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TrkB mutant lacking the amino-terminal half of the extracellular portion acts as a functional brain-derived neurotrophic factor receptor

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Abstract

A series of mutants with deletion in the extracellular portion of TrkB were expressed transiently and stably in mammalian cells to examine the brain-derived neurotrophic factor (BDNF)-binding properties of TrkB. We found that these binding activities were retained by the TrkB deletion mutant (TrkBΔ4) lacking most of the extracellular portion, cysteine-rich cluster 1 and 2, leucine-rich motif and most of the first immunoglobulin-like domain (Ig1). Furthermore, the results of the neurotrophin selectivity, the equilibrium binding constant, auto-phosphorylation and BDNF dependent cell survival indicate that TrkBΔ4 acts as a functional BDNF receptor comparable to wild-type TrkB. Thus, our findings showed that only the carboxyl-terminal half of the extracellular portion of TrkB, which includes the Ig2 domain, is essential for the functional BDNF receptor.

Keywords: Deletion mutant; Ligand binding; Immunoglobulin-like domain; Neurotrophin; Tyrosine kinase receptor

1. Introduction

Neurotrophins display their biological activities through activation of members of the Trk tyrosine kinase receptor family [1–6]. The neurotrophic effect of BDNF is mediated by its specific receptor TrkB [7–12]. Binding of BDNF induces auto-phosphorylation of TrkB, which triggers the subsequent steps in the signal transduction pathway. The extracellular portion of TrkB, which is responsible for BDNF binding, consists of two cysteine-rich clusters (Cys1 and Cys2), a leucine-rich motif (LRM) and two immunoglobulin-like domains (Ig1 and Ig2). Analyses of the binding properties between BDNF and TrkB, using the TrkA-TrkB chimera receptors expressed in mammalian cells [13] and immunoadhesion molecules including the extracellular portion of TrkA-TrkB and TrkC-TrkB chimeras [14], revealed that the Ig2 domain of TrkB confers both specificity and affinity to BDNF. However, TrkB variants with LRM deletion demonstrated that LRM is crucial for the BDNF-binding activity [15] and analyses using maltose-binding protein-TrkB fusion protein expressed in *E. coli* [16,17] showed that the fusion protein with the Ig2 domain of TrkB did not have any binding activities for BDNF. Another study found two regions, LRM-Cys2 and a domain close to the Ig2 domain, to be BDNF-binding domains in

Abbreviations: BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4; Ig, immunoglobulin

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the soluble form of TrkB by affinity separation and chemical cross-linking [18]. These discrepancies may be due to the different experimental conditions. Under some conditions, the extracellular region of TrkB might not preserve its native conformation.

In this report, we evaluated the domain important for BDNF binding by using the membrane-inserted TrkB mutants expressed in mammalian cells, where TrkB mutants are expected to have the same native conformation as wild-type TrkB.

2. Materials and methods

2.1. Wild-type TrkB and TrkB mutants expression constructs

Mouse *trkB* cDNA was cloned in the mammalian expression vector pEF-BOS [19], to yield a construct for the expression of wild-type TrkB (pEF-BOS-*trkB*). The *trkB* cDNA corresponding to the extracellular portion of TrkB was subcloned in pBluescriptII (Stratagene) and sequential deletions on the cDNA sequence were introduced with a deletion mutant kit (Takara). To express all mutants on cell surfaces, the cDNA sequence corresponding to the signal peptide was retained. After confirming the appropriate deletions by nucleotide sequencing, the deleted cDNA fragments of the extracellular domain were replaced with pEF-BOS-*trkB*. The amino acid residues deleted from the TrkB mutants were as follows: TrkBΔ1, Δ3–92; TrkBΔ2, Δ3–160; TrkBΔ3, Δ3–194; TrkBΔ4, Δ3–218; TrkBΔ5, Δ3–262; TrkBΔ6, Δ3–266.

