidium bromide under a UV transilluminator. Expression levels of mRNA were determined using LAS-3000 and MultiGauge (Fujifilm, Tokyo, Japan). Primers for c-fos and β -actin are shown in Table I.

Western blot analysis. The cells treated with or without the chemical reagents were washed with cold phosphate-buffered saline (PBS: 10 mM phosphate, pH 7.4, 100 mM NaCl, and 10 mM KCl) and resuspended in lysis buffer containing 50 mM HEPES, pH 7.3, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail (Sigma chemical). Following incubation on ice for 30 min, and centrifugation at 8,000 x g for 5 min at 4°C, protein concentrations in supernatants were determined by a Bio-Rad protein assay reagent (Hercules, CA). Samples containing 34 μ g protein were immunoprecipitated by anti-TrkB antibody, separated by 7.5% SDS-PAGE and electrotransferred to nitrocellulose membranes (PROTRAN, Scheider & Shuell, Dassel, Germany). Proteins were immunoblotted with appropriate antibodies and visualized using the ECL Western blotting detection system (Boehringer Mannheim, Mannheim, Germany).

Determination of cell number. 293T cells were seeded into 6-well plates at a density of 5x10⁴ cells/well and cultured in RPMI media containing 10% FCS for 24 h and then in serum-free media for 16 h. After the treatment with or without antagonists for 1 h, cells were incubated with 10 ng/ml of BDNF for various periods. Harvested cells were washed with the serum-free media and resuspended 1 ml of serum-free media. Then, 100 μ l of cell suspension were transferred to 96-well plate. The number of viable cells was analyzed by Cell Counting Kit-8 (CKK-8; Dojin, Japan).

Results

Expression and autophosphorylation of TrkB in 293T cells stably expressing TrkB. Expression levels of TrkB mRNA and

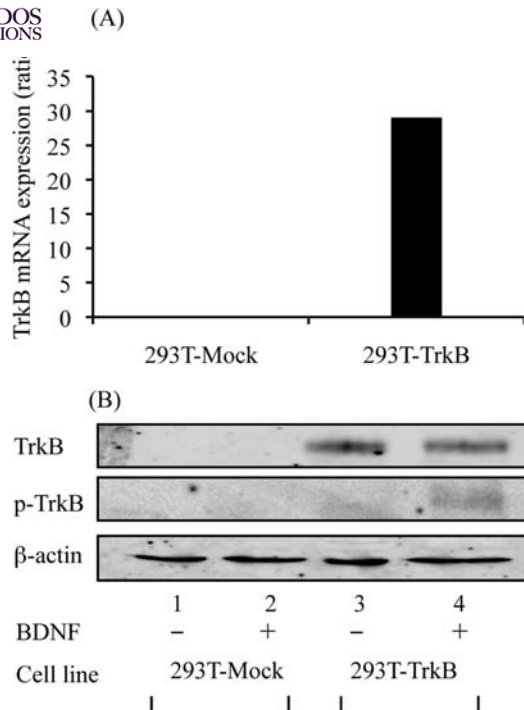
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Figure 1. Expression and autophosphorylation of TrkB in 293T-TrkB and 293T-mock cells. (A) The expression levels are shown as a ratio to GAPDH. TrkB-mock cells showed 0.008 and 293T-TrkB cells were 29.0 of TrkB. (B) Expression and phosphorylation of TrkB protein were detected by Western blot analysis. Cells grown to 70% confluency were serum-starved for 16 h and treated with or without BDNF for 20 min. Aliquots (34 mg protein of the cell lysate) were immunoprecipitated by anti-TrkB antibody and immunoblotted with anti-TrkB and anti-phospho TrkB antibodies (E-6). Lanes: 1, 293T-mock cells; 2, 293T-mock cells treated with BDNF; 3, 293T-TrkB cells; 4, 293T-TrkB cells treated with BDNF.

protein were determined by real-time RT-PCR and Western blot analysis, respectively. 293T-TrkB cells expressed TrkB mRNA 10^4 -fold higher than the 293T-mock cells (Fig. 1A). In Western blot analysis, TrkB protein was detected only in 293T-TrkB cells (Fig. 1B). Moreover, to examine whether expressed TrkB protein could work in the transfected cells, the phosphorylation of TrkB by BDNF stimulation was evaluated by Western blot analysis with anti-phosphoTrkB antibody. Treatment of 293T-TrkB cells with BDNF for 20 min significantly increased the phosphorylation levels of TrkB (Fig. 1B, lane 4).

