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Analysis of Tyrosine Phosphorylation-Dependent Protein-Protein Interactions in TrkB-Mediated Intracellular Signaling Using Modified Yeast Two-Hybrid System[1]

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Activated receptor tyrosine kinases induce a large number of tyrosine phosphorylation-dependent protein-protein interactions through which they mediate their various ligand-exerted functions including regulation of proliferation, differentiation and survival. TrkB receptor tyrosine kinase activated by binding of brain-derived neurotrophic factor (BDNF) also stimulates various protein interactions in a tyrosine phosphorylation-dependent manner in neuronal cells. To examine tyrosine phosphorylation-dependent interactions stimulated by active TrkB, we developed a modified yeast two-hybrid system, which we call the yeast two-and-a-half-hybrid system. In this system, yeast was engineered to express a tyrosine kinase domain of TrkB as an effector, in addition to two fusion proteins with GAL4 DNA-binding and GAL4 activation domains as bait and prey proteins, respectively. Using this system with Shp2 as the bait, we demonstrated that Shp2 interacts directly with BIT/SHPS-1 (also called SIRP) and Grb2 depending on tyrosine phosphorylation mediated by TrkB. Furthermore, we screened an adult human brain cDNA library with the yeast two-and-a-half-hybrid system in order to identify other Shp2-binding proteins in TrkB-stimulated tyrosine phosphorylation signaling. We found that fibroblast growth factor receptor substrate 2β (FRS2β), also called SNT2, interacts with Shp2 dependently on TrkB-mediated tyrosine phosphorylation of FRS2β/SNT2. Therefore, we show that the two-and-a-half-hybrid system is a powerful tool for studying tyrosine phosphorylation-dependent protein-protein interactions in intracellular signaling pathways stimulated by TrkB receptor tyrosine kinase.

Key words: brain-derived neurotrophic factor, FRS2/SNT, neurotrophin, receptor tyrosine kinase, signal transduction.

Various growth factors bind to and activate receptor tyrosine kinases, their specific receptors, initiating their actions (*1*, *2*). The activated receptor tyrosine kinases phosphorylate a variety of proteins including themselves, tyrosine phosphorylation of which is required for both catalytic and signaling activities. These phosphorylated tyrosine residues serve as high-affinity binding sites for many signaling proteins possessing the src homology 2 (SH2) and the phosphotyrosine-binding (PTB) domains (*3–5*). Specificity of these interactions is determined by the amino acid sequences flanking phosphotyrosine residues on phosphorylated proteins and variant residues within the SH2 and PTB domains on the signaling proteins. The growth factors induce a variety of protein-protein interactions through these tyrosine phosphorylation-dependent interactions in their intracellular signaling pathways. The tyrosine phosphorylation-dependent protein interactions are considered to have very important roles in the growth factor-induced intracellular signaling to exert their various effects including regulation of proliferation, differentiation and survival.

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, works as a neurotrophic factor, promoting differentiation and survival in a variety of central nervous system (CNS) and peripheral nervous system (PNS) neurons (*6–10*). BDNF is also involved in regulation of synaptic plasticity in CNS neurons (*11*, *12*). BDNF binds to a receptor tyrosine kinase, TrkB, a member of the Trk family, to exert its effects (*13*, *14*). Following the ligand binding, TrkB is dimerized, activated and autophosphorylated on its tyrosine residues, then it stimulates intracellular signaling pathways, activating a variety of enzymes and effectors, including phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3-K), Shc, Grb2, Ras, Shp2, BIT/SHPS-1 (also called SIRP), and mitogen-activated protein

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kinase (MAPK), *via* protein phosphorylation and protein-protein interactions (*15–18*). However, most of the protein interactions in BDNF-induced intracellular signaling pathways remain unclear.

Shp2, a protein tyrosine phosphatase, is activated by various growth factors including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin (*19*, *20*). Shp2 has two SH2 domains and is activated by association with tyrosine-phosphorylated proteins *via* these domains. In addition, EGF and PDGF stimulate tyrosine phosphorylation of Shp2 (*19*). In response to PDGF, the tyrosine-phosphorylated Shp2 binds to Grb2, leading to Ras activation (*21*, *22*). Studies using phosphatase-inactive Shp2 as a dominant negative mutant indicated that Shp2 works as a positive regulator in growth factor signaling pathways (*20*, *23*, *24*). Previously, we have reported that Shp2 was a component of the signaling pathway of neurotrophins in cultured cerebral cortical neurons and PC12 cells (*17*, *18*). Neurotrophins stimulate interactions of Shp2 with Grb2, BIT/SHPS-1, PI3-K, and several unidentified tyrosine-phosphorylated proteins in these cells. In addition, Shp2 regulates levels of tyrosine phosphorylation of several proteins, including BIT/SHPS-1, in BDNF-stimulated intracellular signaling in cultured cerebral cortical neurons (*25*). Thus, Shp2 interacts with various signaling proteins, thereby regulating the activities and properties of the signaling proteins and Shp2 itself. However, it is an unsettled question what kinds of unidentified interactions with Shp2 exist and function in the signaling pathway induced by neurotrophins.

In the present study, we developed a modified yeast two-hybrid system, which we call the yeast two-and-a-half-hybrid system, to investigate tyrosine phosphorylation-dependent protein-protein interactions in BDNF-induced intracellular signaling. In immunoprecipitation studies, we have shown that Shp2 interacts with Grb2, BIT/SHPS-1, and several unidentified tyrosine-phosphorylated proteins in response to BDNF (*17*, *18*). Here, using the yeast two-and-a-half-hybrid system, we demonstrated that Shp2 interacts directly with tyrosine-phosphorylated BIT/SHPS-1 and that tyrosine-phosphorylated Shp2 associates directly with Grb2. Furthermore, to identify what kinds of proteins show tyrosine phosphorylation-dependent interaction with Shp2 in BDNF-induced intracellular signaling, we screened an adult human brain cDNA library with the yeast two-and-a-half-hybrid system using Shp2 as the bait. We found that fibroblast growth factor receptor substrate 2β (FRS2β), also called SNT2, binds to Shp2 depending on TrkB-stimulated tyrosine phosphorylation of FRS2β/SNT2.

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Analysis and Screening—The yeast strain MaV203 (Gibco BRL) was used. The yeast expressing a fusion protein of the TrkB intracellular domain with glutathione S-transferase (GST) was grown on synthetic complete (SC) medium plates without tryptophan (Trp) but containing a high concentration (2 mM) of methionine (Met). Interactions between Shp2 and BIT/SHPS-1 or Grb2 were assayed on plates of 0.3 mM (low concentration) Met-containing SC medium plate without Trp, leucine (Leu), and histidine (His) but with 25 mM 3-aminotriazole (3AT), the same medium without Leu, Trp, and Uracil (Ura), the same medium without Leu and Trp but with 0.1% (w/v) 5-fluoroorotic acid (5FOA), or the same medium without Leu and Trp. β-Galactosidase was assayed by the colony-lift filter method using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as a substrate in the yeasts growing on 0.3 mM (low concentration) Met-containing SC medium plates without Leu and Trp, according to the yeast supplier's instruction manual (Gibco BRL). To identify Shp2-binding proteins, 4×10^6 transformants were screened on 0.3 mM (low concentration) Met-containing SC medium plates without Trp, Leu, and His but with 25 mM 3AT, using the human brain Matchmarker cDNA library (Clontech).