2.2. Expression of wild-type TrkB and TrkB mutants

COS-1 and NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium (Nikken) containing 10% fetal bovine serum (Intergen) at 37°C in 5% CO_2. Wild-type TrkB and TrkB mutants were transiently expressed in COS-1 cells by transfection of the cDNA constructs using Lipofectamine (Gibco-BRL). After 48 h of transfection, cells were washed with PBS (Nissui) twice and harvested for further analysis. For stable transformants of wild-type TrkB or TrkBΔ4, each construct was co-transfected with pSV2neo into NIH-3T3 cells. After 1 week of G418 treatment (300 μg/ml), several colonies were picked up by the cylinder cloning technique. The expression of wild-type TrkB or TrkBΔ4 was confirmed by Western analysis. The transformants with a high expression level of wild-type TrkB or TrkBΔ4 were used for further experiments.

2.3. Binding assay

Binding assay using membrane fraction was carried out as described [20]. The harvested cells were washed with ice cold T10E1 (10 mM Tris HCl (pH 8.0)/1 mM EDTA). Cells were suspended with T10E1 and homogenized followed by centrifugation at $1000\times g$ for 5 min at 4°C. The supernatant was centrifuged again at $10\,000\times g$ for 30 min at 4°C. The pellet (membrane fraction) was suspended with T10E1 and protein concentration was measured. The binding assay of wild-type TrkB and TrkB mutants transiently expressed in COS-1 cells was carried out using a scintillation proximity assay (SPA, Amersham) system. Briefly, the membrane fraction (equivalent to a protein concentration of 5 μg), pre-coupled with 0.5 mg WGA-SPA-beads, was incubated with 100 pM of ^{125}I-BDNF (NEN) in a 96-well microplate in 100 μl assay solution (20 mM HEPES buffer (pH 7.2), 300 mM KCl, 0.5%BSA). After 4 h of incubation at room temperature, the microplate was counted with a micro-β counter (Wallac). The filter binding assay was performed for the stable transformants of wild-type TrkB and TrkBΔ4. For competition binding assay, the membrane fraction (5 μg) of cells was incubated for 2 h at 4°C with 100 pM of ^{125}I-BDNF with 5 pM to 300 nM unlabeled competitor in the assay solution (20 mM HEPES buffer (pH 7.2), 150 mM KCl, 0.5% BSA). For saturation binding assay, the membrane fraction (5 μg) of cells was incubated for 2 h at 4°C with 16–270 pM of ^{125}I-BDNF in the assay solution. Bound/free separation was carried out using the hydrophilic Durapore PVDF membrane filter (Millipore, pore size 0.45 μm). The filter was washed three times with PBS containing 0.1% BSA and 0.1% protamine sulfate. Radioactivity on the filter was measured with a γ-counter (Aloka). Non-specific binding was determined by incubating the membrane fraction in the presence of 1000-fold excess unlabeled BDNF. The specific binding was calculated by subtracting the

non-specific binding from the total binding. All unlabeled neurotrophins (BDNF, NGF and NT4) were purchased from Peprotech.

2.4. Western analysis

Experiments were carried out at room temperature except when otherwise mentioned. Protein in the Laemmli sample buffer was subjected to SDS-PAGE (MULTI GEL 7.5, Daiichi) and transferred electrically onto a PVDF membrane. For the detection of wild-type TrkB and TrkB mutants, the PVDF membrane was incubated with a blocking solution (5% skim milk and 0.05% Tween 20 in PBS) overnight at 4°C, and then with an anti-Trk antibody (sc-011, Santa Cruz) for 1 h. After washing with PBS/0.05% Tween 20 for 30 min, the membrane was blocked again for 15 min and incubated with HRP-conjugated anti-rabbit IgG antiserum (KPL) for 1 h, followed by washing for 1 h. The signal detection was carried out using ECL system (Amersham). The same method was used for phospho-tyrosine (p-Tyr) detection except for the components of the blocking solution (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2% BSA and 0.05% Tween 20) and the washing solution (10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.05% Tween 20) and anti-p-Tyr antibody (Upstate Biotechnology) and HRP-conjugated anti-mouse IgG antiserum (Cappel) for the first and second antibodies.