uPA and uPAR expression by BDNF stimulation. As it was reported that the expression of uPA and uPAR is correlated with TrkB activation, this regulatory pathway was examined in our cell system. uPA mRNA in 293T-TrkB cells was induced 1.5-fold higher than that in 293T-mock cells after 8 h BDNF treatment. uPAR mRNA expression in 293T-TrkB cells was also induced 3.4-fold higher than that in 293T-mock cells after 12 h BDNF treatment. Thus, activated TrkB induced mRNA expression of both uPA and uPAR (Fig. 2). Moreover, to confirm that uPA and uPAR mRNA expression was correlated with TrkB activation, we used the pharmacological antagonist of TrkB, K252a (11). TrkB-induced expression of uPA and uPAR mRNA was completely blocked by K252a. Therefore, BDNF-induced TrkB activation could

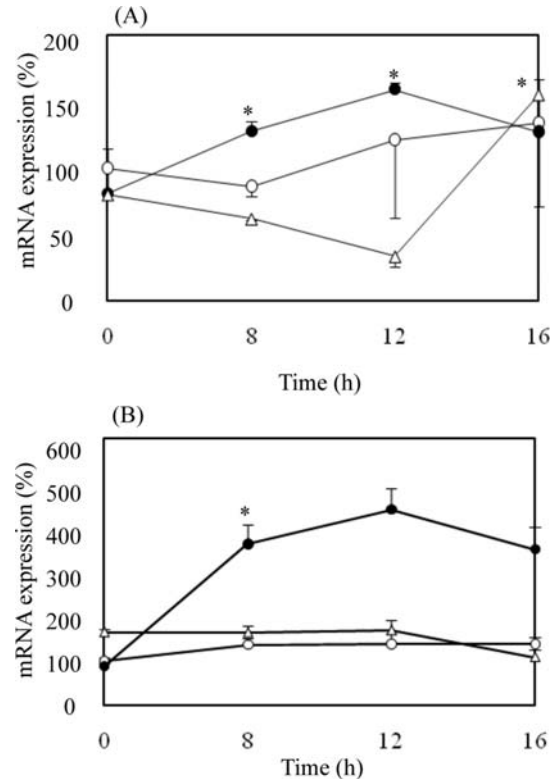


Figure 2. Time course studies of uPA and uPAR mRNA expression. 293T-TrkB and 293T-mock cells were cultured in the presence of BDNF (10 ng/ml) for 0, 8, 12 or 16 h and harvested. TrkB inhibitor K252a was used to confirm correlation between TrkB activation and uPA and uPAR mRNA expression. 293T-TrkB cells were incubated with K252a (200 nM) and then, treated with BDNF for 0, 8, 12 and 16 h and harvested. uPA (A) and uPAR (B) mRNA expression levels were analyzed by quantitative real-time PCR. mRNA expression levels in 293T-mock cells (○), 293T-TrkB cells (●) and K252a-treated 293T-TrkB cells (Δ) were expressed as ratios to the level in 293T-mock (0 h) cells. *, $P < 0.05$.

induce the expression of uPA and uPAR mRNA in 293T-TrkB cells.

BDNF-induced cell survival. It was reported that TrkB plays important roles in regulation of the cell survival in normal peripheral nervous system (12-14). The activation of TrkB was frequently identified in various human cancers, however, its roles are still unclear (5-8). Since 293T-TrkB cells were observed to survive even in serum-starved media in comparison with 293T-mock cells, we evaluated the relationship between TrkB activation and cell survival. It was shown that 70% of 293T-TrkB cells and 43% of 293T-mock cells survived BDNF treatment for 24 h (Fig. 3). In brief, BDNF stimulation induced about 1.7-fold increase of cell survival in 293T-TrkB cells (Fig. 3B), but not in 293T-mock cells. Moreover, this increment was completely reversed by K252a.

To clarify downstream targets of TrkB-signaling associated with cell survival, we used pharmacological antagonists of MEK (PD98059) and PI3K (LY294002), which were reported as downstream candidates of TrkB-signaling (10,14). Though neither PD98059 nor LY294002 could reverse the increment of cell survival by BDNF (data not shown), the combination with both drugs induced 23.5% reduction.