DNA Constructs—cDNA encoding the intracellular domain (a.a. 397–509) of rat BIT/SHPS-1, the full length of human Grb2, or the full length or the phosphotyrosine-binding (PTB) domain-deleted C-terminal region (a.a. 152–492) of human FRS2β/SNT2 was PCR-amplified and inserted into the multiple cloning site (MCS) of the vector pACT2 (Clontech). cDNA encoding the full length of human Shp2 or the fusion protein of rat TrkB intracellular domain (a.a. 487–810) with GST was PCR-amplified and inserted into the MCS1 or 2 of the pBridge (Clontech), respectively. For experiments using HEK 293 cells, the full coding regions of FRS2β/SNT2 and TrkB were cloned respectively into the mammalian expression vectors pcDNA3.1(–)/Myc-His (Invitrogen) and pCXN2 (*26*), the latter a gift from Dr. J. Miyazaki (Osaka University). The point mutations of Shp2, BIT/SHPS-1, FRS2β/SNT2, and TrkB were constructed by PCR-based site-directed mutagenesis (Stratagene).

Preparation of Yeast Protein Extract—Yeasts were grown in SC liquid medium in the absence of Trp, Leu, and Met. Cells were collected by centrifugation, then washed with an ice-cold TE buffer consisting of 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Lysates were prepared from the yeasts by the TCA method according to the *Yeast Protocols Handbook* (Clontech). Briefly, the yeasts were resuspended in an ice-cold TCA buffer consisting of 20 mM Tris-HCl, pH 8.0, 50 mM ammonium acetate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, and 2 μg/ml aprotinin, then equal volumes of ice-cold 20% (w/v) trichloroacetic acid (TCA) and glass beads were added to the suspension. The mixture was vortexed vigorously at 4°C for 15 min, then the supernatant above the settled glass beads was collected as the first cell extract. An ice-cold 1:1 mixture of 20% TCA and the TCA buffer was added to the remaining glass beads, and the mixture was vortexed followed at 4°C for 10 min. The liquid above the glass beads was combined with the first cell extract, and the mixture was centrifuged at 15,000 rpm for 15 min at 4°C. The pellet was resuspended in TCA-Laemmli loading buffer consisting of 3.5% (w/v) SDS, 120 mM Tris-base, 8 mM EDTA, 14% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, and 1 mM PMSF, boiled for 10 min, then centrifuged at 14,000 rpm for 10 min at room temperature. The supernatant was subjected to immunoblotting with anti-phosphotyrosine (4G10; Upstate Biotech.) and anti-TrkB monoclonal antibodies.

Quantitative Assay for β-Galactosidase—Yeasts were grown in SC liquid medium without Trp and Leu in the presence of 0.3 mM Met, and cells were collected by centrifugation, then washed with an ice-cold TE. Lysates were prepared from the yeasts using Y-PER (Pierce), and the

protein concentration of the lysate was determined by the BCA protein assay (Pierce). β-Galactosidase activity in the lysate was measured with chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate according to the yeast supplier's instruction manual (Gibco BRL).

Transfection, Immunoprecipitation, and Immunoblotting in HEK 293 Cells—HEK 293 cells were grown on 60-mm collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) containing 10% (v/v) fetal bovine serum (FBS; Gibco BRL). The cells were transfected with the vectors (0.5 μg each) for expression of TrkB and FRS2β/SNT2 using Lipofectoamine 2000 (Gibco BRL). The medium was replaced with serum-free medium 1.5 days after the transfection. After serum-starvation for 3 h, the cells were stimulated with 2 μg/ml BSA (as a control) or 2 μg/ml BSA plus 100 ng/ml BDNF for 5 min. The cells were then washed once with ice-cold TBS and lysed in a buffer containing 1% (w/v) Triton X-100, 2% (w/v) *n*-octyl-β-D-glucoside, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylarsine oxide (PAO), 20 mM Tris-HCl (pH 7.5), 1 mM PMSF, 5 μg/ml leupeptin, and 2 μg/ml aprotinin. Immunoprecipitation and immunoblotting were performed as described previously (*18*, *27*). Immunoprecipitations of Myc/His-tagged FRS2β/SNT2, Shp2, and TrkB were performed with anti-penta-His monoclonal (Qiagen), anti-Shp2 polyclonal (SantaCruz), and anti-Trk polyclonal (SantaCruz) antibodies, respectively. Protein tyrosine phosphorylation, Myc/His-tagged FRS2β/SNT2, Shp2, TrkB, and phosphorylation of tyrosine residue 504 of TrkB were detected by immunoblotting with anti-phosphotyrosine monoclonal (4G10; Upstate Biotech.), anti-Myc monoclonal (9E10; SantaCruz), anti-Shp2 monoclonal (Transduction Lab.), anti-Trk polyclonal (SantaCruz), and anti-phospho-TrkA (Tyr490) polyclonal (New England Biolabs) antibodies, respectively. A sequence around Tyr 490 of human TrkA is largely conserved in Trk family proteins, and the Tyr 490 corresponds to Tyr 504 of rat TrkB. Immunoblotting for detection of Grb2 in anti-Shp2 and anti-penta-His immunoprecipitates was performed with anti-Grb2 monoclonal (Transduction Lab.) or polyclonal (SantaCruz) antibodies, respectively.

RESULTS

Yeast Two-and-a-Half-Hybrid System, a Modified Two-Hybrid System, for Studying Tyrosine Phosphorylation-Dependent Protein-Protein Interactions—Following activation of TrkB receptor tyrosine kinase by binding of BDNF, various protein-protein interactions are stimulated in a tyrosine phosphorylation-dependent manner (*1*, *15*, *16*). To examine the tyrosine phosphorylation-dependent protein interactions in TrkB-stimulated intracellular signaling, we developed the yeast two-and-a-half-hybrid system, a modified two-hybrid system, in which yeast was engineered to express a tyrosine kinase domain of rat TrkB as an effector, in addition to two fusion proteins with GAL4 DNA-binding and GAL4 activation domains as bait and prey proteins, respectively. In this system, the protein tyrosine phosphorylation is induced by the TrkB tyrosine kinase in the yeast, and the interactions between bait and tyrosine-phosphorylated prey proteins and between prey and tyrosine-phosphorylated bait proteins can both be detected (Fig. 1).