2.5. Surface biotinylation

COS-1 cells, which transiently express wild-type TrkB or TrkB mutants, were washed twice with PBS and harvested with a cell lifter (Costar). The cells were collected by centrifugation at $1000\times g$ for 5 min. The cells were suspended with PBS supplemented with 0.5 mg/ml sulfo-NHS-LC-S-S-biotin (Pierce) and incubated on ice for 30 min. The cells were washed once with ice-cold PBS and incubated on ice with the cultured medium for 10 min, followed by washing with ice-cold PBS. The washed cells were suspended with a lysis buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1% Tween 80 and 10 μg/ml PMSF) and incubated on ice for 10 min. After removing insoluble material by centrifugation at $10\,000\times g$ for 10 min at 4°C, the lysate was incubated with streptavidin-conjugated beads (Dynabeads M-280, Dynal) with gentle rotation for 30 min at 4°C. The beads were recovered by magnetic separation and washed twice with the lysis buffer. The biotinylated protein recovered was suspended in Laemmli sample buffer and boiled 10 min. After removal of the beads magnetically, the sample was analyzed by Western blotting.

2.6. Auto-phosphorylation assay

NIH-3T3 cells stably expressing wild-type TrkB or TrkBΔ4 were incubated with 100 ng/ml BDNF at 37°C for 10 min. The cells were washed twice with ice-cold PBS and harvested with RIPA buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml PMSF and 1 mM orthovanadate). After centrifugation at $1000\times g$ for 10 min at 4°C, the cell lysate (supernatant) was incubated on ice with the anti-Trk antibody for 1 h. Protein G-sepharose was then added to the lysate, and incubated again with gentle rotation at 4°C for 1 h. The immunoprecipitates were collected by centrifugation, washed 4 times with RIPA buffer and suspended in Laemmli sample buffer, followed by Western analysis.

2.7. Survival assay

Survival assay of NIH-3T3 cells stably expressing wild-type TrkB or TrkBΔ4 was carried out and quantified as described previously [15]. Briefly, cells were plated 1000 cells/well in 96-well plates in the cultured medium without serum supplemented with 12.5–200 ng/ml BDNF. After 60 h of incubation, the viability of cells was measured by the MTT assay [21].

3. Results

3.1. BDNF-binding abilities of transiently expressed TrkB mutants

The extracellular portion of TrkB consists of two cysteine-rich clusters (Cys1 and Cys2), a leucine-rich motif (LRM) and two immunoglobulin-like domains (Ig1 and Ig2) (Fig. 1). To determine the important

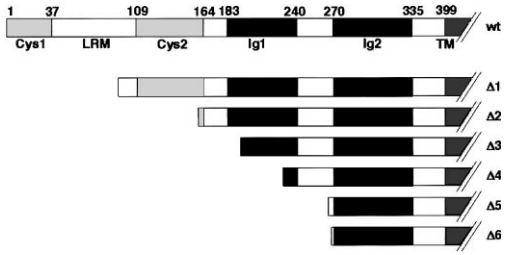

Fig. 1. Schematic illustration of wild-type TrkB and its deletion mutants. A series of constructs encoding deletion mutants of the mouse TrkB were prepared as described in Section 2. Domains of TrkB and their amino acid residues are according to Schneider and Schweiger [25]; the cysteine-rich cluster 1 (Cys1, residues 1–36), leucine-rich motif (LRM, residues 37–108), cysteine-rich cluster 2 (Cys2, residues 109–164), first and second immunoglobulin-like domains (Ig1 and Ig2, residues 183–239 and 270–334, respectively) and transmembrane domain (TM, residues 399–423). The intracellular portion including tyrosine kinase domain is not shown in this illustration.