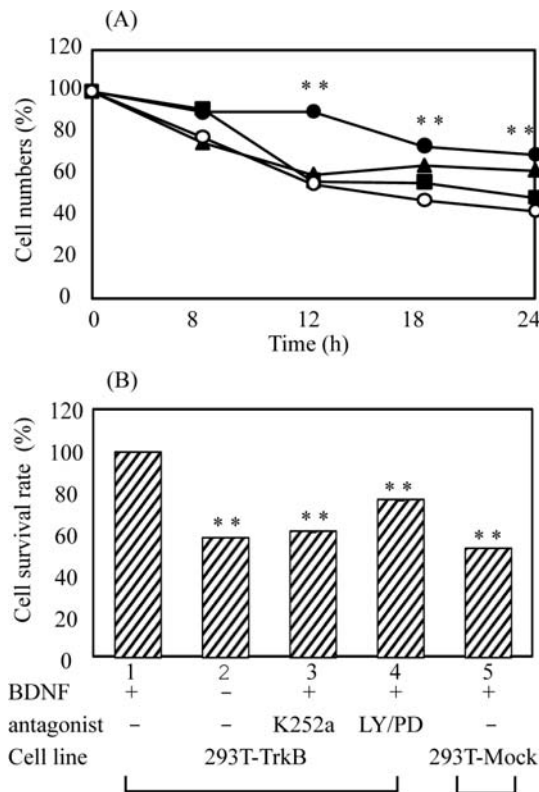


Figure 3. BDNF-induced cell survival. (A) Cells were cultured in serum-free media for 16 h and then treated with K252a (200 nM), PD98059 (50 μ M) or LY294004 (25 μ M) followed by 1 h 10 ng/ml BDNF stimulation. Cells were harvested at 0, 8, 12, 18 or 24 h after BDNF stimulation. Survival rates of 293T-mock cells (○), 293T-TrkB cells (●), K252a-treated 293T-TrkB cells (■) and PD98059/LY294002-treated 293T-TrkB cells (▲) were expressed as ratios to 293T-TrkB cells (0 h) using CCK-8. **, $P < 0.01$. (B) Comparison of cell survival ratio at 24 h after various treatments to 293T-TrkB cells. Survival rate of 293T-TrkB was treated with BDNF (lane 2), BDNF and K252a (lane 3), BDNF and LY294002 (lane 4), and BDNF treated 293T-mock cells (lane 5). **, $P < 0.01$.

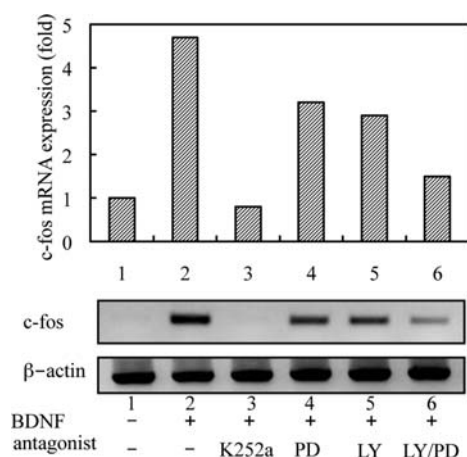


Figure 4. c-fos mRNA expression. Cells were treated without BDNF (lane 1) or with BDNF (lane 2) after the treatment of K252a (lane 3), PD98059 (lane 4), LY294002 (lane 5) or PD98059 and LY294002 (lane 6) for 12 h. RT-PCR was performed and c-fos mRNA levels were determined using MultiGauge.

TrkB-induced expression of c-fos mRNA. We examined the signal pathway of TrkB-associated cell survival. MAP kinase

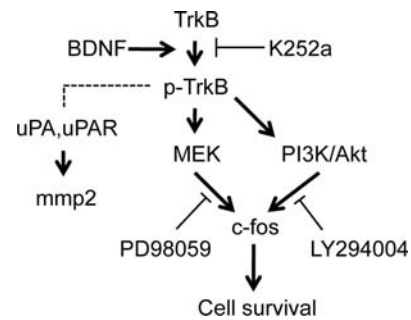


Figure 5. Schematic representation of TrkB signaling. Following BDNF binding, TrkB was activated by autophosphorylation. Activated TrkB induced c-fos-associated cell survival through MEK and PI3K/Akt pathway. Tyrosine kinase inhibitor; K252a, MEK inhibitor; PD98059, P13K inhibitor; LY294002, urokinase-type plasminogen activator receptor; uPAR, urokinase-type plasminogen activator; uPA and matrix metalloproteinase 2; mmp2.

and PI3K pathways have reported to be correlated with cell survival through tumor-associated gene expression. Moreover, BDNF was previously reported to induce the expression of c-fos (5,10), and then c-fos mRNA expression was suspected to be induced by TrkB activation.