We expressed a fusion protein of TrkB intracellular domain with glutathione S-transferase (GST) as the effector in the yeast, using the pBridge vector. cDNA encoding the fusion protein of the TrkB intracellular domain with GST (GST-TrkB) was inserted into the multiple cloning site (MCS) 2 of the pBridge vector, which is located downstream of the *Met25* promoter and a nuclear localization sequence (NLS). Expression of the GST-TrkB possessing NLS is regulated by the *Met25* promoter and is attenuated in the presence of a high concentration (more than 1 mM) of methionine (Met). Dimerization of the GST-TrkB fusion proteins through dimerization of the GST moiety was thought to result in activation of GST-TrkB when expressed in yeast. In addition, as a control, we used a kinase ac-

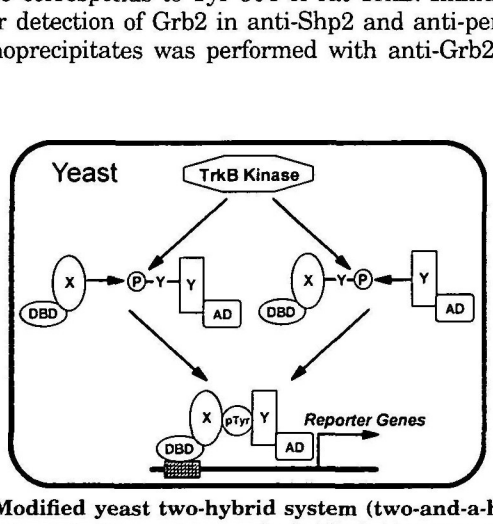

Fig. 1. **Modified yeast two-hybrid system (two-and-a-half-hybrid system).** In the yeast two-and-a-half-hybrid system, yeast expresses the tyrosine kinase domain of TrkB as an effector, in addition to two fusion proteins with GAL4 DNA-binding domain (DBD) and GAL4 activation domain (AD) as bait and prey proteins, respectively. Using this system, interactions between bait and tyrosine-phosphorylated prey proteins and between prey and tyrosine-phosphorylated bait proteins can both be investigated. X and Y are proteins fused with DBD and AD, respectively. In this study, X is Shp2, and Y is BIT/SHPS-1, Grb2, or human brain cDNA library.

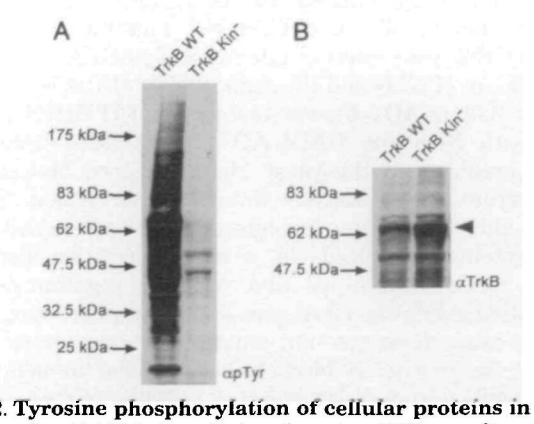

Fig. 2. **Tyrosine phosphorylation of cellular proteins in yeast expressing TrkB intracellular domain.** cDNA encoding the fusion protein of the intracellular domain of TrkB wild type (TrkB WT) or kinase-deficient mutant (TrkB Kin–) with GST was inserted into the yeast expression vector pBridge. These vectors were introduced into yeast MaV203. The yeast was lysed, and the lysate was immunoblotted with anti-phosphotyrosine (A) and anti-TrkB monodonal (B) antibodies, as described under "EXPERIMENTAL PROCEDURES." The arrowhead on the right of the immunoblot indicates the fusion protein of the intracellular domain of TrkB with GST. Molecular masses are shown on the left of the immunoblots.

tivity-deficient mutant of GST-TrkB (GST-TrkBkin$^-$) in which the lysine residue on the ATP-binding site in the kinase domain of TrkB was replaced by an asparagine residue. To examine whether the expression of the GST-TrkB induces protein tyrosine phosphorylation in yeast, lysates prepared from yeast expressing the wild type or the kinase activity-deficient mutant of GST-TrkB were immunoblotted with anti-phosphotyrosine antibody. The expression of wild-type GST-TrkB (GST-TrkBwt), but not GST-TrkBkin$^-$, stimulated protein tyrosine phosphorylation in the yeast (Fig. 2A). A protein with an apparent molecular mass of about 65 kDa, which corresponded to the calculated molecular mass of GST-TrkB, showed an intense tyrosine phosphorylation in the yeast expressing GST-TrkBwt. In addition, anti-TrkB monoclonal antibody recognized the 65-kDa protein, although the amount of the 65-kDa protein in the yeast expressing GST-TrkBkin$^-$ was larger than in that expressing GST-TrkBwt (Fig. 2B). These results indicate that the expression of GST-TrkBwt stimulates tyrosine phosphorylation of proteins including GST-TrkB itself in the yeast.

Tyrosine Phosphorylation-Dependent Interactions of Shp2 with BIT/SHPS-1 and Grb2 in the Yeast—We previously reported that Shp2 interacts with BIT/SHPS-1 (also called SIRP) and Grb2 in response to BDNF in cultured cerebral cortical neurons and TrkB-expressing PC12 cells (*17*, *18*). To examine whether Shp2 interacts directly with BIT/SHPS-1 and Grb2 in a TrkB-induced tyrosine phosphorylation-dependent manner, we performed the two-and-a-half-hybrid assay in the GST-TrkBwt- or kin$^-$-expressing yeast, using the full-length Shp2 as a bait and the intracellular domain of BIT/SHPS-1 or the full-length Grb2 as a prey (Fig. 3). Human full-length Shp2 cDNA was cloned into MCS1 of the pBridge vector possessing the sequences for GST-TrkBwt or kin$^-$. MCS1 is located downstream of the sequence for GAL4 DNA-binding domain (GAL4 DBD), and a fusion protein of Shp2 with GAL4 DBD is expressed in the yeast under the control by the constitutive *ADH1* promoter, then targeted to the nucleus by NLS, which is an intrinsic part of the GAL4 DBD. cDNAs encoding the intracellular domain of rat BIT/SHPS-1 and the full-length human Grb2 were inserted into MCS of pACT2, which lies downstream of NLS and the sequence for GAL4 activation domain (GAL4 AD). Fusion proteins of BIT/SHPS-1 and Grb2 with NLS and GAL4 AD are expressed from the *ADH1* promoter in the yeast. Here, we used MaV203, a yeast strain that possesses three reporter genes, *HIS3*, *URA3*, and *lacZ*. When the interaction between bait and prey proteins is induced, the yeast can grow on medium lacking histidine or uracil, and expresses β-galactosidase. In addition, when the yeast containing the interacting proteins is cultured on medium containing 5-fluoroorotic acid (5FOA), its growth is blocked, because the induction of *URA3* leads to conversion of 5FOA to 5-fluorouracil, which is toxic. MaV203 was transformed simultaneously with pACT2 containing BIT/SHPS-1 or Grb2 and with the pBridge containing both Shp2 and GST-TrkBwt or kin$^-$. The transformants were plated on low concentration (0.3 mM) methionine-containing leucine- and tryptophan-minus Synthetic Complete (SC) medium, on the same medium lacking histidine but containing 25 mM 3-aminotriazole (3AT), on the medium lacking uracil, or on the medium plus 0.1% 5FOA. β-Galactosidase was detected by the colony-lift filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as a substrate with the yeast grown on the low methionine-containing SC medium plate. As a result, interactions of Shp2 with Grb2 and BIT/SHPS-1 were observed in the yeasts expressing GST-TrkBwt, but not in those expressing GST-TrkBkin$^-$, when plated on the SC medium plate lacking histidine but containing 25 mM 3-aminotriazole (3AT) (Fig. 3). The results of the assay for β-galactosidase also showed the TrkB tyrosine kinase activity-dependent interactions of Shp2 with Grb2 and BIT/SHPS-1, although the activity in the yeast expressing Grb2 was greater than that in the yeast expressing BIT/SHPS-1, indicating that Shp2 interacts with Grb2 more intensely than with BIT/SHPS-1 in the yeast. On the other hand, the yeast expressing BIT/SHPS-1 could not grow on plates of SC medium lacking uracil, although that expressing Grb2 grew on the same medium in a TrkB tyrosine kinase-dependent manner. However, the yeast expressing BIT/SHPS-1 showed growth inhibition dependently on TrkB tyrosine kinase activity on plates of SC medium plus 0.1% 5FOA, as did that expressing Grb2, although extent of the