region of TrkB for BDNF binding, several TrkB mutants with sequential deletions from the amino-terminal side (Fig. 1) were transiently expressed in the COS-1 cells and their BDNF-binding abilities were examined. The specific binding of ^{125}I-BDNF, which was comparable to that of wild-type TrkB, was observed in TrkBΔ2–Δ4 and a slightly weaker BDNF binding was observed in TrkBΔ1 (Fig. 2A). Whereas no significant BDNF binding was observed in TrkBΔ5 or TrkBΔ6 (Fig. 2A). Since these results may be due to inefficient biosynthesis of some TrkB mutants, we next examined the expression of TrkB mutants. The membrane samples used for the binding assay were subjected to Western analysis using an anti-Trk antibody that recognizes the intracellular region of TrkB. As shown in Fig. 2B, each TrkB mutant was expressed appropriately at the same level as wild-type TrkB and also migrated in agreement with each deletion. The broad signal observed was most likely due to differences in glycosylation because the extracellular portion of TrkB is known to be heavily glycosylated [22]. We further examined whether the TrkB mutants were expressed on the cell surface. Cell surface biotinylation was performed using sulfo-NHS-LC-S-S-biotin and the biotinylated proteins were isolated from the cell lysates using streptavidin-conjugated beads, followed by Western analysis using anti-Trk antibody. The results shown in Fig. 2C confirmed the appropriate presence of each mutant on the cell surface as wild-type TrkB. Therefore, removal of amino-terminal region from TrkB mutants did not appear to interfere with biosynthesis and transport of TrkB mutants to the cell surface.

We considered that the results of the specific binding of ^{125}I-BDNF (Fig. 2A) referred the BDNF-binding ability of each TrkB mutant. Transiently expressed TrkBΔ4, lacking most of the extracellular

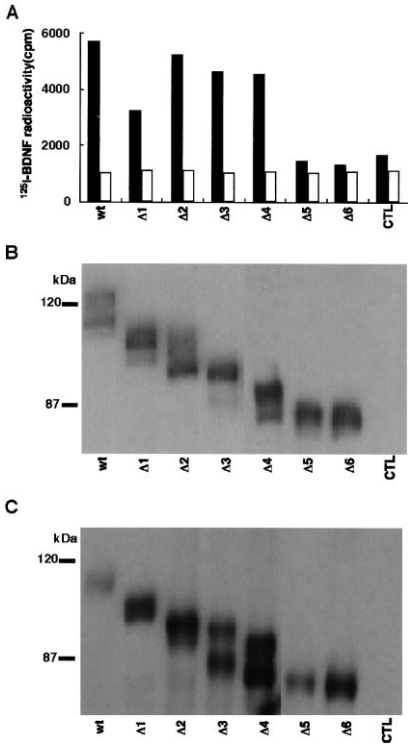

Fig. 2. BDNF-binding abilities of the transiently expressed TrkB mutants. (A) Wild-type TrkB or TrkB mutants were transiently expressed in COS-1 cells and the cell membrane fractions were used for ^{125}I-BDNF binding. The cell membrane fraction prepared from vector-transfected COS-1 cells was used as a negative control (CTL). The radioactivity of ^{125}I-BDNF (cpm) in each sample was measured in the absence (total binding, filled bar) or presence (non-specific binding, open bar) of 1000-fold excess unlabeled BDNF. The binding assay was performed in duplicate and the means are shown. (B) Detection of the TrkB mutants. The cell membrane fractions for the binding assay were subjected to Western analysis using an anti-Trk antibody. Molecular weight marker (Bio-Rad) was used to estimate the signal size. (C) Cell surface expression of trkB mutants. The transfected cells were biotinylated using sulfo-NHS-LC-S-S-biotin, an activated biotin for cell surface labeling. The biotinylated protein was precipitated from the cell lysate by streptavidin-conjugated beads. The precipitates were analyzed by Western blot using anti-Trk antibody.