Fig. 4 showed that c-fos mRNA expression was increased up to 4.7-fold in the presence of BDNF comparing to that without BDNF in 293T-TrkB cells. This induction was completely suppressed by K252a. Based on this observation, we investigated further downstream targets of TrkB pathway using antagonists. Neither PD98059 nor LY294002 could block TrkB-induced c-fos mRNA expression, but the combination treatment with both drugs showed partial suppression (31.9%).

uPA might induce c-fos mRNA expression in ovarian cancer cells (15,16). However, the mRNA expression of c-fos by TrkB activation was not suppressed by inhibition of uPA/uPAR pathway with anti-uPA neutralizing antibody in 293T-TrkB cells (data not shown).

Discussion

Expression of TrkB and BDNF is usually detected in primary neuroblastomas with an unfavorable outcome such as highly invasive and metastatic capacity (2,5,6). We investigated the molecular mechanisms how TrkB expression contributed to cell survival.

To analyze TrkB signaling pathway and its function, we established a stable cell line, which expressed TrkB mRNA and functional protein. Cell survival was increased in 293T-TrkB cells after 24 h BDNF treatment and BDNF-induced survival of 293T-TrkB cells was suppressed by TrkB inhibitor. These results strongly suggested that TrkB could mediate BDNF-induced cell survival.

In 293-TrkB cells, the mRNA expression of uPA and uPAR, which are known as targets of TrkB (17), were induced to a higher level than that in 293T-mock cells by BDNF treatment. Moreover, suppression of the uPA/uPAR expression by K252a strongly supported the participation of TrkB in the gene expression of uPA/uPAR.



is well-known as a proteinase receptor and recently to be associated with cell migration, adhesion, differentiation and proliferation through intracellular signaling (12,16,18). Therefore, to examine the association of uPAR to BDNF-induced cell survival, uPAR signaling was suppressed by anti-uPA antibody, which could inhibit uPA activity. However, anti-uPA antibody could not suppress BDNF-induced cell survival. Taken together, uPA/uPAR signaling did not participate in BDNF-induced cell survival.

The next question was which pathway mediated TrkB signaling. In ovarian cancer cells, BDNF stimulation rapidly activated TrkB and Akt, and TrkB inhibitor dramatically inhibited BDNF-induced TrkB and Akt activation (9,14). It suggested that TrkB-activated Akt, a downstream of PI3K. Since Akt is known to exert anti-apoptotic effects, Akt could mediate BDNF-induced cell survival. Although neither PI3K antagonist (LY294002) nor MEK antagonist (PD98059) could hardly reduce the TrkB-induced survival rate, a combination of both antagonists suppressed the BDNF-induced cell survival. These results indicated that BDNF-induced cell survival was in part, mediated by both MEK and PI3K pathways in 293T-TrkB cells.

Several downstream candidates of MEK or PI3K were screened. In this study, c-fos mRNA expression was dramatically increased by BDNF, and K252a completely suppressed this increment in 293T-TrkB cells. c-fos is known to exert oncogenic activity and expressed in various tumor cells. Thus, up-regulation of c-fos mRNA expression by BDNF could play a certain role of TrkB-induced cell survival. We supposed that PI3K/Akt or MEK-signaling pathway activation by TrkB could induce c-fos mRNA expression. Neither PD98059 nor LY294002 could block TrkB-induced c-fos mRNA expression. However, the combination treatment with both drugs partially blocked TrkB-induced c-fos mRNA expression. Taken together, the increment of c-fos expression by TrkB activation was mediated in part by both PI3K/Akt and MEK pathways.

In conclusion, TrkB could play a certain role in c-fos-associated cell survival through both MEK and PI3K pathway. uPA/uPAR which could be induced by TrkB were not involved in cell survival (Fig. 5). Further study is required to clarify the relationship of TrkB activation to c-fos expression.

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