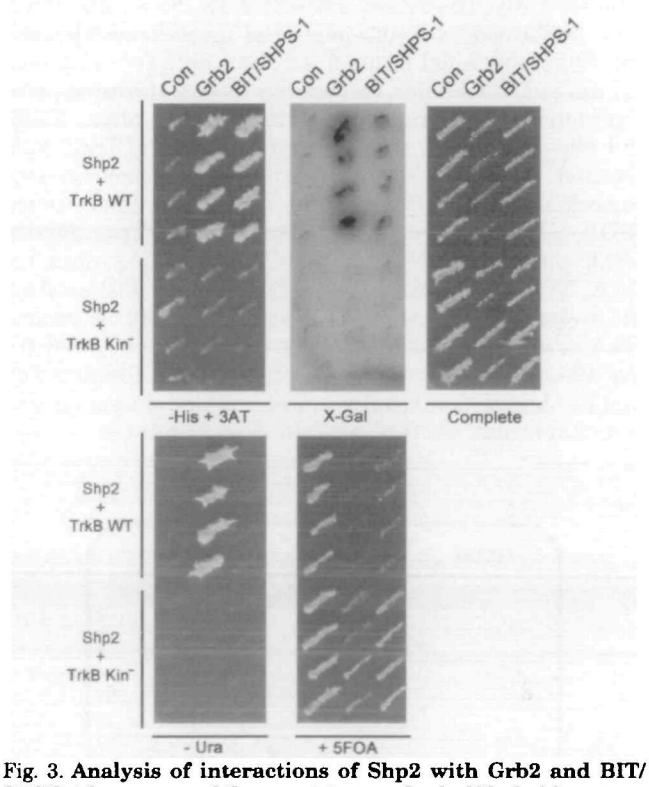

Fig. 3. **Analysis of interactions of Shp2 with Grb2 and BIT/SHPS-1 by means of the yeast two-and-a-half-hybrid system.** cDNA encoding the full-length Grb2 (Grb2) or the intracellular domain of BIT/SHPS-1 (BIT/SHPS-1) was inserted or not inserted (Con) into the yeast expression vector pACT2. These vectors were introduced into the yeasts expressing both full-length Shp2 and the GST fusion protein with the intracellular domain of TrkB wild type (TrkB WT) or kinase-deficient mutant (TrkB Kin$^-$). Four independent transformants were streaked on plates of SC medium without Leu, Trp, and His but with 25 mM 3AT (− His + 3AT), on the medium without Leu, Trp, and Ura (− Ura), on the medium without Leu and Trp but with 0.1% 5FOA (+ 5FOA) or on the medium without Leu and Trp (Complete), or were subjected to β-galactosidase assay by the colony-lift filter method using X-Gal as a substrate (X-Gal), as described under "EXPERIMENTAL PROCEDURES."

growth inhibition in the yeast expressing BIT/SHPS-1 was lower than that in the yeast expressing Grb2. These results indicate that the *URA3* reporter gene is expressed in the yeast expressing BIT/SHPS-1, although the expression of the *URA3* reporter gene is weaker in the yeast expressing BIT/SHPS-1 than in that expressing Grb2, reflecting the strength of the interactions of Shp2 with these proteins.

Next, to determine whether the interactions of Shp2 with BIT/SHPS-1 and Grb2 are due to phosphorylated tyrosine residues, we changed the tyrosine residues to phenylalanine residues on Shp2 and BIT/SHPS-1, then employed the two-and-a-half-hybrid system with these phenylalanine mutants. BIT/SHPS-1 has four tyrosine residues in its intracellular domain, which are phosphorylated and are involved in binding of BIT/SHPS-1 to Shp2 in insulin-, EGF-, and BDNF-induced intracellular signaling (*28–30*). All four tyrosine residues of BIT/SHPS-1 were changed to phenylalanine (4F). On the other hand, Grb2 binds to phosphorylated tyrosine residues 542 and 580 on Shp2 in PDGF-stimulated intracellular signaling *in vitro* (*21*). These tyrosine residues were mutated to phenylalanine individually (Y542F and Y580F) and together (Y542/580F). Using the mutated BIT/SHPS-1 and Shp2, we performed the two-and-a-half-hybrid assay (Fig. 4). As a result, the yeast expressing 4F mutant of BIT/SHPS-1 showed no growth on the SC medium plate lacking histidine, no activity of β-galactosidase, and no growth inhibition on the SC medium plate containing 5FOA, in contrast to that expressing wild-type BIT/SHPS-1 (Fig. 4A). These results indicate that the interaction of Shp2 with BIT/SHPS-1 in the yeast is dependent on the TrkB-stimulated phosphorylation of the tyrosine residues on BIT/SHPS-1. In the yeast expressing the mutants of Shp2, their interactions with Grb2 were reduced in comparison with that in the yeast expressing wild-type Shp2 (Fig. 4B). The Shp2 Y542F mutant showed strong attenuation in the interaction with Grb2, while the Y580F mutant showed moderate attenuation. In the yeast expressing the Y542/580F mutant, the growth on the medium lacking histidine or uracil, the growth inhibition on the medium containing 5FOA, and the β-galactosidase expression were completely abolished. These results indicate that interaction of Shp2 with Grb2 requires the TrkB-stimulated phosphorylation of tyrosine residues 542 and 580 on Shp2. Therefore, it seems reasonable to suppose that TrkB tyrosine-phosphorylates BIT/SHPS-1 and Shp2, then the phosphorylated BIT/SHPS-1 and Shp2 directly bind to Shp2 and Grb2, respectively. In addition, this system allowed us to observe interactions both between bait and tyrosine-phosphorylated prey proteins, corresponding to Shp2 and BIT/SHPS-1, respectively, and between tyro-