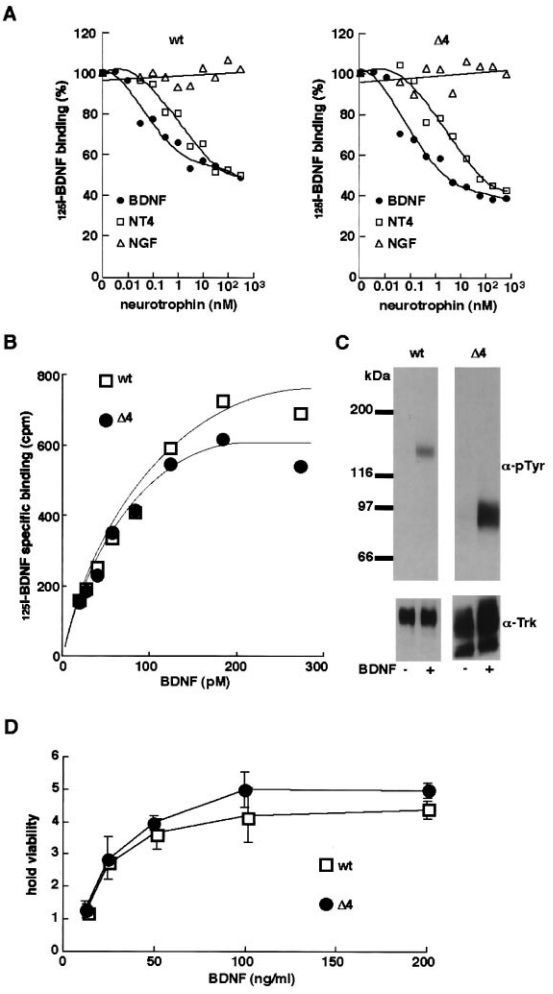

Fig. 3. TrkBΔ4 acts as a functional BDNF receptor comparable to wild-type TrkB. (A) Comparison of the neurotrophin selectivity. NIH-3T3 cell membrane stably expressing wild-type TrkB or TrkBΔ4 was used for competition binding assay. The radioactivity of ^{125}I-BDNF (cpm) was measured in the presence of the various concentration of unlabeled competitor, BDNF (closed circle), NGF (open triangle) and NT-4 (open square). The binding assay was performed in duplicate and means are shown. (B) Saturation binding assay of wild-type TrkB and TrkBΔ4. Representative results are shown. The membrane fraction prepared from NIH-3T3 cells stably expressing wild-type TrkB or TrkBΔ4 were incubated with various concentrations of ^{125}I-BDNF and specific binding was determined as described in Section 2. Experiments were performed in duplicate and the means of specific binding for wild-type TrkB (open squares) and TrkBΔ4 (closed circles) were plotted on the graph. Similar results were obtained in three independent experiments. (C) BDNF-induced auto-phosphorylation assay. NIH-3T3 cells stably expressing wild-type TrkB or TrkBΔ4 were incubated in the absence (−) or presence (+) of 100 ng/ml BDNF for 10 min. The cells were lysed and subjected to the immunoprecipitation using the anti-Trk antibody. The immuno-precipitates were analyzed by Western blotting using an anti-phosphotyrosine antibody (upper panel) or the anti-Trk antibody (lower panel). Molecular weight marker (Bio-Rad) was used to estimate the signal size. (D) Survival assay. NIH-3T3 cells stably expressing wild-type TrkB or TrkBΔ4 were cultured in serum-free medium supplemented various concentration of BDNF. The viability without BDNF was determined as 1. The viability was measured by MTT assay. The survival assay was performed in triplicate and means and standard deviations are shown.
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portion, had the BDNF-binding ability comparable to wild-type TrkB.

3.2. Comparison of TrkBΔ4 with wild-type TrkB

To further investigate the properties of TrkB mutants, the constructs for wild-type TrkB and TrkBΔ4 were stably transfected into the NIH-3T3 cell. We prepared the cell membrane fractions from these stable transformants and examined the ligand selectivity of TrkBΔ4. The competition binding assay was performed using several unlabeled neurotrophins that interact with the Trk family (Fig. 3A). There are no significant differences between TrkBΔ4 and wild-type TrkB in ligand selectivity; both had a specific binding ability to BDNF and NT-4, but no binding ability to NGF. We further examined the equilibrium binding constant (K_d) of the interaction of ^{125}I-BDNF with wild-type TrkB and TrkBΔ4 by saturation binding assay. The binding properties with increased ^{125}I-BDNF showed that both wild-type TrkB and TrkBΔ4 had ^{125}I-BDNF binding with saturation (Fig. 3B). The K_d value analyzed by the Scatchard plot from three independent experiments were 84 ± 42 pM for wild-type TrkB, which was in the same range previously reported [11,23]. Surprisingly, TrkBΔ4 showed a K_d value of 91 ± 10 pM, which was quite similar to that of wild-type TrkB. The BDNF-induced auto-phosphorylation assay was also carried out to examine whether TrkBΔ4 is activated to induce signaling by BDNF. After exposure to BDNF for 10 min, the stable transformants expressing wild-type TrkB and TrkBΔ4 were harvested. The lysed cells were immunoprecipitated by the anti-Trk antibody, and the precipitates were subjected to Western analysis using anti-phosphotyrosine antibody. Both wild-type TrkB and TrkBΔ4 were significantly autophosporylated by BDNF stimulation (Fig. 3C). Fi-