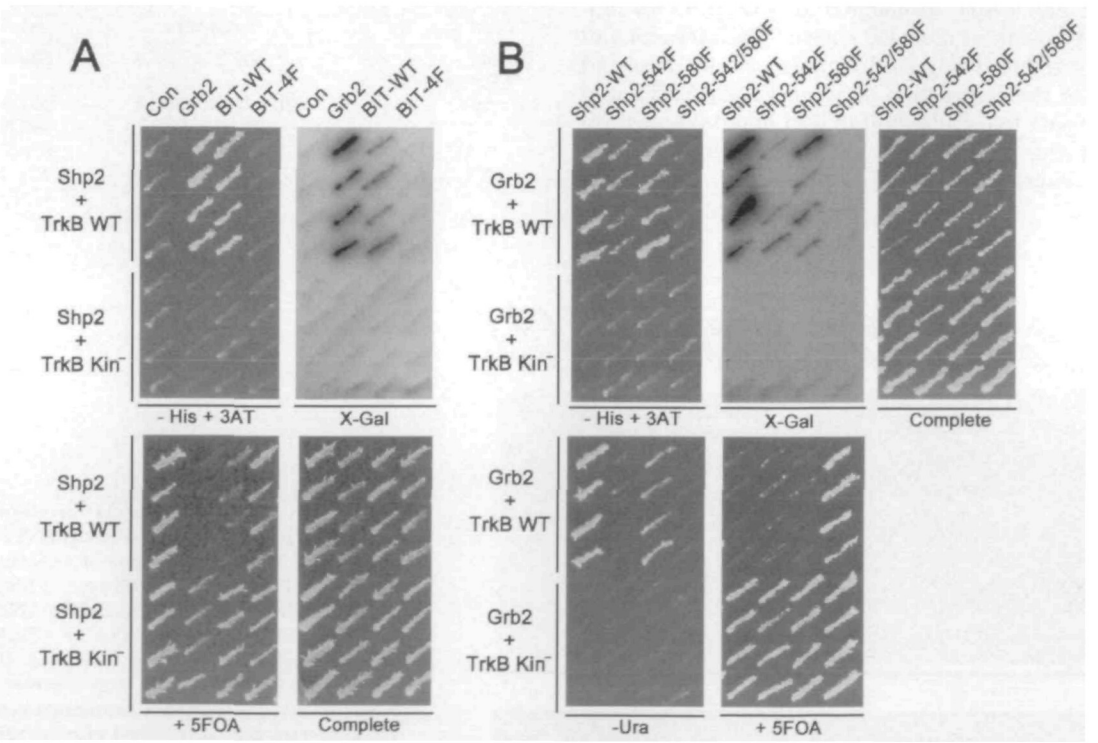

Fig. 4. **Tyrosine phosphorylation-dependent interactions of Shp2 with Grb2 and BIT/SHPS-1 in yeast.** (A) cDNA encoding the full-length Grb2 or the intracellular domain of BIT/SHPS-1 wild type (BIT-WT) or 4F mutant (BIT-4F) was inserted or not inserted (Con) into the yeast expression vector pACT2. These vectors were introduced into the yeast expressing both the full length Shp2 and the GST fusion protein with the intracellular of TrkB wild type (TrkB WT) or kinase deficient mutant (TrkB Kin⁻). (B) cDNA encoding Shp2 wild type (Shp2-WT), or Shp2 Y542F (Shp2-542F), Y580F (Shp2-580F) or Y542/580F (Shp2-542/580F) mutant was inserted into the yeast expression vector pBridge containing the sequence of the GST fusion protein with the intracellular domain of TrkB wild type (TrkB WT) or kinase-deficient mutant (TrkB Kin⁻). These vectors were introduced into the yeast expressing the full-length Grb2. (A and B) Four independent transformants were streaked on plates of SC medium without Leu, Trp, and His but with 25 mM 3AT (− His + 3AT), on the medium without Leu, Trp, and Ura (− Ura), on the medium without Leu and Trp but with 0.1% 5FOA (+ 5FOA) or on the medium without Leu and Trp (Complete), or were subjected to β-galactosidase assay by the colony-lift filter method using X-Gal as a substrate (X-Gal), as described under "EXPERIMENTAL PROCEDURES."

sine-phosphorylated bait and prey proteins, corresponding to Shp2 and Grb2, respectively.

Identification of Tyrosine Phosphorylation-Dependent Interaction of Shp2 with FRS2β/SNT2 Using the Yeast Two-and-a-Half-Hybrid System—We have previously reported that several unidentified proteins interact with Shp2 in response to neurotrophins in cultured cerebral cortical neurons and PC12 cells (*18*). To identify other Shp2-binding proteins in BDNF-induced intracellular signaling, we screened a yeast expression library prepared from adult human brain in the two-and-a-half-hybrid system using Shp2 as a bait. We obtained four positive clones. Two of these clones encoded fibroblast growth factor receptor substrate 2β (FRS2β), also called SNT2 (*31*, *32*). The remaining two clones encoded BIT/SHPS-1. Thus, we newly found that FRS2β/SNT2 binds to Shp2 depending on the activity of TrkB tyrosine kinase in the yeast system (Fig. 5).

We postulated that SH2 domains of Shp2 mediate the interaction between Shp2 and FRS2β/SNT2, because there are putative Shp2-binding sites in the C-terminal region of FRS2β/SNT2. To identify the tyrosine residues of FRS2β/SNT2 that mediate its association with Shp2, we performed the yeast two-and-a-half-hybrid experiments employing a series of FRS2β/SNT2 mutants in which individual or multiple tyrosine residues were changed to phenylalanine (Fig. 5). As a result, we found that tyrosine residues 417 and 455 were both necessary for the interaction between FRS2β/SNT2 and Shp2, although the FRS2β/SNT2 mutated only at tyrosine residue 455 showed the interaction in a remarkably reduced degree. Furthermore, we assessed the strength of the interaction between FRS2β/SNT2 and Shp2 by the yeast two-and-a-half-hybrid liquid β-galactosidase assay, using chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate (Table I). As a result, Y417F and Y455F phenylalanine mutants of FRS2β/SNT2 were found to show reduced but still detectable interactions. In the double mutant Y417/455F, however, the interaction was completely abolished. These results indicate that the TrkB-mediated interaction between FRS2β/SNT2 and Shp2 requires both phosphotyrosine residues 417 and 455 on FRS2β/SNT2.

Tyrosine Phosphorylation-Dependent Interaction of Shp2 with FRS2β/SNT2 in HEK 293 Cells—Next, we confirmed the interaction between FRS2β/SNT2 and Shp2 in HEK 293 cells. We coexpressed Myc/His-tagged FRS2β/SNT2 with TrkB in HEK 293 cells (Fig. 6). The expression of TrkB stimulated tyrosine phosphorylation of TrkB itself without the treatment with BDNF, although the addition of

TABLE I. **Assessment of the degree of interaction between FRS2β/SNT2 and Shp2 by the yeast two-and-a-half-hybrid liquid β-galactosidase assay.** cDNA encoding PTB domain-deleted form of FRS2β/SNT2 wild type (WT), or FRS2β/SNT2 Y417 (Y417F), Y455F (Y455F), or Y417/455F (Y417/455F) mutant was inserted into an yeast expression vector pACT2. These vectors were introduced into the yeasts expressing both full-length Shp2 and the GST fusion protein with the intracellular domain of TrkB wild type (TrkB WT) or kinase-deficient mutant (TrkB Kin$^-$) The activities (μmol/mg protein) of β-galactosidase were measured using chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate, as described under "EXPERIMENTAL PROCEDURES." The values represent the means ± SD of four individual cultures.

	TrkB WT	TrkB Kin$^-$
Con.	607.7 ± 24.0	629.3 ± 71.3
FRS2β/SNT2		
WT	70,672.1 ± 802.1	991.1 ± 91.2
Y417F	21,670.0 ± 632.1	886.5 ± 103.2
Y455F	3,958.1 ± 198.9	1,078.1 ± 55.4
Y417/455F	1,055.6 ± 170.2	1,108.6 ± 44.2

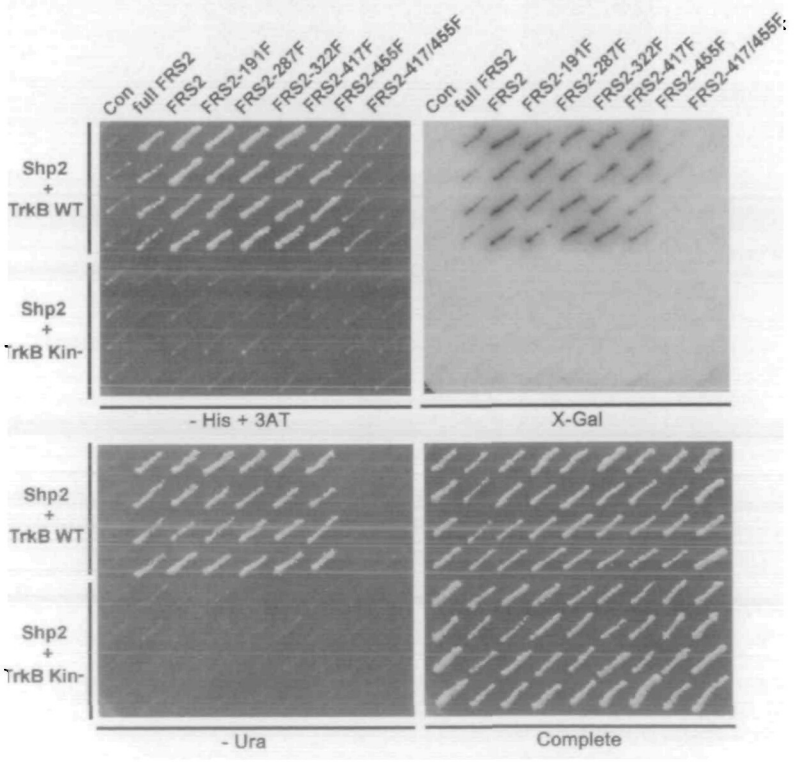

Fig. 5. **Tyrosine phosphorylation-dependent interaction of Shp2 with FRS2β/SNT2 in yeast.** cDNA encoding the wild type of full-length FRS2β/SNT2 (full FRS2), or the wild type (FRS2) or Y191F (FRS2-191F), Y287F (FRS2-287F), Y322F (FRS2-322F), Y417F (FRS2-417F), Y455F (FRS2-455F), or Y417/455F (FRS2-417/455F) mutant of the PTB domain-deleted FRS2β/SNT2 was inserted or not inserted (Con) into the yeast expression vector pACT2. These vectors were introduced into the yeast expressing both the full-length Shp2 and the GST fusion protein with the intracellular domain of TrkB wild type (TrkB WT) or kinase-deficient mutant (TrkB Kin$^-$). Four independent transformants were streaked on plates of SC medium plate without Leu, Trp, and His but with 25 mM 3AT (− His + 3AT), on the medium without Leu, Trp, and Ura (− Ura) or on the medium without Leu and Trp (Complete), or were subjected to β-galactosidase assay by the colony-lift filter method using X-Gal as a substrate (X-Gal), as described under "EXPERIMENTAL PROCEDURES."

BDNF slightly increased the tyrosine phosphorylation of TrkB (Fig. 6A). This result indicates that overexpression of TrkB can induce activation of TrkB in a ligand-independent manner in HEK 293 cells. The tyrosine phosphorylation of FRS2β/SNT2 was stimulated dependently on the TrkB activation (Fig. 6B). Consistent with the tyrosine phosphorylation of FRS2β/SNT2, interaction of Shp2 with FRS2β/SNT2 was also observed. It has been reported that FRS2α/SNT1, another member of the FRS2/SNT family, associates with Grb2 in response to fibroblast growth factor (FGF) and nerve growth factor (NGF) (*33*). We observed that FRS2β/SNT2 also interacted with Grb2 dependently on the TrkB activation (Fig. 6B). Furthermore, to explore the possibility that tyrosine residues 417 and 455 on FRS2β/SNT2 function as the binding sites for Shp2, we coexpressed Y417F or Y455F phenylalanine mutants and the Y417/455F double phenylalanine mutant of Myc/His-tagged FRS2β/SNT2 with TrkB. The active TrkB induced somewhat reduced tyrosine phosphorylation of all of the phenylalanine mutants of FRS2β/SNT2 in comparison with tyrosine phosphorylation of the wild type (Fig. 6B). The interactions of the Y417F and Y455F mutants with Shp2 were reduced but not completely abolished, but the double mutant showed no interaction with Shp2. On the other hand, the interactions of FRS2β/SNT2 with Grb2 were not affected in any phenylalanine mutants of FRS2β/SNT2. In an immunoprecipitation study with anti-Shp2 antibody, the interaction of Shp2 with FRS2β/SNT2 was also completely abolished in the double mutant of FRS2β/SNT2 (Fig. 6C). In addition, the amount of Grb2 in the anti-Shp2 immunoprecipitate corresponded to that of FRS2β/SNT2, indicating that Shp2 and Grb2 can simultaneously bind to FRS2β/SNT2, namely, that Grb2 associates with Shp2 *via* binding to FRS2β/SNT2 in HEK 293 cells.