nally, the BDNF-dependent cell survival assay was carried out. The transformants expressing wild-type TrkB and TrkBΔ4 were cultured in serum-free medium supplemented various concentration of BDNF, and the viability of cells was examined by the MTT assay. Consistent with the binding assay and auto-phosphorylation assay, the BDNF dependent survival of TrkBΔ4 was the same level as wild-type TrkB (Fig. 3D).

The results of the ligand selectivity, the equilibrium binding constant, auto-phosphorylation and BDNF-dependent cell survival indicate that TrkBΔ4, which lacks Cys1 and Cys2, LRM and most of Ig1 in the extracellular portion, acts as a functional BDNF receptor comparable to wild-type TrkB.

4. Discussion

The importance of the Ig2 domain of TrkB for BDNF binding has been reported by several groups [13,14,18]. This importance was also confirmed with other Trk receptors, TrkA and TrkC [14,24]. These results agree with our findings that TrkB mutants, TrkBΔ1–Δ4, possessed significant BDNF-binding activities, besides we could not detect any BDNF binding in TrkBΔ5 or TrkBΔ6 both of which contain the whole Ig2 domain. The absence of the BDNF-binding activity was also observed using the cell membrane sample stably expressing TrkBΔ5 (data not shown). Our results suggest that not only the Ig2 domain, but also the internal region between Ig1 and Ig2 plays an important role in BDNF binding. Deletion of the internal region between Ig1 and Ig2 might destroy the native conformation of the Ig2 domain.

In transient expression in COS-1 cells, wild-type TrkB was found to migrate with an estimated molecular weight (MW) of 120 kDa (Fig. 2B), which was slightly lower than the native TrkB of 145 kDa [8]. This observation was most likely due to the transiently expressed TrkB being less glycosylated. In spite of partial glycosylation, transiently expressed wild-type TrkB and TrkB mutants in COS-1 cells do not affect the BDNF-binding activities (Fig. 2A). However, as partial glycosylation of TrkB may affect some BDNF-induced biological activities, stable transformants expressing wild-type TrkB and TrkBΔ4 were prepared for further experiments. The estimated MW of stably expressed wild-type TrkB and TrkBΔ4 were approximately 145 and 96 kDa, respectively, which were larger than those transiently expressed. This result suggests that stably expressed wild-type TrkB and TrkBΔ4 are sufficiently glycosylated.

We found BDNF-binding, BDNF-induced auto-phosphorylation properties and BDNF-dependent survival using stable transformants of wild-type TrkB or TrkBΔ4. We showed that TrkBΔ4 acts as a functional BDNF receptor and the amino-terminal region of the extracellular portion including Cys1, LRM, Cys2 and Ig1 domains was not essential. But this result did not agree with the reports that LRM of TrkB plays an important role in BDNF binding [15–17]. Ninkina et al. reported no BDNF-binding activity of TrkB variants lacking only LRM expressed in NIH-3T3 which was the same as our expression system [15]. Interestingly, we also obtained that TrkBΔ1 which lacks Cys1 and most of LRM had a slightly weaker BDNF-binding ability than wild-type TrkB and TrkBΔ2–Δ4 (Fig. 2A). From these observations, it seems that lacking of LRM, Cys2 interferes with the binding of BDNF. Further study of the precise structure of TrkB should clarify role of LRM and Cys1 and Cys2.

Our results clearly demonstrated that TrkBΔ4 lacking the amino-terminal region of the extracellular portion including Cys1, LRM, Cys2 and Ig1 domains acts as a functional BDNF receptor comparable to wild-type TrkB.

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