FRS2β/SNT2 contains a PTB domain in its amino-termi-

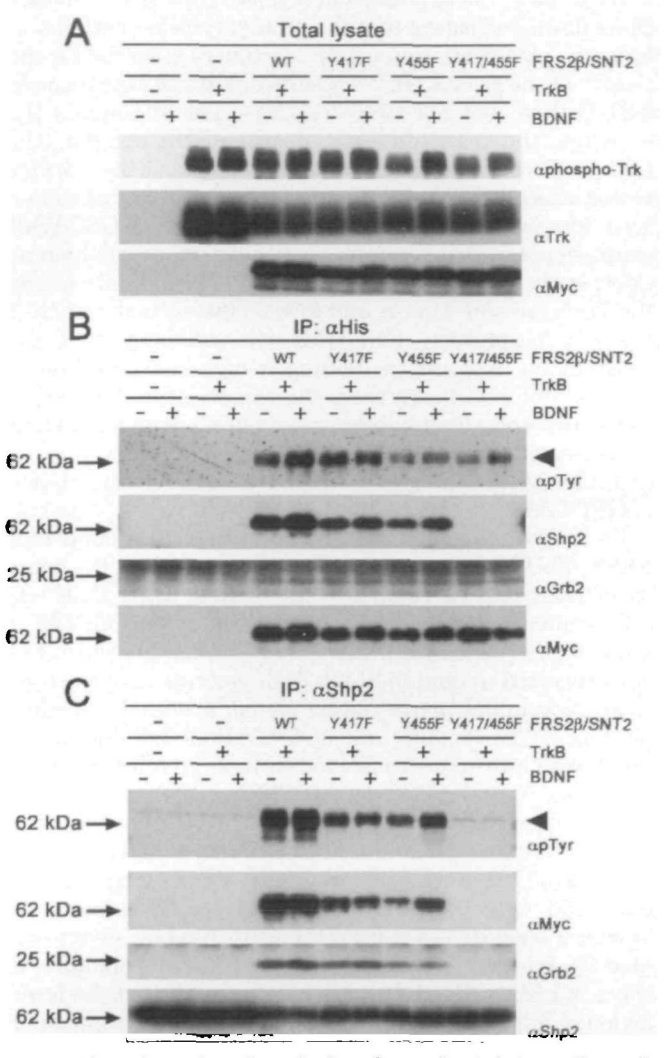

Fig. 6. **Tyrosine phosphorylation-dependent interaction of FRS2β/SNT2 with Shp2 in HEK293 cells.** HEK 293 cells expressing (+) or not expressing (−) the wild type of full-length TrkB and/or the wild type (WT) or Y417F (Y417F), Y455F (Y455F), or Y417/455F (Y417/455F) mutant of the Myc/His-tagged full length FRS2β/SNT2 were incubated with 2 μg/ml BSA (−) or 2 μg/ml BSA plus 100 ng/ml BDNF (+) for 5 min, and lysed. (A) The lysates were directly analyzed by Western blotting with anti-phospho-Trk polyclonal (αphospho-Trk), anti-Trk polyclonal (αTrk), or anti-Myc monoclonal (αMyc) antibodies. (B and C) Myc/His-tagged FRS2β/SNT2 (B) and Shp2 (C) were immunoprecipitated with anti-penta-His monoclonal and anti-Shp2 polyclonal antibodies, respectively. The immunoprecipitates were analyzed by Western blotting with anti-phosphotyrosine monoclonal (αpTyr), anti-Myc monoclonal (αMyc), anti-Shp2 monoclonal (αShp2), or anti-Grb2 polyclonal (αGrb2 in B) or monoclonal (αGrb2 in C) antibodies. Arrowheads on the right of the immunoblots indicate tyrosine-phosphorylated FRS2β/SNT2. Molecular masses are shown on the left of the immunoblots.

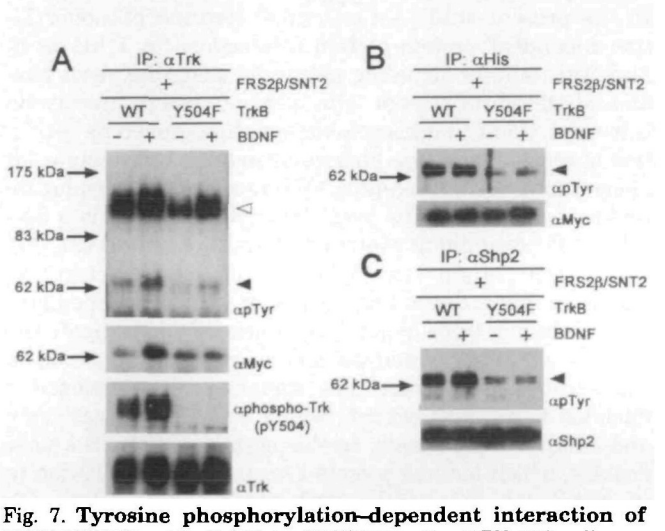

Fig. 7. **Tyrosine phosphorylation–dependent interaction of FRS2β/SNT2 with TrkB in HEK293 cells.** HEK 293 cells expressing both the wild type of Myc/His-tagged full-length FRS2β/SNT2 and the wild type (WT) or Y504F mutant (Y504F) of full-length TrkB were incubated with 2 μg/ml BSA (−) or 2 μg/ml BSA plus 100 ng/ml BDNF (+) for 5 min, then lysed. TrkB (A), Myc/His-tagged FRS2β/SNT2 (B), and Shp2 (C) were immunoprecipitated with anti-Trk polyclonal, anti-penta-His monoclonal, and anti-Shp2 polyclonal antibodies, respectively. The anti-Trk immunoprecipitates (A) were analyzed by Western blotting with anti-phosphotyrosine monoclonal (αpTyr), anti-Myc monoclonal (αMyc), anti–phospho-Trk polyclonal (αphospho-Trk), or anti-Trk polyclonal (αTrk) antibodies. The anti-His (B) and anti-Shp2 (C) immunoprecipitates were analyzed by Western blotting with anti-phosphotyrosine monoclonal antibody (αpTyr), anti-Myc monoclonal (αMyc in B), or anti-Shp2 monoclonal (αShp2 in C) antibodies. The open arrowhead on the right of the immunoblot indicates tyrosine-phosphorylated TrkB. Closed arrowheads on the right of the immunoblots indicate tyrosine-phosphorylated FRS2β/SNT2. Molecular masses are shown on the left of the immunoblots.

nus region. The PTB domain was first identified in the adapter protein Shc as a protein module that binds to the tyrosine-phosphorylated NPXY sequence (*5*). TrkB has the NPXY motif in its juxtamembrane domain. Therefore, we examined whether the TrkB-induced FRS2β/SNT2 signaling requires tyrosine residue 504, which lies in the NPXY motif of TrkB. In HEK 293 cells expressing the Y504F mutant of TrkB, the tyrosine phosphorylation of FRS2β/SNT2 and the interactions of FRS2β/SNT2 with Shp2 and TrkB were remarkably reduced (Fig. 7). We confirmed that the tyrosine phosphorylation of the Y504F mutant of TrkB was not detected with anti-phospho-Trk antibody, which reacts only with the phosphorylated tyrosine residue 504, but the phosphorylation of other tyrosine residues on the Y504F mutant was detected with anti-phosphotyrosine antibody. However, the total level of tyrosine phosphorylation of the mutant TrkB was less than that of the wild type of TrkB (Fig. 7A).

In summary, FRS2β/SNT2 binds to TrkB through the tyrosine residue 504 of TrkB, and is tyrosine-phosphorylated by active TrkB. It then works as an adapter protein for Shp2 and Grb2. Shp2 binds to FRS2β/SNT2 through tyrosine residues 415 and 455 of FRS2β/SNT2, and other tyrosine residues of FRS2β/SNT2 mediate the interaction of FRS2β/SNT2 with Grb2.

DISCUSSION

In the present study, we examined tyrosine phosphorylation-dependent protein-protein interactions in TrkB-mediated intracellular signaling pathways using the yeast two-and-a-half-hybrid system. This kind of protein interaction is a major event in intracellular signaling evoked by activation of receptor tyrosine kinases including Trk (receptor for neurotrophin), EGF receptor, FGF receptor and insulin receptor (*1*, *2*, *15*, *16*). The yeast two-hybrid system is a useful tool for examining protein-protein interactions (*34*, *35*). However, the original two-hybrid system cannot detect protein interaction depending on post-translational modifications including tyrosine phosphorylation. To investigate the tyrosine phosphorylation-dependent protein interaction in TrkB-stimulated intracellular signaling, we developed a modified two-hybrid system, which we call the yeast two-and-a-half-hybrid system. In this system, the TrkB kinase domain, which induces protein tyrosine phosphorylation in the yeast, was coexpressed with two fusion proteins with GAL4 DNA–binding and GAL4 activation domains as bait and prey proteins, respectively. Using this system, we showed that Shp2 interacts directly with BIT/SHPS1 and Grb2 dependently on the tyrosine phosphorylation stimulated by the TrkB in the yeast. Furthermore, we identified FRS2β/SNT2 as a Shp2-binding protein by means of a screening of the human brain cDNA library using the two-and-a-half-hybrid system with Shp2 as the bait. Therefore, we suggest that the two-and-a-half-hybrid system is a powerful tool for studying the tyrosine phosphorylation-dependent protein-protein interactions in TrkB-stimulated intracellular signaling.

In the yeast two-and-a-half-hybrid system, we expressed a fusion protein of TrkB intracellular tyrosine kinase domain with GST in addition to the bait and prey proteins. When we expressed only the TrkB intracellular domain rather than the fusion protein with GST, we could not detect any increase in the protein tyrosine phosphorylation in the yeast (data not shown). This result indicates that the activation of the TrkB tyrosine kinase in the yeast requires the fused GST moiety. It is known that GST forms a dimer (*36*, *37*). In addition, activation of TrkB is induced by its dimerization through the binding of its ligand, BDNF (*16*, *38*). The GST moiety of the GST-TrkB fusion protein may induce dimerization of the fusion protein, resulting in the activation of the TrkB tyrosine kinase.

When $p60^{v\text{-}src}$ is expressed in a yeast, growth of the yeast slows down, indicating that the protein tyrosine phosphorylation by $p60^{v\text{-}src}$ interferes with machinery essential for the growth of the yeast (*39*). We observed that the expression of GST-TrkBwt, but not GST-TrkBkin$^-$, also attenuated the growth of the yeast (data not shown). cDNA encoding the GST-TrkB was inserted into the cloning site of the pBridge vector, which is located downstream of the *Met25* promoter. Therefore, we were able to control the level of GST-TrkB expression by changing the concentration of methionine (Met) in the medium. To suppress the growth inhibition by the TrkB tyrosine kinase, and to keep the normal growth of the yeast expressing GST-TrkB, we ordinarily grew the yeast in the medium containing a high concentration (2 mM) of Met, which represses the expression of GST-TrkB. In the two-and-a-half-hybrid assay and screening, the yeast was cultured in the medium containing a low concentration (0.3 mM) of Met in order to induce a moderate expression of GST-TrkB.

Using the two-and-a-half-hybrid system, we found that FRS2β/SNT2 associates with Shp2 depending on the activity of TrkB, and we confirmed this finding using HEK 293 cells expressing FRS2β/SNT2 and TrkB. FRS2β/SNT2 is structurally related to FRS2α/SNT1 (*31*, *32*, *40*), which has been reported to bind to Shp2. Both proteins are composed of an N-terminal myristylation signal, a phosphotyrosine-binding (PTB) domain, and a C-terminal tail containing five tyrosine residues conserved in both proteins. Tyrosine residues 436 and 471 of mouse FRS2α/SNT1 are involved in its interaction with Shp2 (*40*). We showed that the interaction of Shp2 with human FRS2β/SNT2 required tyrosine residues 417 and 455, which correspond to tyrosine residues 436 and 471 of mouse FRS2α/SNT1, respectively. Tyrosine residues other than the Shp2-binding sites mediated the interaction of FRS2β/SNT2 with Grb2 similarly to those of FRS2α/SNT1. In addition, we found that the interaction of Shp2 with Grb2 was dependent on the association of FRS2β/SNT2 with Shp2 in HEK 293 cells expressing FRS2β/SNT2 and TrkB, although the interaction of FRS2β/SNT2 with Grb2 was independent of the binding of FRS2β/SNT2 to Shp2. These results indicate that FRS2β/SNT2 binds simultaneously to both Shp2 and Grb2, namely, that a Shp2-FRS2β/SNT2-Grb2 complex is formed dependently on the activation of TrkB in HEK 293 cells.

In PC12 cells, FRS2α/SNT1 is tyrosine-phosphorylated in response to NGF and FGF, both of which induce neuronal differentiation (*33*). However, it is not tyrosine-phosphorylated in response to EGF or insulin, neither of which induces the neuronal differentiation. FRS2α/SNT1 is also tyrosine-phosphorylated in response to BDNF in cultured neuronal cells (*41*). In the present study, we showed the TrkB activity-dependent interaction of FRS2β/SNT2 with Shp2 by the yeast two-and-a-half-hybrid system and the expression experiment using HEK 293 cells. Furthermore,

the activation of TrkB stimulated the interaction of FRS2β/SNT2 with Grb2 and TrkB in HEK 293 cells expressing FRS2β/SNT2 and TrkB. These results indicate that FRS2β/SNT2 as well as FRS2α/SNT1 is involved in BDNF-induced intracellular signaling mediated by TrkB. Therefore, we suggest that both the members of the FRS2/SNT family have important roles in intracellular signaling pathways stimulated by neurotrophins including BDNF.

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